Pulsed Field Gradient Nuclear Magnetic Resonance Diffusion Study on Bicellar Mixtures Containing Pluronic F68

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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2011

Abstract

Described in this report is stimulated echo pulsed field gradient (STE-PFG) $^1$H nuclear magnetic resonance (NMR) diffusion on neutral and negatively charged magnetically aligned bicelles incorporating the Pluronic tri-block copolymer F68. Bicelles are model lipid membrane systems composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC).

Pluronic F68 incorporated into neutral bicellar mixtures (q= [DMPC]/[DHPC]= 4.5) exhibited resonance intensity decays that are non-exponential and diffusion-time dependent, i.e. non-Gaussian diffusion. In contrast, Pluronic F68 incorporated in negatively charged bicellar mixtures, containing 1 mol% 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), showed the F68 intensity decays that are exponential and diffusion-time independent, viz., Gaussian diffusion. The implication may be that neutral bicellar mixtures incorporating Pluronic F68 consist of extended lamellae composed of meshed ribbon structures, while negatively charged bicellar mixtures incorporating Pluronic F68 consist of perforated lamellae. Pluronic F68 incorporated into the bicelles reports these morphological differences through its diffusion.
Acknowledgments

I am grateful for the opportunity to work under Professor Peter Macdonald. With his continuous advice, guidance and patience on my project, I have learned more than I thought possible in my work. I would like to thank Dr. Kaz Nagashima for his unflagging efforts in teaching me how to use the NMR spectrometer and helping me carry out experiments.

I would also like to thank my colleagues Qasim Saleem and Rohan Alvares for numerous discussions and suggestions about my project, sports and food, and moral support before and after any public speaking event. Thanks to Sameer Al-Abdul Wahid for his constant support and answering his phone when I call for help. To my colleagues in the Kanelis lab, thanks for making me laugh everyday.

Finally, I want to thank my parents for their continuous understanding and encouragement. To my two little sisters, thank you for being my biggest inspiration, aggravation and cheerleaders.
# Table of Contents

Abstract..........................................................................................................................ii
Acknowledgements ...........................................................................................................iii
Table of Contents ...............................................................................................................iv
List of Tables .......................................................................................................................vi
List of Figures .....................................................................................................................vii
List of Symbols ..................................................................................................................x
List of Abbreviations .........................................................................................................xii

1 Introduction .....................................................................................................................1
   1.1 Lipids and Membranes ...............................................................................................2
   1.2 Bicelles ......................................................................................................................3
   1.3 Pluronic Triblock Copolymers ....................................................................................8
      1.3.1 Properties and Uses of Pluronics .......................................................................8
      1.3.2 The modes of incorporation of Pluronics into the Lipid Bilayer .........................9
   1.4 Molecular Diffusion ..................................................................................................12
      1.4.1 Methods of Measuring Diffusion by Fluorescence ...........................................14
         1.4.1.1 Fluorescence Recovery After Photobleaching .............................................14
         1.4.1.2 Fluorescence Correlation Spectroscopy ......................................................16
         1.4.1.3 Single Particle Tracking ...........................................................................18
      1.4.2 Methods of Measuring Diffusion by NMR .........................................................20
         1.4.2.1 Pulsed Field Gradient Spin Echo Measurements .........................................20
         1.4.2.2 $^1$H Magic Angle Spinning ......................................................................23
         1.4.2.3 Exchange Spectroscopy ...........................................................................24
         1.4.2.4 One-Dimensional Exchange Spectroscopy by Sideband Alternation ..........25
   1.5 Goals of this research ...............................................................................................28

2 Experimental Section ....................................................................................................29
   2.1 Materials ..................................................................................................................29
   2.2 Preparation of Bilayered Micelles ...........................................................................29
   2.3 Binding Assay ...........................................................................................................30
   2.4 NMR Spectroscopy ................................................................................................30
      2.4.1 Magnetic Alignment of Bicelles ........................................................................31
      2.4.2 $^{31}$P NMR .......................................................................................................31
      2.4.3 Proton NMR .....................................................................................................31
      2.4.3 Inversion Recovery Experiments ......................................................................32
      2.4.4 Measurement of Lateral Diffusion Coefficient .................................................32
      2.4.5 Data Processing ...............................................................................................32
3 Results and Discussion

3.1 $^{31}$P NMR Assessment of Bicelle Magnetic Alignment

3.2 Lateral Diffusion Measurements

3.2.1 Self-Diffusion of Polyethylene glycol 20,000

3.2.2 Self-Diffusion of Polyethylene glycol 12,000

3.2.3 Self-Diffusion of Pluronic F68

3.2.4 Diffusion of Pluronic F68 Incorporated in Bicellar Mixtures

3.3 Binding Assay

4 Conclusions

5 References
List of Tables

Table 2.1: A summary table of sample compositions used in the project...........................................30
Table 3.1: The $^{31}$P chemical shifts of $q = 3.5$ bicellar mixtures......................................................35
Table 3.2: The $^{31}$P chemical shifts of $q = 4.5$ bicellar mixtures......................................................35
Table 3.3: The effective ratios of planar-to-curved phospholipid populations, $q^*$.............................37
Table 3.4: The number of moles of F68 present as the bound and free in the binding assay based on the PPO and PEO peaks.................................................................61
List of Figures

Figure 1.1: Various lipid structures at various ratios of long chain lipid: short chain lipid
Figure 1.2: Magnetic alignment of bicelles with their bilayer normals orthogonal and parallel to the direction of the applied magnetic field
Figure 1.3: $^{31}$P spectrum of magnetically aligned DMPC/DHPC (q=4.5) bicelles at 30°C
Figure 1.4: A- Schematic of the spanned conformation of Pluronic F68 in a lipid bilayer
       B- Schematic of the U-conformation of Pluronic F68 in a lipid bilayer
Figure 1.5: A schematic diagram of a FRAP instrument
Figure 1.6: A schematic experimental setup for FCS measurements
Figure 1.7: The basic Stejskal-Tanner pulsed field-gradient pulse sequence
Figure 1.8: Stimulated echo pulsed field gradient pulse sequence
Figure 1.9: The basic pulse sequence of an EXSY experiment
Figure 1.10: The pulse sequence of an ODESSA experiment
Figure 3.1: The $^{31}$P NMR spectrum for the neutral bicellar sample at 30°C
Figure 3.2: The $^{31}$P NMR spectrum for the negatively charged bicellar sample at 30°C
Figure 3.3: A schematic cross section of a DMPC/DHPC bicelle
Figure 3.4: STE PFG $^1$H NMR spectra of PEG 20,000
Figure 3.5: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG20,000 in 10mM Tris buffer at 25°C
Figure 3.6: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG20,000 in 10mM Tris buffer at 25°C at lower receiver gain levels
Figure 3.7: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG12,000 in 10mM Tris buffer at 25°C
Figure 3.8: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG12,000 in 10mM Tris/100 mM KCl solution at 25°C
Figure 3.9: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG12,000 in 10mM Tris buffer at 25°C at various number of transients (nt)
Figure 3.10: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 1.75wt% sample of F68 in 10mM Tris buffer at 25°C
Figure 3.11: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 1.75 wt% sample of F68 in 10mM Tris buffer at 25°C at various number of transients (nt)
Figure 3.12: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris buffer at 25°C
Figure 3.13: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 0.01wt% sample of F68 in 10mM Tris buffer at 25°C
Figure 3.14: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 1.75 wt%, 0.10 wt% and 0.01 wt% sample of F68 at 25°C for $\Delta=400$ ms.

Figure 3.15: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 in 10mM Tris buffer at 15°C.

Figure 3.16: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 in 10mM Tris buffer at 35°C.

Figure 3.17: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 at 15°C, 25°C and 35°C at $\Delta= 500$ ms.

Figure 3.18: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 in 10mM Tris/100mM KCl solution at 15°C.

Figure 3.19: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 in 10mM Tris/100mM KCl solution at 25°C.

Figure 3.20: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 in 10mM Tris/100mM KCl solution at 35°C.

Figure 3.21: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10 wt% sample of F68 in 10mM Tris/100mM KCl at 15°C, 25°C and 35°C at $\Delta= 500$ ms.

Figure 3.22: F68 Structure.

Figure 3.23: Mass spectrum of F68.

Figure 3.24: STE PFG $^1$H NMR spectra of magnetically aligned bicelles (q=4.5) in D$_2$O incorporating 0.4 mol% F68 as a function of increasing gradient pulse amplitude ($\Delta=100$ ms).

Figure 3.25: From left to right, $^1$H spectra of magnetically aligned bicelles at 30°C (q=4.5) in D$_2$O solution incorporating 0.4 mol% F68 without and with pre-saturation of the water resonance.

Figure 3.26: Semilogarithmic plot of STE-PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles with 1 mol% DMPG.

Figure 3.27: Schematic of the morphology changes undergone by neutral (DMPC/DHPC/F68) and charged (DMPG/DMPC/DHPC/F68) bicellar mixtures.

Figure 3.28: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles.

Figure 3.29: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles with 1 mol% DMPG and 100mM KCl.

Figure 3.30: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles with 100mM KCl.

Figure 3.31: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles with 5 mol% cholesterol.
Figure 3.32: The plot of the number of moles of bound F68 in vesicles with respect to moles free in solution based on PEO and PPO peaks versus the molar ratio of F68 added relative to POPC.
List of Symbols

c_L total lipid concentration
D diffusion coefficient
D’ mutual diffusion coefficient
D_\perp diffusion perpendicular to the bilayer normal
D_|| diffusion parallel to the bilayer normal
D_{zz} apparent diffusion coefficient
dC/dx solute- concentration gradient
f the frictional factor
g gradient pulse amplitude
G integer number of rotation periods
J flux of solute molecules per unit cross-section area
k_B the Boltzmann’s constant
P1 \pi/2 pulse in ODESSA pulse sequence
P2, P3 pulses in ODESSA pulse sequence that interconvert transverse and longitudinal polarization
q ratio of long chain: short chain phospholipid
r_{hl} hydrodynamic radius
t time
t_1 half of rotation period
T absolute temperature
T_1 longitudinal relaxation time
T_2 transverse relaxation time
T_m transition temperature
T_R spinning period
\eta medium viscosity
\Delta t change in time
\tau_1 second delay time
\tau_2 first and third delay time
\tau_m mixing period
\Delta diffusion time
\Delta_r reduced diffusion time
γ magnetogyric ratio
δ gradient pulse duration
θ angle between the bilayer normal and the direction of the applied magnetic field
List of Abbreviations

CHAPSO 3-(cholamidopropyl) dimethylammonio-2-hydroxy-1-propanesulfonate
CMC  Critical micelle concentration
DHPC 1,2-dihexanoyl-sn-glycero-3-phosphocholine
DLPC 1,2-dilauryl-sn-glycero-3-phosphocholine
DMPC 1,2-dimyrystoyl-sn-glycero-3-phosphocholine
DMPG 1,2-dimyrystoyl-sn-glycero-3-phospho-(1’-rac-glycerol)
DMPS 1,2-dimyrystoyl-sn-glycero-3-phospho-L-serine
DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
EO  ethylene oxide
ESI-MS  electrospray ionization mass spectrometry
EXSY  exchange spectroscopy
FCS  Fluorescence correlation spectroscopy
FID  free induction decay
FRAP  Fluorescence recovery after photobleaching
GPC  gel permeation chromatography
GPC/ESI-MS  gel permeation chromatography electrospray ionization mass spectrometry
HLB  hydrophilic-lipophilic balance
MAS  Magic angle spinning
NMR  Nuclear magnetic resonance
NOE  nuclear overhauser effect
NOESY  nuclear overhauser enhancement spectroscopy
ODESSA  one-dimensional exchange spectroscopy by sideband alternation
PEG  polyethylene glycol
PEO  poly(ethylene oxide)
PFG NMR  pulsed field gradient nuclear magnetic resonance
PFG STE  pulsed field gradient stimulated echo
PMT  photomultiplier
PO  propylene oxide
PPO  poly(propylene oxide)
rf  radio frequency
SEC  size exclusion chromatography
SPT single particle tracking
TIRF total internal reflection fluorescence
Chapter 1
Introduction

Biological membranes provide an environment required for many vital biochemical processes such as signalling, compartmentalization, hosting of enzyme complexes, and folding and the activity of proteins. A biological membrane consists of proteins and lipids. A lipid bilayer, one of the main components of a biological membrane, provides mechanical stability and low permeability to ions and large molecules. Singer and Nicholson in 1972 introduced the biological significance of the lateral mobility of the membrane constituents in their fluid mosaic model. According to the model, all membrane components can freely diffuse along the plane of the membrane and the rate of their diffusion determines the kinetics of the membrane-associated biochemical reactions.

The diffusion of molecules in the complex cellular membrane is affected by membrane inhomogeneities and interactions with cytoplasm. For deeper understanding of how membrane composition and structure influence the lateral mobility of its constituents, simple model membrane systems that can mimic real biological systems are necessary. The diffusion coefficients can be experimentally measured by a range of techniques and can also be theoretically determined by numerical simulations. This can help verify and refine the models of membrane structure on an atomic scale.

In this report, various techniques of measuring the diffusion coefficient of the constituents in these membranes are described as well as several models of biological membranes. Among several structures of self-assembled membrane lipids in water, bicelles are considered to be a clearly defined model biological membrane and have been used in various biophysical studies. Fluorescence recovery after photobleaching, fluorescence correlation spectroscopy and single particle tracking have been used to measure lipid and protein diffusion in membranes while nuclear magnetic resonance (NMR) techniques such as pulsed-field gradient spin echo NMR, magic angle spinning, and exchange spectroscopy have also been used in measuring the diffusion coefficient.
1.1 Lipids and Membranes

Phospholipids are one of the broad classes of lipids that are found in biological membranes. These compounds have a range of polar groups that are esterified to the phosphoric acid moiety of the molecule and they are called the “head” group. The long hydrocarbon fatty acid chains in these compounds are called the “tail” group. The nature of their fatty acid chains contribute significantly to the chemical and physical nature of these phospholipids and the membranes that they constitute.

An important characteristic of membrane lipids is their amphipathic character indicated by the polar or hydrophilic head group and the non-polar or hydrophobic group. These amphipathic lipids spontaneously form various molecular assemblies in aqueous media reducing the contact between the solution and hydrophobic chains. These polar head groups are positioned on the surface of the membranes where they are exposed to water while the non-polar regions are sequestered from water.

Monolayers at an air-water interface are arranged such that the lipid tails are in the air and the polar head is in the solution. Micelles bury the non-polar tail in the centre of a spherical structure, orienting the polar head group to the outside (Figure 1.1). A critical micelle concentration, CMC, characterizes the micelle formation of amphipathic molecules. At CMC and above, micelles form. When monolayers are stacked back-to-back, a bilayer is formed. When phospholipids are added to water, they form stable bilayers spontaneously. These bilayers can be very large in area ($10^8 \text{ nm}^2$) in contrast to micelles. Since the exposure of the edges of the bilayer to water is energetically highly unstable, they coil around themselves and form closed unilamellar or multilamellar vesicles. Unilamellar vesicles, also called liposomes, are formed when the phospholipids are arranged in a single lipid bilayer. The amphipathic character of phospholipids, the interaction of the polar group with water, and the burial of the hydrophobic chains inside govern the formation of these vesicles.
1.2 Bicelles

Among the molecular assemblies formed by phospholipids, bicelles are disc-shaped, bilayered mixed micelles bearing a membrane structure\(^2\-^5\). They are mainly composed of long-chain and short-chain phosphatidylcholines. One of the most common examples is the mixture of 1,2-dimyristoyl-\textit{sn}-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-\textit{sn}-glycero-3-phosphocholine (DHPC) (Figure 1.1). The long-chain phospholipid, DMPC, forms the central planar region of the bilayer while the short-chain phospholipid, DHPC forms the edge regions or the rim of the bicelle, shielding the hydrophobic tails of the long chain lipids from water\(^4\).

Bicelle size is dependent upon the molar ratio of long chain versus short chain phospholipid (q) (Figure 1.1) and the total phospholipid concentration (\(c_L\))^4. DMPC, the long chain phospholipid component, has been combined with other phospholipids that have identical chain lengths but different head groups such as dimyristoyl phosphatidylserine (DMPS) and dimyristoyl phosphatidylglycerol (DMPG). This can change the charge characteristics of the interface and provide flexibility in the composition of phospholipids\(^4\). In order to alter the total bilayer thickness, according to Whiles and coworkers\(^3\), bicelles can be prepared with dipalmitoyl phosphatidylcholine (DPPC) or dilauryl phosphatidylcholine (DLPC)^4. Other than DHPC, the rim of the bicelle can be prepared with a bile-salt derivative such as 3-(cholamidopropyl) dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPSO).

As the lipid molar ratio and the lipid concentration determine the size and properties of bicelles, a range of model phases can be formed in order to imitate a variety of diverse biological systems\(^4\). Bicelles form discoidal lipid aggregates whose combined mass of about 4080 kDa and whose diameters are typically 50 nm when the long- to short-chain ratio is low (q < 3) (Figure 1.1) and total lipid concentration \(c_L\) is between 15-25 % w/w\(^4\). For high q values, at temperatures above the transition temperature, bicelles have shown formation of edge-to-edge contacts while below the transition temperature, these bicelles show the standard bicelle morphology\(^5\).
Bicelles with q ratios greater than 2.5 tend to align spontaneously in the magnetic field such that their bilayer normals are orthogonal to the direction of the applied magnetic field at temperatures above the transition temperature of the long chain phospholipid ($T_m$) (Figure 1.2). The magnetic alignment can be assessed by $^{31}P$ NMR spectroscopy. In the $^{31}P$ NMR spectrum, (Figure 1.3) the presence of two well-resolved, narrow resonances characterizes magnetically aligned bicelles. The more intense lower frequency resonance at $-11.7$ ppm corresponds to DMPC while the less intense higher frequency resonance at $-3.5$ ppm corresponds to DHPC. The ratio of the integrated intensities of each peak matches up with their q- value. The magnetic alignment can be changed with normals parallel to the direction of the applied magnetic field.
(Figure 1.2) by doping the bicelles with paramagnetic ions, aromatic molecules, and some membrane proteins.\(^5\)

Figure 1.2: Magnetic alignment of bicelles with their bilayer normals orthogonal and parallel with respect to the applied magnetic field (Adapted from Sanders, C.R., Hare, B.J., Howard, K.P., Prestegard, J.H. (1994). *Prog. NMR Spectrosc.* 26, 421-444)\(^6\)
Figure 1.3: $^{31}$P NMR spectra of DMPC/DHPC ($q=4.5$) bicelles. This $^{31}$P spectrum was taken at 30°C. Bicelles yield two distinct narrow NMR resonances when magnetically aligned.

Bicelles form an unaligned phase when the amount of short chain lipids is increased ($q < 1$ and $c_L \sim 5$-15% w/w)$^4$. These bicelles have shown to be smaller in size (252 kDa and 8 nm) than their counterparts that can be aligned and they also contain the discoidal morphology with isolated lipid pools$^4$. They are stable over a range of phospholipid ratios ($q = 0.05$-0.5) and temperatures (15°C – 37°C). They can exist in a wide range of sample conditions and have a viscosity similar to the solution state of proteins, so they are also suitable for high-resolution NMR studies of proteins. Bicelles provide a model system in which the interaction between protein and membrane can be studied. The aligned-phase bicelles, $q > 2.5$, can be used in solid-state NMR studies to resolve the orientation of the protein and the phospholipids while the unaligned phase bicelles can be used to perform structural studies of proteins$^4$.$^7$-$^10$.

Bicelles are characterized by a structure that is in-between a lipid vesicle and a classical mixed micelle$^5$. In contrast to lipid vesicles, bicelles do not contain inner aqueous compartments and are often optically clear$^5$. Mixed micelles have very small planar surfaces compared to bicelles. Contrary to mixed micelles, bicelles preserve a bilayered area that can contribute to mimic several dynamic and conformational properties of liquid crystalline phase bilayers$^4$.$^5$. The planar core region of the bicelles, formed from long-chain phospholipids, tends to better resemble a fragment of natural membrane and tends to be a better membrane model than micelles to study membrane-associated biomolecules$^4$.

Bicelles have been used as a medium to reconstruct and characterize integral membrane proteins. It has been found that certain membrane proteins can be incorporated into bicelles preserving the native protein structure, the protein function, and the bicelle morphology while some membrane proteins can interact with bicelles, disrupt the morphology and perturb the native folding of the protein$^5$. Thus it is important to develop DMPC-based bicelle systems compatible with proteins by preparing them to closely resemble native membranes. While
bicelles have been widely used as membrane mimetics for a broad range of biophysical investigations, it must be noted that actual biomembranes are composed of phosphatidylcholines with unsaturated acyl chains as well as those with saturated ones\(^2\). The unsaturated C=C bond causes disorder in the composition of the phospholipid molecules in the membrane and thus changes the fluidity of the bilayered membrane. Therefore bicelles can be prepared similar to native membranes by using lipids with longer acyl chains, introducing some unsaturated chains, adding net negative charge on the bicellar mixtures and adding some cholesterol\(^5\).
1.3 Pluronic Triblock Copolymers

1.3.1 Properties and Uses of Pluronics

Pluronic triblock copolymers, also known as poloxamers, consist of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks arranged in A-B-A tri-block structure PEO-PPO-PEO\textsuperscript{13}. They are synthesized via step-wise anionic polymerization by sequential addition of propylene oxide (PO) and ethylene oxide (EO) monomers in the presence of alkaline catalyst, e.g. sodium or potassium hydroxide\textsuperscript{14}. The reaction is initiated from the PO block followed by the growth of EO chains at both ends of the PO block. This procedure is known to produce polymers with a relatively low polydispersity index\textsuperscript{14}.

These non-ionic triblock copolymers are commercially available in a number of different PEO and PPO chain lengths. Block copolymers with different numbers of EO and PO units can be characterized by their hydrophilic-lipophilic balance, HLB. Their molecular size, hydrophilicity and lipophilicity vary by the number of EO and PO units. When dissolved in water at concentrations above the CMC, these copolymers organize into micelles. The micellization of Pluronics in water is endothermic\textsuperscript{15}. The PPO blocks have lower solubility in water relative to the two PEO blocks; thus hydrophobic PPO blocks are separated from the aqueous exterior, and form the core of the micelles. The hydrophilic PEO chains form the exterior of the micelle. The PPO core of these micelles can be used for the storage of numerous water-insoluble drugs\textsuperscript{13}. The PEO blocks reduce unfavourable drug interactions with the surroundings. It has been reported that the incorporation of drugs into Pluronic micelles can improve the solubility and the stability of drugs\textsuperscript{13}.

The CMC of Pluronics is very sensitive to temperature. The CMC can change by 3 orders of magnitude when the temperature is changed by 20K\textsuperscript{15}. Pluronics in their monomeric form can be transitioned to micelles by increasing the temperature by 20K at constant concentration. According to Walderhaug et al\textsuperscript{14,16}, a commercial sample of Pluronic triblock copolymer F68 can transit from a fluid to a gel around 37°C at high concentrations (>20 wt%)\textsuperscript{6}. Pluronic F68 is a
triblock copolymer with 30 repeating units of propylene oxide flanked by 76 repeating units of ethylene oxide on both sides\textsuperscript{14}. The gelation process of the block copolymer, which is observed when warmed from ambient temperature to body temperature, involves the formation of a molecular network of interconnected micelles\textsuperscript{14,16}.

The high efficiency of Pluronics and their relatively low toxicity makes them ideal for applications in the fields of medicine and pharmacology\textsuperscript{17,18}. Due to their amphiphilic character, Pluronic triblock copolymers demonstrate surfactant properties such as ability to interact with hydrophobic surfaces and biological membranes\textsuperscript{13}. Their interactions with biological materials such as substrate-supported monolayers, phospholipid vesicles and liposomes have recently been investigated\textsuperscript{19}. Batrakova \textit{et al}\textsuperscript{13} have reported that Pluronic triblock copolymers incorporate into membranes and changed the microviscosity. The polymer induces a reduction in ATP levels in cancer cells and eliminates drug sequestration within cytoplasmic vesicles\textsuperscript{13}. Pluronics have potential as inexpensive and readily available substitutes for more expensive lipid-grafted polymers for drug delivery as well as for sealing damaged or permeabilized cell membranes after injury\textsuperscript{19}.

1.3.2 The Modes of Incorporation of Pluronics into the Lipid Bilayer

Pluronics are known to be incorporated readily into lipid bilayers\textsuperscript{17-25}. The introduction of Pluronic triblock copolymers in the lipid bilayer may cause interactions between the short hydrophobic PPO chains and the acyl chains of the phospholipids and thus create disruptions in the DMPC packing\textsuperscript{22}. The interaction between the Pluronic and the lipid bilayers can also result in a decrease in membrane microviscosity, an increase in the rate of lipid flip-flop, and alterations in the membrane size and shape\textsuperscript{19}. If the PPO block does not have a proper length, it will simply migrate into the membrane or adsorb on to the bilayer surface while the PEO blocks will remain on the membrane exterior. Increasing the PPO block length may allow the polymer to fully span the bilayer. However, in order to achieve the spanning conformation, the length of the PPO block must be near the dimensions of the acyl chain length of the lipid bilayer (Figure
1.4.A. The type of the relationship between the polymer and the model membrane (i.e. adsorption on the bilayer surface, partial insertion and full spanning of the bilayer) is determined by the hydrophobic PPO block length and the PEO chain length

Figure 1.4: Schematics of two possible conformations of Pluronic F68 in a lipid bilayer-bound state are given. (A) This represents the spanned conformation where F68 is incorporated into the membrane with the PPO block residing through the hydrophobic
region while leaving the PEO blocks dangling outside of the membrane. (Adapted from Soong. R.; Nieh. M.P.; Nicholson. E.; Katsaras. J.; Macdonald. P.M. (2010) *Langmuir*, 26(4), 2630-2638)²⁶ (B) This represents the U-conformation where PPO block may be incorporated in one leaflet of the bilayer while both the PEO blocks are dangling in solution to one side of the bilayer.

There are two main modes of incorporation of Pluronics into bilayers discussed in the literature¹⁹-²². First the hydrophobic PPO block may adopt a coil conformation in one leaflet of the bilayer while both the PEO blocks are dangling in solution to one side of the bilayer (U- (Figure 1.4.B) or V-conformation). When Pluronics are added to lipid bilayers during preparation, they may be incorporated into the membrane by sandwiching the PPO block within the hydrophobic region while leaving the PEO blocks pointed inside and outside of the membrane (spanned conformation) (Figure 1.4.A). According to Kastarelos and co-workers²⁰, the spanned conformation is unlikely as a mode of incorporation if Pluronics were added to preformed vesicles or membranes as the hydrophilic PEO block has to be taken through the hydrophobic region of the membrane²⁰,²². However the question of whether these Pluronics remains in one leaflet of bilayer in a U- or V-conformation or spans the bilayer is still an ongoing debate. Experiments have shown that depending on the method of preparation, the mode of incorporation tends to vary²⁰,²²,²³. Also a combination of these models may exist in a given sample²⁰. Measurements of the lateral diffusion coefficient of the Pluronic may answer the question because different diffusion rates may be observed for each conformation.
1.4 Molecular Diffusion

Self-diffusion can be described as the net result of the particles or molecules in solution undergoing a thermal motion-generated random-walk process\(^{27}\). Self-diffusion data provide information about molecular organization and phase structure. Self-diffusion rates are susceptible to structural changes and to binding and association phenomena especially for colloidal and macromolecular systems in solution\(^{27}\). Experimental self-diffusion coefficients are directly related to the lateral molecular displacement in the laboratory frame and require no additional analysis. Typical self-diffusion coefficients in liquid systems at room temperature range from about \(10^{-9}\) m\(^2\)s\(^{-1}\) for small molecules to \(10^{-12}\) m\(^2\)s\(^{-1}\) for large polymers\(^{27}\). The magnitude of diffusion coefficient can be specified by

\[
D = \frac{k_B T}{f}, \quad \text{(Equation 1)}
\]

where \(k_B\) represents the Boltzmann’s constant, \(T\) represents the absolute temperature and \(f\) represents the frictional factor. The \(f\) value can be given by the Stokes equation for a spherical particle of hydrodynamic radius \(r_H\) in a medium of viscosity \(\eta\):

\[
f = 6\pi \eta r_H. \quad \text{(Equation 2)}
\]

Equations 1 and 2 can be combined to obtain the Stokes- Einstein equation:

\[
D = \frac{k_B T}{6\pi \eta r H}. \quad \text{(Equation 3)}
\]

The mutual diffusion coefficient characterizes the relaxation of concentration gradients in a non-equilibrium two-component system according to Fick’s Law,

\[
J = -D’(dC/dx), \quad \text{(Equation 4)}
\]

where the flux of solute molecules per unit cross-section area is given by \(J\), the mutual diffusion coefficient is given by \(D’\), and the solute- concentration gradient is given by \(dC/dx\).
In a two-component system, there is one mutual diffusion coefficient and depending on the composition it may come close to either of the self-diffusion coefficients of the components. For the case of multi-component diffusion in non-equilibrium systems, there are $(n-1)^2$ different diffusion coefficients characterizing the relative fluxes of species in an n-component mixture\textsuperscript{27}.
1.4.1 Methods of Measuring Diffusion by Fluorescence

1.4.1.1 Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP) or (micro)photolysis is a fluorescence technology, nowadays widely used for measuring the translational diffusion coefficient of fluorescent molecules. The technique involves photobleaching fluorescent molecules in an area by a light beam and measuring the recovery of the fluorescence using a highly attenuated light beam as the surrounding unbleached areas diffuse into the bleached area\textsuperscript{28}. Based on the recovery of fluorescence in the bleached area, the diffusion coefficient can be obtained. Diffusion coefficient is equal to:

\[ D = \frac{r^2}{4\tau} \]  

(Equation 5)

where \( r \) is the radius of the circular beam and \( \tau \) is the time required for the bleached area to recover half of its original intensity\textsuperscript{29}.

A typical FRAP measuring instrument (Figure 1.5) requires a strong bleaching light source and an attenuated one for monitoring fluorescence before and during the fluorescence recovery process\textsuperscript{28}. A laser source is frequently used for bleaching while a less powerful laser light or light from a mercury lamp is used for monitoring\textsuperscript{28}. Ideally a single laser source is used as a bleaching and a monitoring source\textsuperscript{28}. This can be accomplished by adding a neutral density filter to control the intensity of the single laser source or by using a dual beam splitter to split the laser beam into a low and high intensity beam where a shutter interrupts the high intensity beam path and protects the photomultiplier (PMT)\textsuperscript{28}. The laser beam is aimed at the microscope and towards the sample, and the fluorescence intensity during recovery is detected either directly by a PMT signal or by analysis of camera images taken during recovery\textsuperscript{28}.

FRAP has become an important method of studying molecular mobility in biological samples and of studying diffusion in all kinds of environments such as polymer solutions, gels, and other matrices\textsuperscript{28,29}. In FRAP, samples do not have to be placed between membranes or
brought in contact with osmotically active solutions. Also one can study mobility and interactions in small, intact samples\textsuperscript{28}. FRAP has been effectively used to determine the translational mobility of various solutes in cytoplasm as well as the mobility of molecules in tissues\textsuperscript{28}. There has been widespread application of FRAP in studying the lateral diffusion in cell membranes as it has been established as a highly adaptable and sensitive technique\textsuperscript{28,29}. Therefore FRAP is used in research laboratories as well as pharmaceutical laboratories for research on mobility of macromolecular drugs, the mobility and binding of antitumor drugs, and the mobility of drugs in membranes prior to transmembrane penetration\textsuperscript{28}.

**Figure 1.5: A schematic diagram of a FRAP instrument (Adapted from Lustyik. G. (2001) Current Protocols in Cytometry, 2 (2) 12)\textsuperscript{30}.

FRAP allows for microscopic measurements of samples for details on molecular motions and interactions in a particular part of the sample. The speed of the experiments, their spatial and time resolution, and ability to measure \textit{in vivo} and \textit{in vitro} samples are some of their main advantages. One of the constraints of this technique is that it can only be used to measure the mobility of proteins and other constituents in the cell membranes when the observation times and length scales are fixed\textsuperscript{28}. Also the choice of fluorophore is influenced by the accessible excitation
source and hydrophilicity of the medium in which the fluorophore is to be dissolved; it is also a trade-off between its photostability and instability. Furthermore the use of high intensity light may cause some damage to the biological samples. Some of the events that can be caused by high illumination intensities are cross-linking of membrane proteins, loss of enzymatic activity and breakdown of the cell. Another concern related to FRAP is whether bleaching can lead to a local increase in the temperature which in turn can affect the mobility of the molecules.

1.4.1.2 Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a technique based on the statistical analysis of fluorescence intensity signal fluctuations detected from fluorophores in a thermodynamic system. FCS is usually used to determine chemical and photophysical rate constants, local concentrations, molecular aggregations, and most importantly, translational and rotational diffusion coefficients. FCS has been used to determine diffusion coefficients of molecules in planar systems including surfactant bilayers or monolayers on air-water or oil-water interfaces and biological membranes.

In confocal FCS, one or more parallel laser beams are typically used as excitation sources of the fluorescence microscope. As shown in Figure 1.6, the excitation beam is focused onto the diffraction-limited spot by a dichroic mirror. The confocal pinhole in the emission channel blocks any fluorescent light not originating from the focal regions providing tight axial resolution resulting in a small detection volume (~1 fL). Diffusion of fluorophores in the detection volume and photophysical reactions cause fluctuations in the emission which is focused onto the detector such as an avalanche photodiode or a photomultiplier tube with single photon sensitivity. Parameters of interest such as the diffusion coefficient or the concentration, can be obtained by fitting the experimental results onto a mathematical model function.
High single fluorophore sensitivity has made FCS an alternative to FRAP which requires high fluorophore concentrations. Compared to FRAP, FCS requires a lower concentration of fluorescent probes and lower laser power. When compared to highly sensitive single molecules techniques such as single particle tracking (Section 1.4.1.3), FCS provides quick and reliable experimental readout without lengthy analysis of data. The sub-micrometer spatial resolution provided by FCS proves to be valuable in measuring intracellular mobility-related parameters. FCS also offers higher dynamic performance and increased sensitivity as a result of its temporal resolution being limited to a millisecond time scale.

In FCS, the detection volume has to be positioned with a vertical accuracy of about 100 nm; if not, the deviation of the laser can lead to an unnecessary enlargement of the detection
area\textsuperscript{32}. Optical artefacts such as astigmatism, refractive index mismatch and saturation can alter the focal volume and obstruct the determination of the detection area, which is essential for quantitative measurements\textsuperscript{32}. Slow diffusion in membranes cause the fluorophores to occupy the detection volume longer, causing strong photobleaching that leads to apparent reduction in the measured diffusion times\textsuperscript{32}. Thus low power lasers have to be used in membrane FCS but this produces a weak signal that requires long measurement times. The physical properties of the membranes may be changed by heat production and electrochemical alterations of the lipids even when using moderate excitation lasers\textsuperscript{32}. Furthermore, special care must be taken when choosing a fluorophore for FCS experiments. Their ability to partition into the lipid bilayer, high quantum efficiency, large absorption cross-section and photostability are some of the criteria that suitable fluorophores must meet for successful FCS experiments\textsuperscript{32}.

1.4.1.3 Single Particle Tracking

Computer-enhanced video microscopy is used in single particle tracking (SPT) to follow the motion of proteins or lipids on the plane of a membrane\textsuperscript{34,35}. Fluorophores such as organic dyes, quantum dots, or fluorescent proteins, and submicrometer particles such as polymer latex beads or colloidal gold coated with specific antibodies or ligands can be used as labels in the molecules of interest\textsuperscript{36}. Individual molecules or small clusters are observed with a typical spatial resolution of tens of nanometers and average time resolution of tens of milliseconds. In SPT, the diffusion coefficient $D$ may be measured from the trajectory of an individual particle\textsuperscript{36}. Monte Carlo calculations are used to examine the statistical distribution of single-trajectory diffusion coefficients. These distributions can assess the heterogeneity of the membrane as well as any obstacles that may hinder the diffusion in the membrane\textsuperscript{36}.

Particles are imaged in bright field mode with enhanced contrast and launched onto a video camera although total internal reflection fluorescence (TIRF) microscopy can be used since only a thin plane of the sample is illuminated\textsuperscript{35}. This reduces the background fluorescence and allows for the single particles to be detected in the membrane. The video images are processed, recorded in real time and the videos are analyzed digitally\textsuperscript{35}. In every image of the
movie, the \( x,y \) positions of each single-particle trajectory is determined and the displacements at \( \Delta t \) time intervals are calculated. Depending on the type of motion, the mean square displacement can be related to the diffusion coefficient through different models\(^\text{35}\).

SPT can detect deviations from Brownian diffusion such as confined diffusion, anomalous diffusion or directed flow of particles\(^\text{35}\). A major advantage of SPT is that it has the ability to determine modes of motion in individual molecules. The spatial resolution is several orders of magnitudes higher than FRAP so that motion in small domains can be characterized with adequate time resolution. While FRAP averages over thousands of diffusing molecules, SPT measures individual trajectories and can resolve various subpopulations that are hard to differentiate by FRAP. In SPT, for each membrane component, definitive specificity in the measurement of motion can be obtained\(^\text{34}\).
1.4.2 Methods of Measuring Diffusion by NMR

Nuclear magnetic resonance has become a unique means for the study of molecular dynamics in chemical and biological systems. The two main methods of measuring the self-diffusion coefficients by NMR are the analysis of relaxation data and pulsed field gradient NMR (PFG NMR)\textsuperscript{37}. In solution state, relaxation measurements are susceptible to motions happening in the picosecond to nanosecond time scale while in PFG measurements, motion is detected over millisecond to second time scale\textsuperscript{37}.

1.4.2.1 Pulsed-Field Gradient Stimulated Echo Measurements

Pulsed field gradient nuclear magnetic resonance provides a rapid and non-invasive method of measuring translational motion\textsuperscript{37}. A technical requirement is that the species of interest gives a narrow resonance that is able to withstand long delays in the pulse sequence. The experiment involves imposing a pulsed linear gradient of magnetic field across the sample such that the frequency of the nuclear spin resonance becomes transiently position-dependent.

Figure 1.7: The basic Stejskal-Tanner pulsed field-gradient pulse sequence (Adapted from Stilbs. P. (1987). *Prog NMR Spectrosc.*, 19, 1-45)\textsuperscript{27,38}.
The basic 90°-180° echo experiment was originated by Carr and Purcell\textsuperscript{39} and is named the Hahn echo sequence. Stejskal and Tanner\textsuperscript{40} improved the static field gradient spin echo experiment (Figure 1.7) in the mid sixties in the form of the pulsed field gradient technique\textsuperscript{27}. The magnetic field remains largely homogeneous during the experiment. The dispersion and refocusing of spins occur in two identical field gradient pulses. To separate the echo attenuation due to diffusion from that due to the transverse relaxation, the experiment is conducted with fixed intervals between the radio frequency (rf) pulses\textsuperscript{27}. The spin echo is detected in a relatively homogeneous magnetic field hence there is no need for very rapid, broadband electronic circuitry in the spectrometer.

![Figure 1.8: Pulse sequence for a diffusion experiment using stimulated echo and pulsed field gradient (PFG STE) (Adapted from Soong. R., Macdonald. P.M. (2007). Biochimica et Biophysica Acta, 1768, 1805-1814)\textsuperscript{42,43}.](image)

Hahn demonstrated that when three 90° rf pulses are applied to a spin system in thermal equilibrium, there are as many as five spin echoes can result\textsuperscript{41}. The stimulated echo occurs during the interval after the third rf pulse (Figure 1.8) which happens to be the same duration as the interval between the first two rf pulses\textsuperscript{41}. According to Hahn, the relaxation attenuation of the stimulated echo has a $T_1$-dependence during the second and third rf pulses while the Hahn echo and other echoes attenuate according to $T_2$. For example, when chemical exchange occurs, the apparent $T_2$ may become much shorter than $T_1$ and then it is more valuable to use the stimulated echo technique than the 90°-180° echo technique\textsuperscript{27}. In the situations where $T_1 > T_2$, longer $\Delta = \tau_1$.
+ τ₂ can be used in diffusion measurements since Δ is limited by T₁. This allows for measurements in slower diffusion⁴²,⁴³.

The pulse sequence for the stimulated echo pulsed field gradient experiment is given in Figure 1.8. The first 90° rf pulse flips the magnetization onto the xy-plane. The spins then precess with their precessing axis on the xy-plane, while attaining a range of phase angles and losing phase coherence. The second 90° pulse at time t = τ₂, stores the current phase angles of the spins in the z-direction where they are not affected by the field gradients and relax in the longitudinal direction. The third 90° pulse at time t = τ₂ + τ₁ restores the phase angles with reversed sign so that they can precess to form an echo during the second τ₂ interval. During the first delay time τ₂, as shown in Figure 1.8, a gradient pulse of amplitude g and duration δ encodes the spins according to their position along the direction of the applied magnetic field gradient, i.e. along the z-direction. This magnetization is stored along the z-direction during the second delay time, τ₁, when longer diffusion times Δ are allowed by taking advantage of T₁ being greater than T₂. The second gradient pulse during the third delay time τ₂ decodes the position along the direction of the applied gradient accordingly. The stimulated echo intensity that results during the third delay time τ₂ decreases with increasing diffusion during the time Δ⁴².

\[ I = I_o \exp \left( -\frac{2\tau_2}{T_2} \right) \exp \left( -\frac{\tau_1}{T_1} \right) \exp \left[ -D \left( \gamma g \delta \right)^2 \left( \Delta - \delta/3 \right) \right] \] (Equation 6)

The echo intensity of the PFG NMR decays according to Equation 6. D gives the isotropic diffusion coefficient, Δ is the experimental diffusion time, γ represents the magnetogyreric ratio, and T₁ and T₂ are the longitudinal and the transverse relaxation times respectively. Either the gradient pulse amplitude g or duration δ can be incremented for extracting the diffusion coefficient D from the echo.

For a molecule incorporated inside a lipid bilayer, the diffusion coefficient becomes anisotropic and the diffusion can be described by a diffusion tensor having tensor elements D⊥ and D∥. These tensor elements D⊥ and D∥ correspond to diffusion perpendicular and parallel to the bilayer normal. PFG NMR measures the effective diffusion coefficient as a function of the
relative orientation of the applied gradient and the normal to the membrane. Then the measured diffusion coefficient becomes

\[ D_{zz} = D_{\perp} \sin^2 \theta + D_{\parallel} \cos^2 \theta \]  (Equation 7)

Where \( \theta \) is the angle between the bilayer normal and the direction of the applied field gradient.

When the bicelles are aligned in the magnetic field with their bilayer normal perpendicular to the direction of the external magnetic field, and when the field gradient is applied along the \( z \)-direction parallel to \( B_0 \), \( \theta \) becomes 90°, \( \sin^2 \theta \) becomes 1, and \( \cos^2 \theta \) becomes 0. Then Equation 6 becomes \( D_{zz} = D_{\perp} \) which correspond to the lateral diffusion within the bilayer. Using PFG NMR, the lateral diffusion of molecules in the plane of the lipid bilayer can be measured.

### 1.4.2.2 \(^1\)H Magic Angle Spinning

Spinning the samples at frequencies in the kilohertz (kHz) range around an axis that is tilted at an angle of 54.7° with respect to the magnetic field is called magic angle spinning (MAS). In the 1980s, several laboratories reported that MAS on lipid membranes generate well-resolved NMR spectra of lipids. The MAS technique eliminates the line broadening of lipid resonances caused by residual dipolar couplings and/or chemical shift anisotropy, and variations in bulk magnetic susceptibility. Dipolar coupling has a strong angular dependence in the form of \( (3 \cos^2 \theta - 1) \) where \( \theta \) is the angle between the dipolar-coupled spins and the static magnetic field. Thus spinning the sample at 54.7°, magic angle, to the static magnetic field apparently eliminates the dipolar couplings \( (3 \cos^2 \theta - 1 = 0) \).

Semi-solid membrane samples are loaded in cylindrical volumes of Teflon inserts inside MAS rotors. MAS at moderate spinning frequencies reduces the line-width of lipid proton resonances to 3-20 Hz from several kHz values. A very stable magnetic field is needed to achieve narrow resonance line-widths in a long experiment such as in multidimensional experiments. It is best to use higher spinning frequencies although sufficient resolution may be achieved even at lower frequencies. Spinning at frequencies of 10 kHz or higher gives negligible
intensities of spinning sidebands for all lipid signals thus simplifying the data analysis\textsuperscript{46}. However higher spinning frequencies can result in heating of sample temperature, which requires the supply of a very cold gas to the rotor bearings.

By implementing a gradient coil in a high-resolution MAS probe, Maas \textit{et al}\textsuperscript{47} in 1996 introduced gradient enhanced NMR spectroscopy\textsuperscript{48}. According to Maas \textit{et al}, the magnetic field gradient coil is aligned along the spinner axis that is oriented along the magic angle. This avoids rotational averaging of the magnetic field gradient and ensures that rotating, non-diffusing spins experience the same gradient strength\textsuperscript{48}. Gradient coils in the MAS probe allow for PFG experiments to be conducted on semi-solid samples. PFG MAS experiments are advantageous in determining the diffusion coefficients of samples in small amounts, while the effects of dipolar fields and line broadening due to chemical shift anisotropy, are reduced\textsuperscript{48}.

\textsuperscript{1}H MAS NMR can be combined with PFG techniques to select and suppress signals\textsuperscript{49} or to study the diffusion of semi-solid samples that have short relaxation times\textsuperscript{50}. The NMR signals of fast diffusing components can be selectively attenuated to simplify spectral interpretation\textsuperscript{45}. To attenuate signals with short T\textsubscript{2} relaxation times, PFG diffusion weighting and spin-echo sequences can be combined to form an experiment called spin-echo enhanced diffusion filtered spectroscopy to select just the signals that show slower self-diffusion rates\textsuperscript{45}.

\subsection{1.4.2.3 Exchange Spectroscopy}

When the equilibrium population of dipolar-coupled spins are perturbed, the resonance intensities are modulated depending on the durations of their interaction and the through space distance due to the so-called nuclear Overhauser effect (NOE)\textsuperscript{51}. Meanwhile, species that exchange molecular structures within the time scale comparable to the longitudinal relaxation time, typically show two separate resonances with different chemical shifts and intensities, both of which are also dependent on the observation time in the experiment. These two phenomena can be detected simultaneously by a single 2D NMR experiment\textsuperscript{51}. A nuclear Overhauser enhancement spectroscopy (NOESY) experiment then provides cross-peaks due to NOE and
cross-peaks between mutually exchanging positions\textsuperscript{51}. A modified NOESY pulse sequence has been established to suppress the diagonal signals, which has enabled the observation of the NOEs between nuclei having similar chemical shifts\textsuperscript{52}. The same modified NOESY pulse sequence can also be used to study the dynamic or chemical exchange processes, in which case it is called Exchange spectroscopy (EXSY) experiment.

![Figure 1.9: The basic pulse sequence of an EXSY experiment (Adapted from Jeener. J., Meier. B.H., Bachmann. P., Ernst. R.R. (1979). J. Chem. Phys., 71(11), 4546-4553.)\textsuperscript{53}]

During the preparation period (Figure 1.9), the spin system is placed in the desired starting conditions. Then the spins evolve at their characteristic frequencies during the evolution period, $t_1$\textsuperscript{53}. After the second rf pulse, information between spins are transferred during the mixing period, $\tau_m$. Lastly during the detection period, the receiver is turned on and the free induction decay (FID) is detected and stored. At short mixing times, the resulting 2D spectrum provides a qualitative map of the kinetic matrix of an exchange process\textsuperscript{53}. The EXSY experiment can be used not only to explain the qualitative aspects of exchange, but also to plot complex exchange networks, and clarify exchange mechanisms\textsuperscript{51}. It has also been used to quantitatively determine the rate constants derived from the computational analysis of diagonal- and cross-peak intensities or collecting EXSY spectra over a range of mixing times\textsuperscript{51}.

EXSY can be used to track the diffusion-mediated changes in molecular orientation\textsuperscript{43}. The presence of an anisotropic interaction such as chemical shift, or dipolar interaction can be used to measure the translational diffusion. In this method, the resonance frequency is related to
the molecular orientation with regard to the direction of the magnetic field\textsuperscript{43}. Lateral diffusion of molecules around the radius of curvature of a spherical lipid vesicle alters the molecular orientation with respect to the magnetic field allowing EXSY data to provide lateral diffusion coefficients of the molecules\textsuperscript{43}.

1.4.2.4 One-Dimensional Exchange Spectroscopy by Sideband Alternation

Slow molecular reorientation or spin exchange of chemically equivalent nuclei can be detected by a one-dimensional magic angle spinning (1D MAS) experiment called one-dimensional exchange spectroscopy by sideband alternation, acronymed as ODESSA\textsuperscript{54,55}. In ODESSA, the experiment achieves the resolution by slow magic angle spinning so that the powder pattern corresponding to all the chemical sites gets broken into a series of sidebands to obtain the signals from each site\textsuperscript{54}.

In the ODESSA pulse sequence (Figure 1.10), the preparation period or the variable delay, $t_2$, is fixed as a half of a rotation period, $t_2 = T_R/2$ where $T_R$ is the spinning period. It is followed by a mixing time, $\tau_m$, corresponding to a G integer multiples of the rotation period, $\tau_m = G T_R$, and a detection period $t$\textsuperscript{55}. As shown in Figure 1.10 below, P1 is a $\pi/2$ pulse or cross polarization, and P2 and P3 pulses exchange transverse and longitudinal polarization\textsuperscript{55}. The $t_2$ delay creates magnetization related to each spinning sideband to be polarized in different directions by the mixing time, $\tau_m$. During the mixing time, dynamic processes redistribute the polarization between several sidebands that lead to a $\tau_m$-dependent MAS spectrum from which the kinetic parameters for the dynamics processes can be derived\textsuperscript{54,55}. 
ODESSA provides a faster means by which motional correlation times can be obtained by simple exponential fitting of integrated peak intensities as a function of mixing time\(^5\). These motional correlation times can be related to various rate coefficients. By means of a 1D experiment, ODESSA provides information on exchange between equivalent nuclei in the solid state\(^5\). The ODESSA technique can be performed accurately if the experiment is conducted on-resonance, and only one isotropic chemical shift (and consequently only one series of sidebands) is present\(^5\). The spinning rate must be kept faster than the dynamic process to acquire adequate number of kinetic points, and it must be kept slower than the overall magnetic anisotropy for observable sidebands\(^5\).
1.5 Goals of this Research

Prepared in this project are neutral, negatively charged and charge-shielded bicellar mixtures incorporating Pluronic F68. These bicellar mixtures spontaneously align in the magnetic field of the NMR magnet. DMPC/DHPC bicelles, which have a net negative magnetic susceptibility anisotropy, align in a magnetic field such that the normal to the plane of the lipid bilayer becomes perpendicular to the direction of the magnetic field.

Pluronic F68 was chosen as a reporter polymer since it has an amphiphilic character and is known to interact with hydrophobic surfaces and biological membranes. It is soluble in water and is commercially available at a low cost. The length of the PPO block of F68 is sufficient to span the acyl chain region of the lipid bilayer. By measuring the lateral diffusion of F68 incorporated in the bicellar mixtures, one should be able to answer the question whether the Pluronic is in the U- or V-conformation or the spanned conformation in the lipid bilayer.

In this study, PFG NMR diffusion method was employed because the measurement is rapid and non-invasive. First, the diffusion coefficient of polyethylene glycol (PEG), a homo-polymer composed only of ethylene oxide units, PEG 20,000, was determined. Second, the same lateral diffusion of the homo-polymer with smaller molecular weight (PEG 12,000) was examined to compare the difference due to the molecular weights. Next, the diffusion of F68 free in solution was measured as a blank experiment. Lastly, the F68 diffusion in the magnetically aligned bicelles was studied. The temperature was varied from 15°C to 35°C which corresponds to the temperature range where the annealing procedure was conducted on the bicelles to promote the magnetic alignment. In addition, the influence of salt content in the sample was examined. Furthermore, the influence of bicelle morphology on the apparent diffusion coefficients of F68 was examined by changing bicelle compositions.
Chapter 2
Experimental Section

2.1 Materials

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Alabaster, AL. Pluronic F68, PEG samples as well as all other biochemicals and reagents were purchased form Sigma-Aldrich, Oakville, ON, Canada. All phospholipids and chemicals were used without further purification.

2.2 Preparation of bicelles

Neutral bicellar mixtures were prepared at a lipid content of 25 wt% in D$_2$O solution (10 mM Tris, pH 7.4). They were composed of DMPC, DHPC, and Pluronic F68 ([DMPC]/[DHPC] = 4.5 and 0.4 mol % F68 relative to DMPC). Negatively charged bicellar mixtures contained an additional DMPG at 1 mol% relative to DMPC. The samples were buffered in a solution of 10 mM Tris, 100 mM KCl, pH 7.4. Two samples were made at different cholesterol contents of 5 and 10 mol% relative to DMPC. The desired quantities of DMPC, DHPC, DMPG, cholesterol and F68 were dissolved in the appropriate volume of D$_2$O solution. This was followed by a few cycles of freezing, thawing and gentle vortexing until a clear solution was obtained. All bicellar samples were then stored at 4 °C for about 24-48 hours before NMR experiments.
<table>
<thead>
<tr>
<th>Bicelle Composition</th>
<th>q-value</th>
<th>Aqueous Component</th>
<th>F68 Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC/DHPC</td>
<td>3.5</td>
<td>10 mM Tris, pH 7.4</td>
<td>0.4 mol% relative to DMPC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Tris, 100 mM KCl pH 7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>10 mM Tris, pH 7.4</td>
<td>0.4 mol% relative to DMPC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Tris, 100 mM KCl pH 7.4</td>
<td></td>
</tr>
<tr>
<td>DMPC/DHPC/DMPG</td>
<td>3.5</td>
<td>10 mM Tris, pH 7.4</td>
<td>0.4 mol% relative to DMPC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Tris, 100 mM KCl pH 7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>10 mM Tris, pH 7.4</td>
<td>0.4 mol% relative to DMPC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Tris, 100 mM KCl pH 7.4</td>
<td></td>
</tr>
<tr>
<td>DMPC/DHPC / cholesterol</td>
<td>4.5</td>
<td>10 mM Tris, pH 7.4</td>
<td>0.4 mol% relative to DMPC</td>
</tr>
</tbody>
</table>

Table 2.1: A summary table of sample compositions used in the project.

2.3 Binding Assay

Multilamellar vesicles (MLVs) were prepared at 20 mg lipid / mL in 10 mM Tris, pH 7.4 solution. Each sample in the assay was composed of varying amounts of Pluronic F68 at 0 mol%, 0.004 mol%, 0.01 mol%, 0.04 mol%, 0.1 mol% and 0.4 mol% relative to POPC. The desired quantities of POPC and F68 were dissolved in the appropriate volume of buffer solution. This was followed by a few cycles of freezing, thawing and gentle vortexing until a homogeneous solution was obtained. The samples were centrifuged at 8000 rpm for 5 minutes. The supernatant was removed and kept for further analysis while the residue left was re-suspended in buffer solution and centrifuged at 8000 rpm for 5 minutes. The supernatant was discarded and the residue left was dissolved in D$_2$O. As a chemical-shift reference 1 µL of 100 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) was added to each sample. The $^1$H NMR spectra of Pluronic F68 bound MLV samples and the first supernatant samples were obtained.
2.4 NMR spectroscopy

All NMR spectra were recorded on a Varian Infinity 500 MHz NMR spectrometer using a Varian 5mm switchable broadband liquid probe equipped with a gradient coil along the z-direction.

2.4.1 Magnetic Alignment of Bicelles

The bicellar mixtures were transferred into a 5 mm NMR tube at 4 °C and placed in the probe-head set in the bore of an 11.7 T superconducting magnet. The sample temperature was then raised to 35 °C. An annealing procedure was performed to enhance the magnetic alignment of the sample, which involved repetitive cycles of the temperature between 20 and 35 °C with 10 minutes of equilibration at each temperature.

2.4.2 $^{31}$P NMR

The degree of the magnetic alignment was evaluated by $^{31}$P NMR spectroscopy. $^{31}$P NMR spectra were recorded at 202.31 MHz using a single pulse excitation, quadrature detection, complete phase cycling of the pulses, and WALTZ proton decoupling during the signal acquisition. Typical acquisition parameters are as follows: a 90° pulse length of 9.5 µs, a recycle delay of 10 s, a spectral width of 40 kHz, and an 8K data size. The spectra were processed with an exponential multiplication equivalent to 50 Hz line broadening prior to Fourier transformation. Once the magnetic alignment was deemed acceptable, the sample temperature was kept at 30 °C.

2.4.3 Proton NMR

$^1$H NMR spectra were recorded at 499.788 MHz. Typical acquisition parameters were as follows: a 90° pulse length of 20.5 µs, a recycle delay of 5 s, a spectral width of 10 kHz, and a 2-K data size. Pre-saturation irradiation pulse of 600 ms at saturation power of 28 was applied to suppress HDO resonance before pulsing and data acquisition. The transmitter offset was set at
the water resonance frequency. The spectra were processed with an exponential multiplication equivalent to 2 Hz line broadening prior to Fourier transformation, and were referenced to DSS.

### 2.4.4 Inversion Recovery Experiments

Proton T$_1$ relaxation times were measured using a conventional inversion recovery sequence followed by double gradient echo for water suppression$^{57}$. The transmitter offset was set at the water resonance frequency and the solvent peak was suppressed.

### 2.4.5 Measurement of Lateral Diffusion Coefficient

$^1$H NMR diffusion experiments were recorded at 499.78 MHz using the stimulated echo (STE) pulsed field gradient (PFG) procedure$^{41}$, 90°-τ$_2$-90°-τ$_1$-90°-τ$_2$-echo. The radio frequency pulses were phase cycled to cancel out unwanted echoes in the NMR experiment$^{58}$. Typical acquisition parameters are as follows: a 90° pulse length of 20.5 μs, a recycle delay of 5 s, a spectral width of 10 kHz, and a 4K data size. The field gradient pulses were applied along the longitudinal (z) direction. The gradient pulses were of duration δ and of strength g. The amplitude of the gradient pulses was incremented while their duration was kept constant at 5 ms. The diffusion time, i.e. the separation between the two gradient pulses, was $\Delta = \tau_1 + \tau_2$ and the reduced (effective) diffusion time was $\Delta_r = \Delta - \delta/3$. The reduced diffusion time, $\Delta_r$, was set between 50 and 500 ms. A pre-saturation irradiation pulse of 600 ms at saturation power of 28 was applied before pulsing and data acquisition. The transmitter offset was set at the water resonance frequency. The spectra were processed with an exponential multiplication equivalent to 2 Hz line broadening prior to Fourier transformation.

### 2.4.6 Data Processing

All NMR data were processed using MestReC NMR processing software (Mestrelab Research, Escondido, CA). $^1$H NMR spectra were processed using Fourier transform in MestReC with line broadening of 2 Hz.
Chapter 3
Results and Discussion

3.1 $^{31}$P NMR Assessment of Bicelle Magnetic Alignment

It is important to ensure the quality of magnetic alignment of the bicelles before starting the diffusion measurements. One can use either $^{31}$P or $^2$H NMR to assess the quality of the alignment. $^{31}$P NMR is a suitable means of measuring the quality and direction of magnetic alignment of bicelles as phospholipids require no specific labelling to facilitate the acquisition of $^{31}$P NMR spectra.

The $^{31}$P spectra were obtained by placing the bicellar mixtures in the magnet of the NMR spectrometer and a typical result obtained is shown in Figure 3.1. In the absence of paramagnetic ions, bicelles have a negative magnetic susceptibility anisotropy and thus align magnetically with the normal of the planar bilayer oriented at 90° relative to the direction of the magnetic field. As shown in the $^{31}$P NMR spectrum for the neutral bicellar sample at 30°C, (Figure 3.1) the presence of two well-resolved, narrow resonances is characteristic of magnetically aligned bicelles. The more intense lower frequency resonance at -12.49 ppm corresponds to DMPC while the less intense higher frequency resonance at -4.05 ppm corresponds to DHPC. The ratio of the integrated intensities of each peak represents their q-value; in this case it is 4.5. The $^{31}$P peak for DMPG in the negatively charged bicellar sample is barely seen on the spectrum at around -8 ppm (Figure 3.2). This is due to the small amount (1 mol% relative to DMPC) of DMPG that was added to the sample and the chemical shift of DMPG being closer to DMPC resonance. The $^{31}$P chemical shifts for DHPC, DMPG and DMPC for all four bicellar samples of q = 3.5 are given in Table 3.1.
Figure 3.1: The $^{31}$P NMR spectrum for the neutral bicellar sample composed of DMPC + DHPC at $q = 4.5$ of 25 w/w% lipid/water acquired at 30°C. The chemical shifts are listed in Table 1.

Figure 3.2: The $^{31}$P NMR spectrum for the negatively charged bicellar sample composed of DMPC + DHPC with 1 mol% DMPG at $q = 4.5$ of 25 w/w% lipid/water acquired at 30°C. The chemical shifts are listed in Table 1.
When the q value is increased from 3.5 to 4.5, the $^{31}$P NMR resonances of DMPC, DMPG and DHPC shift to lower frequencies (Tables 3.1 and 3.2). The decrease of DMPC chemical shift is attributed to the increase in the bicelle order parameter $S_{bicelle}$ where increasing q value or rather decreasing the amount of DHPC increases the bicelle ordering$^{59}$. The DHPC chemical shift represents the fraction of DHPC present within the planar bilayer regions relative to the fraction of DHPC present within the curvy regions$^{59,60}$. This shift in the $^{31}$P resonances to lower frequencies can be due to the affinity of DHPC to move into the planar region of the bicelle and become miscible with DMPC$^{59}$.

<table>
<thead>
<tr>
<th>Components</th>
<th>DHPC (ppm)</th>
<th>DMPG (ppm)</th>
<th>DMPC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC+DHPC+DMPG+F68</td>
<td>-2.878</td>
<td>-7.045</td>
<td>-10.675</td>
</tr>
<tr>
<td>DMPC+DHPC+F68</td>
<td>-2.9843</td>
<td></td>
<td>-10.3212</td>
</tr>
<tr>
<td>DMPC+DHPC+DMPG+F68+salt</td>
<td>-2.45</td>
<td>Not resolved</td>
<td>-9.84</td>
</tr>
<tr>
<td>DMPC+DHPC+F68+salt</td>
<td>-2.9361</td>
<td></td>
<td>-10.5625</td>
</tr>
</tbody>
</table>

Table 3.1: The $^{31}$P chemical shifts of DHPC, DMPG, and DMPC peaks for the negatively charged and neutral bicellar mixtures (q = 3.5).

<table>
<thead>
<tr>
<th>Components</th>
<th>DHPC (ppm)</th>
<th>DMPG (ppm)</th>
<th>DMPC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC+DHPC+DMPG+F68</td>
<td>-3.4670</td>
<td>-7.9078</td>
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<td>DMPC+DHPC+F68</td>
<td>-4.0462</td>
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<td>-12.4933</td>
</tr>
<tr>
<td>DMPC+DHPC+DMPG+F68+salt</td>
<td>-4.2876</td>
<td>Not resolved</td>
<td>-12.7829</td>
</tr>
<tr>
<td>DMPC+DHPC+F68+salt</td>
<td>-3.9014</td>
<td></td>
<td>-12.445</td>
</tr>
</tbody>
</table>

Table 3.2: The $^{31}$P chemical shifts of DHPC, DMPG, and DMPC peaks for the negatively charged and neutral bicellar mixtures (q = 4.5).

The reduction of the amount of DHPC in the curved regions decreases the proportion of curved regions. This is because of the highly mobile DHPC in the curvy edge regions of the bicelle undergoes fast exchange between the planar region and the highly curved toroidal pore region of the bicelles while DMPC molecules stay confined in the planar region (Figure 3.3)$^{61}$. 
An effective ratio of planar-to-curved phospholipid populations, $q^*$, can be calculated (Table 3.3) from the observed chemical shifts of DHPC and DMPC by

$$q^* = \frac{(q + \omega^*)}{(1 - \omega^*)}$$  

(Equation 8)

where $q = [\text{DMPC}]/[\text{DHPC}]$ and $\omega^* = \omega_{\text{DHPC}}/\omega_{\text{DMPC}}$ where $\omega_{\text{DHPC}}$ and $\omega_{\text{DMPC}}$ correspond to observed chemical shifts of DHPC and DMPC respectively$^{50}$. The effective ratios of planar-to-curved phospholipid populations for negatively charged and neutral bicellar mixtures are listed in Table 3.3.

Figure 3.3: A schematic cross section of a DMPC/DHPC bicelle in the perforated lamellae morphology. The green phospholipids correspond to DMPC that forms the planar region while the red phospholipids correspond to DHPC in the curved toroidal pore regions of the lamellae (Adapted from Soong, R., Macdonald, P.M. (2005) *Biophys. J.*, 88, 255–268)$^{43}$. 
### Table 3.3: The effective ratios of planar-to-curved phospholipid populations, $q^*$, and $\omega^* = \omega_{\text{DHPC}}/\omega_{\text{DMPC}}$ based on the observed chemical shifts of DHPC and DMPC peaks for the negatively charged and neutral bicellar mixtures at 30°C.

<table>
<thead>
<tr>
<th>Components</th>
<th>$q=3.5$</th>
<th></th>
<th>$q=4.5$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC+DHPC+DMPG+F68</td>
<td>0.270</td>
<td>5.16</td>
<td>0.297</td>
<td>6.82</td>
</tr>
<tr>
<td>DMPC+DHPC+F68</td>
<td>0.289</td>
<td>5.33</td>
<td>0.324</td>
<td>7.13</td>
</tr>
<tr>
<td>DMPC+DHPC+DMPG+F68+salt</td>
<td>0.250</td>
<td>4.99</td>
<td>0.335</td>
<td>7.28</td>
</tr>
<tr>
<td>DMPC+DHPC+F68+salt</td>
<td>0.278</td>
<td>5.23</td>
<td>0.313</td>
<td>7.01</td>
</tr>
</tbody>
</table>

As Table 3.3 demonstrates, despite the composition of the bicellar mixture, the $q^*$ value increases as the $q$ value increases. This is indicative of the shift in the equilibrium distribution of DHPC moving away from the curved regions towards planar regions of the bicelle as $q$ value increases$^{59}$. The redistribution of DHPC from the curved regions of the bicelle may make the curved regions less stable and may alter the morphology from discoidal to chiral ribbons or perforated lamellae. However $^{31}P$ NMR alone is not able to provide the distinction between these morphologies. A combination of techniques such as small angle neutron scattering (SANS), polarized optical microscopy, cryo-EM, and diffusion NMR have shown that bicellar mixtures containing negatively charged amphiphiles such as DMPG have a perforated lamellae morphology while neutral bicellar mixture shows a interconnected ribbon morphology$^{59,62}$. 
3.2 Lateral Diffusion Measurements

3.2.1 Self-Diffusion of Polyethylene glycol 20,000

A diffusion NMR experiment was conducted on a sample of polyethylene glycol (PEG) standard 20,000. PEG 20,000 is a polymer made up of ethylene oxide monomers. This PFG NMR measurement was taken by varying the gradient amplitude or the g-value from equation 6, from 0.11 T/m to 3.45 T/m. Based on the STE PFG $^1$H NMR measurements taken, it is evident that the signal intensities of the PEO resonance of PEG 20,000 at 3.6 ppm decays rather slowly with increasing gradient amplitude while the water resonance decays extremely fast (Figure 3.4). Given its larger size, it is expected that the PEG 20,000 diffuses slowly while water molecules diffuse extremely fast.

![Figure 3.4: STE PFG $^1$H NMR spectra of PEG 20,000 in D$_2$O as a function of increasing gradient pulse amplitude ($\Delta=500$ ms)](image)

The diffusion coefficient can be obtained from the slope of the semilogarithmic plot of normalized integrated intensities versus $(g\gamma\delta)^2(\Delta-\delta/3)$. This plot should provide a straight line for simple Gaussian behaviour. When measurements were taken at various diffusion times, 50 ms ≤
Δ ≤ 500 ms and the semilogarithmic normalized intensities were plotted against the set of known constants in Equation 6, the diffusive intensity decays of PEG 20,000 were virtually linear and overlapped (Figure 3.5) indicating no diffusion time dependence and a single effective diffusion coefficient for each situation. The apparent diffusion coefficient was determined to be $5.26 \times 10^{-12}$ m$^2$s$^{-1}$. The determination of the diffusion coefficient of PEG 20,000 using PFG NMR from the straight line in the semilogarithmic plot of $I/I₀$ versus $(g\gamma\delta)^2(\Delta-\delta/3)$ demonstrates simple Gaussian diffusion behaviour.

![Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG20,000 in 10mM Tris buffer at 25°C.](image)

Figure 3.5: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG20,000 in 10mM Tris buffer at 25°C.

The absolute intensities at each gradient amplitude were extremely large at an order of magnitude of 5 to 6. At the longest diffusion time at the highest g-value, the PEG 20,000 had only decayed to about 20% of its original intensity. The diffusion decays were receiver gain independent as shown by the overlapping intensity decays in Figure 3.6 where STE PFG $^1$H NMR measurements taken at three different receiver gain values (gain = 8, 2, 0).
Figure 3.6: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG20,000 in 10mM Tris buffer at 25°C at lower receiver gain levels.
3.2.2 Self-Diffusion of Polyethylene glycol 12,000

In Figures 3.7 and 3.8, PEG 12,000, which is a smaller polymer in size, compared to PEG 20,000 shows faster diffusive decay compared to PEG 20,000. The plot of PEG12,000 is vastly different from that of PEG20,000. In these figures, the plot shows a curved decay as opposed to the linear decay that was expected. Linear diffusion decay represents one diffusion coefficient while the curved diffusion decay represents a broad range of diffusion coefficients. Based on the earliest data points the apparent diffusion coefficient of PEG 12,000 in Tris buffer was found to be approximately $5.39 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ and in the presence of salt the apparent diffusion coefficient was $6.23 \times 10^{-11} \text{ m}^2\text{s}^{-1}$. The diffusion coefficient of PEG 12,000 in Tris buffer solution is slightly faster than that in Tris buffer with 100 mM KCl solution. The presence of ions appears to make the polymer diffuse slower because of the increase in the solvent polarity and decrease in the polymer solubility.

![Figure 3.7: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG12,000 in 10mM Tris buffer at 25°C.](image-url)
Figure 3.8: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG12,000 in 10mM Tris/100 mM KCl solution at 25°C.

At the longest diffusion time and at the highest g-value, the PEO resonance of PEG 12,000 had decayed to more than 99% of its original intensity. The absolute intensities at these lowest data points were rather small at an order of magnitude of 2. Hence the reduced signal to noise at these increments could be the cause of the curved behaviour. There could also be polydispersity present in the PEG 12,000 polymer sample. The non-linearity of the gradient field and the heterogeneity of the $B_1$ field can also be factors contributing to the curvature in the diffusive decays. Since the PEG 20,000 sample showed linear decay under the same conditions, the non-linearity of the gradient field and the heterogeneity of the $B_1$ field could be eliminated as factors for the curvature. In order to correct the signal-to-noise issue, the number of transients collected was increased from 16 to 1600 where the 100-fold increase in the number of transients increases the signal-to-noise ratio by ten-fold. The resulting decays showed the same curvature and the delays were overlapped (Figure 3.9). Despite the ten-fold increase in the signal-to-noise ratio, the curvature was not eliminated. Thus it can be assumed that the curvature is not due to any signal-to-noise issues. Then the PEG 12,000 polymer sample must have high polydispersity.
present to give a range of diffusion coefficients that contribute to the curvature present in the diffusion decay.

Figure 3.9: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of PEG12,000 in 10mM Tris buffer at 25°C at 16 and 1600 transients (nt) for Δ= 500 ms.
3.2.3 Self-Diffusion of Pluronic F68 in aqueous solution

The diffusion coefficient of Pluronic triblock copolymer F68 at 1.75 wt% was measured at the same conditions as the PEG samples. At 1.75 wt% the Pluronic F68 is well below its critical micelle concentration and expected to behave in a monomeric form. A PFG NMR experiment was conducted on the 1.75 wt% sample of F68 by varying the gradient amplitude from 0.11 T/m to 3.45 T/m. The measurements were taken at various diffusion times, $50 \text{ ms} \leq \Delta \leq 500 \text{ ms}$ and the semilogarithmic normalized intensities were plotted against a set of known constants (Figure 3.10).

![Figure 3.10: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 1.75wt% sample of F68 in 10mM Tris buffer at 25°C.](image)

This sample also indicated similar diffusive decay as the PEG 12,000 sample in that there is significant curvature observed. The apparent diffusion coefficient of $6.36 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ was obtained from the early data points. Similar to PEG 12,000, to correct the signal to noise issue, the number of transients collected at $\Delta= 500 \text{ ms}$ was increased from 16 to 64 to 256. The observed decays all overlapped and remained at the same degree of curvature for each number of transients (Figure 3.11). Thus it can be assumed that the curvature is not due to any signal-to-noise issues.
Figure 3.11: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 1.75 wt% sample of F68 in 10mM Tris buffer at 25°C for Δ= 500 ms and at various number of transients (nt).

The persistent curvature may be due to several different diffusion coefficients. A fraction of the Pluronic F68 in the 1.75 wt% sample may become micellized and it would be diffusing slower as the micelles are larger than an individual Pluronic F68. The amount of Pluronic F68 was reduced to 0.1 wt% to reduce the amount of micellelized Pluronic F68 (Figure 3.12). Though the 0.1 wt% sample shows slightly faster decay than the 1.75 wt% sample, the curvature was still present. The amount of Pluronic F68 was further reduced to 0.01 wt% (Figure 3.13). As the amount of Pluronic F68 was reduced, the apparent diffusion coefficient kept increasing from $6.36 \times 10^{-11}$ m$^2$/s$^{-1}$ to $7.62 \times 10^{-11}$ m$^2$/s$^{-1}$ to $8.06 \times 10^{-11}$ m$^2$/s$^{-1}$ for 1.75 wt%, 0.10 wt% and 0.01 wt% respectively indicating a faster diffusion at lower amounts of Pluronic F68. The diffusion of a F68 micelle is slower than that of F68 in its monomeric form. As the amount of F68 decreases in each of the samples, the amount of F68 micelles present decreases and then the diffusion coefficient of the sample increases. The Pluronic sample also becomes less viscous as the concentration of F68 keeps decreasing. Then the polymer molecules will diffuse faster. The remaining curvature can then be a result of the high polydispersity present in the polymer sample. Though the apparent diffusion coefficient at early data points seem to a slightly different
from each other, in the Figure 3.14 it shows that the behaviour at the three concentrations may not be that different.

**Figure 3.12:** Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris buffer at 25°C.

**Figure 3.13:** Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 0.01wt% sample of F68 in 10mM Tris buffer at 25°C.
Figure 3.14: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 1.75wt%, 0.10wt% and 0.01wt% sample of F68 in 10mM Tris buffer at 25°C for \( \Delta = 400 \text{ms} \).

The 0.10 wt% Pluronic F68 sample in 10 mM Tris buffer was run at three different temperatures 15 °C (Figure 3.15), 25 °C (Figure 3.12), and 35 °C (Figure 3.16) to see if the critical micellization temperature had an impact on the curvature. Pluronic triblock copolymers are known to have cmc values that are sensitive to temperature. Changing the temperature by 20 °C is known to completely micellize the polymer from free polymer\textsuperscript{22}. The curvature was the least pronounced at the lowest temperature (Figure 3.15). The diffusion is also the slowest at the lower temperature (15 °C). As the temperature increases by 10 °C, the diffusion of F68 becomes faster (Figure 3.17) and the diffusion decays become highly curved (Figures 3.12 3.16). As the temperature was increased, the apparent diffusion coefficient kept increasing from \( 5.03 \times 10^{-11} \) m\(^2\)s\(^{-1} \) to \( 6.77 \times 10^{-11} \) m\(^2\)s\(^{-1} \) to \( 9.29 \times 10^{-11} \) m\(^2\)s\(^{-1} \) for 15 °C, 25 °C and 35 °C respectively indicating a faster diffusion at higher temperatures (Figure 3.17). Concentrated F68 solutions at 37 °C have been reported to form gels\textsuperscript{14,16}. There may be some gel formation at high temperatures even at the lower concentrations that were used. Thus Pluronics coexisting in gel and solution states at 35 °C can also give rise to the biexponential diffusion and hence the curvature. With increasing temperature, free polymer in solution becomes more hydrophilic and diffuses faster. At the same time the polymers in micelles increases slightly and contribute to the curvature.
Figure 3.15: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris buffer at 15°C.

Figure 3.16: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris buffer at 35°C.
Figure 3.17: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris buffer at 15°C, 25°C and 35°C at Δ= 500ms.

To observe the effect of salt on the diffusion of F68 free in solution, a 0.10 wt% F68 sample was prepared in 10 mM Tris/ 100 mM KCl solution. Diffusion measurements were taken of this sample at three different temperatures 15 °C (Figure 3.18), 25 °C (Figure 3.19), and 35 °C (Figure 3.20). Similar to the 0.10 wt% Pluronic F68 sample in 10 mM Tris buffer, this sample at 15 °C also showed the slowest diffusion and the least curvature. As the temperature increases by 10 °C, the diffusion of F68 in salt becomes faster (Figure 3.21) and the diffusion decays become highly curved (Figures 3.18 and 3.19). As the temperature was increased, the apparent diffusion coefficient kept increasing from $5.18 \times 10^{-11} \text{m}^2\text{s}^{-1}$ to $6.86 \times 10^{-11} \text{m}^2\text{s}^{-1}$ to $8.74 \times 10^{-11} \text{m}^2\text{s}^{-1}$ for 15 °C, 25 °C and 35 °C respectively indicating a faster diffusion at higher temperatures (Figure 3.21). When these decays were compared to those of the 0.10 wt% Pluronic F68 sample in 10 mM Tris buffer, the values fall within the error of each other. This indicates that the presence of KCl does not seem to favour micellization.
Figure 3.18: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris/100mM KCl solution at 15°C.

Figure 3.19: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris/100mM KCl solution at 25°C.
Figure 3.20: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris/100mM KCl solution at 35°C.

Figure 3.21: Semilogarithmic plot of normalized STE-PFG NMR intensity decays of a 0.10wt% sample of F68 in 10mM Tris/100mM KCl solution at 15°C, 25°C and 35°C at Δ=500ms.
In this report, Pluronic F68 was investigated at different number of transients, concentrations, temperatures, and with or without salt. The range of self-diffusion coefficients of F68 that was observed in solution was not altered with the increase in the signal-to-noise level. As the concentration of F68 in solution increases the concentration-induced micellization causes the diffusion decays to become highly curved. Pluronic F68 goes from being molecularly dispersed mostly as a free polymer at 0.01 wt% to being mostly micellized at 1.75 wt%. As the concentration decreases the mostly free polymer shows faster diffusion than the micellized F68 at higher concentrations. As the temperature increases, the diffusion of F68 becomes faster as the ratio of free polymer in solution become more hydrophilic while the amount of polymer micelles increases only slightly. The addition of salt does not favour micellization and causes similar diffusion compared to F68 free in solution. The persistent curvature in every case, even in the cases where there is little or no micellization at low concentration and low temperature is due to the polydispersity of the polymer.

Polymers are mixtures of molecules of different sizes. Synthetic polymers always contain a certain polydispersity that is usual from the polymerization process. Electrospray ionization mass spectrometry (ESI-MS) is a technique used to determine the molecular weight of polymers. A mass spectrum of a polymer sample represents the distribution of ions by mass of the sample. Pluronics are synthetically made polymers. They are polydisperse in their overall size and the ratio of the block sizes. The lengths of each PEO and PPO block tend to vary (Figure 3.22) corresponding to a range of overall molecular weights. For a monodisperse sample, there will be one peak in the mass spectrum representing the average molecular weight. The average molecular weight of F68 is reported to be between 8350- 8400 g/mol\textsuperscript{14}. There is however a range of molecular weights shown in the mass spectrum of F68 (Figure 3.30). The mass spectrum of F68 in Figure 3.30 shows one peak that falls between 8350-8400 g/mol at 8391 g/mol. The peak nearest to 8391 g/mol is at 8286 g/mol. This corresponds to one EO monomer and one PO monomer. The peaks with the highest relative abundance are at 8741 g/mol and 8898 g/mol. Their difference of 157 g/mol can correspond to one EO monomer and two PO monomers. Thus this mass spectrum of Pluronic F68 shows the high polydispersity of the copolymer.
It should be concluded that prior to using Pluronic F68 in NMR studies, it should be purified. Size exclusion chromatography (SEC) or gel permeation chromatography (GPC) is the most common method for purifying polymers and determining molecular weight distributions. GPC combined with ESI mass spectrometry (GPC/ESI-MS) can provide accurate information on chemical composition and molecular weight distribution. GPC/ESI-MS results in a decrease of the polydispersity of the polymer. Thus samples such as F68 with broad molecular weight distributions can have their polydispersity reduced by such methods.

Figure 3.22: Structure of F68

Figure 3.23: Mass spectrum of F68
3.2.3 Diffusion of Pluronic F68 Incorporated in Bicellar Mixtures

Negatively charged bicellar mixture was composed of DMPC, DHPC, and DMPG incorporating F68. After ensuring the magnetic alignment of the bicelles by $^3$P NMR, $^1$H STE PFG NMR diffusion measurements were taken of this optically clear sample at various diffusion times, $50 \text{ ms} \leq \Delta \leq 500 \text{ ms}$, at $30^\circ \text{C}$. Figure 3.24 shows a series of $^1$H STE PFG NMR spectra of magnetically aligned bicellar mixture ($q = 4.5$) containing 0.4 mol% F68 as a function of the gradient pulse amplitude $g$. There are three well-resolved resonances at 4.67 ppm, 3.66 ppm and 1.12 ppm correspond to HDO, F68 EO methylenes, and F68 PO methyls respectively. Resonances such as the DMPC choline methyl protons are not present in the $^1$H spectra as a result of their short $T_2$ time relative to the echo delay time $\tau_2$ used in the experiment.

Figure 3.24: STE PFG $^1$H NMR spectra of magnetically aligned bicelles ($q=4.5$) in D$_2$O incorporating 0.4 mol% F68 as a function of increasing gradient pulse amplitude ($\Delta=100 \text{ ms}$)
Even though the sample contains 75wt% water, the HDO peak is smaller compared to the EO and PO peaks due to the pre-saturation of the water peak. Comparison of the ordinary \(^1\)H spectrum and the water peak suppressed \(^1\)H spectrum of the magnetically aligned bicelles at 30°C (q=4.5) in D\(_2\)O solution incorporating 0.4 mol% F68 is shown in Figure 3.25. Despite its height, the HDO peak decays fast with increasing g while the two F68 peaks are decaying much more slowly. This indicated that F68 is a large molecule trapped within or between the lipid bilayers.

![Figure 3.25](image)

**Figure 3.25:** From left to right, \(^1\)H spectra of magnetically aligned bicelles at 30°C (q=4.5) in D\(_2\)O solution incorporating 0.4 mol% F68 without and with pre-saturation of the water resonance.

The semilogarithmic normalized intensities of the EO peak were plotted against a set of known constants, \((g\gamma\delta)^2(\Delta-\delta/3)\) (Figure 3.26). The EO and PO peaks both showed the same diffusion behaviour. The diffusion decays show some curvature but from the early linear data points the diffusion coefficient can obtained to be \(3.22\times10^{-11}\) m\(^2\)s\(^{-1}\). The diffusion coefficient of free F68 in solution at 30°C was found to be \(1.05\times10^{-10}\) m\(^2\)s\(^{-1}\). The diffusion coefficient of F68 incorporated in negatively charged bicellar mixture is less than a third of the value of the diffusion coefficient of free F68 in solution. This indicates that the F68 in the negatively charged bicellar mixture diffuses slower than free F68. This suggests that the incorporation of F68 in the bicelles may be in the spanned conformation. However when this apparent diffusion coefficient
is compared with that of a PEG with similar molecular weight trapped between the lamellae of negatively charged bicellar mixture\textsuperscript{62}, the diffusion coefficients appear to be similar. Thus it can then be assumed that the Pluronic may not be spanned in the bilayer rather confined in the lamellae of the negatively charged bicellar mixture.

![Figure 3.26: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of F68 incorporated at 0.4 mol\% into magnetically aligned negatively charged bicelles with 1 mol\% DMPG (q=4.5)](image)

The data plots of semilogarithmic normalized intensities of the EO peak at each diffusion time $\Delta$ overlaps (Figure 3.26). The degree of curvature increases as the $(g\gamma\delta)^2(\Delta-\delta/3)$ value increases. This can be explained by the high polydispersity in the F68 polymer as well as the poor signal-to-noise ratio at lower intensities. The displacement of F68 in the bicellar mixture by diffusion can be determined by the root mean square (rms) displacement $<z^2>^{1/2}$.

$$<z^2>^{1/2} = (4D\Delta)^{1/2} \quad \text{(Equation 9)}$$
Thus for the case of $\Delta = 50$ ms the rms displacement was found to be $2.5 \times 10^{-6}$ m. This distance indicates that these negatively charged bicellar mixture might take on a lamellar morphology (Figure 3.27) where unobstructed diffusion is possible.

For the neutral bicellar mixture containing no DMPG, the diffusion decays of F68 EO peak shows non-Gaussian diffusion. The semilogarithmic plot of normalized intensities of the EO peak versus $(g \gamma \delta)^2(\Delta - \delta/3)$ shows non-linear delays and not overlapping decays at different diffusion times (Figure 3.28). This non-overlapping nature is obvious at short diffusion times while at longer diffusion times, the highly curved decays are beginning to overlap. The diffusion time dependence of the non-linear decays indicates that diffusion is in one-dimensional curvilinear path$^{26,63}$. This curvilinear diffusion of F68 in neutral bicellar mixtures indicate that the lengths of the bicellar mixtures have to be long, and based on previous small angle neutron scattering (SANS) data, the neutral bicellar mixtures result in ribbon-like structures (Figure 3.27)$^{26}$. At long diffusion times, due to considerable branching or interconnectedness of one-dimensional curvilinear paths, the diffusion becomes diffusion time-independent and shows Gaussian behaviour.

Figure 3.27: Schematic of the morphology changes undergone by neutral (DMPC/DHPC/F68) and charged (DMPG/DMPC/DHPC/F68) bicellar mixtures (Reprinted from Soong, Nieh, Nicholson, Katsaras, and Macdonald (2010) Langmuir 26: 2630-2638.)$^{26}$
It was expected that the negative charge would be screened by salt when the negatively charged bicellar mixture was prepared in buffer solution containing 100 mM KCl. If the negative charges were screened, then the bicellar mixture would become charge neutral. Then the diffusion decays of the charge screened negatively charged bicellar mixture would be identical to that of neutral bicellar mixtures. This is the result for the negatively charged bicellar mixtures prepared in 10 mM Tris/100 mM KCl solution (Figure 3.29). This figure resembles the diffusion decay of neutral bicellar mixture. The negatively charged bicellar mixture containing salt diffuses slower relative to the negatively charged bicellar mixture containing no salt. Similarly, the salt containing neutral bicellar mixture (Figure 3.30) diffuses slower than the neutral bicellar mixture containing no salt. Salt may make the gap between lipid bilayers narrower limiting the diffusion of Pluronic. Thus the samples containing salt, the neutral bicellar mixtures containing salt and the negatively charged bicellar mixture containing salt, result in previously mentioned ribbon-like structures where the diffusion of F68 occurs in one-dimensional curvilinear paths.
Figure 3.29: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles with 1 mol% DMPG and 100mM KCl (q=4.5)

Figure 3.30: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of F68 incorporated at 0.4 mol% into magnetically aligned bicelles with 100mM KCl (q=4.5)
There were two DMPC/DHPC bicellar mixtures prepared containing 5 and 10 mol% cholesterol relative to DMPC and 0.4 mol% F68 relative to DMPC. They both contained macroscopic inhomogeneities in the sample unlike the clear DMPC/DHPC bicellar mixtures lacking cholesterol. It is known that cholesterol is a major component in mammalian cell membranes and it influences the lateral organization of membrane constituents\(^\text{46}\). This causes formation of lipid clusters and rafts. Interactions between cholesterol and phospholipids can alter membrane properties\(^\text{46}\). It is known that an increase in the cholesterol concentration can cause an increase in the lipid chain order because of the molecular structure of cholesterol. Hydrogen bonds to the lipid polar group and van der Waals interactions between acyl chains and rings of cholesterol could be the reasons for the increase in the lipid chain order\(^\text{46}\). The semilogarithmic plot of normalized intensities of the EO peak versus \((g\gamma\delta)^2(\Delta-\delta/3)\) of this 5 mol% cholesterol sample shows non-linear delays and not overlapping decays at different diffusion times (Figure 3.31). The plot shows similar decays relative to the neutral bicellar mixture without cholesterol but this plot also shows faster decay and less curvature compared to the neutral bicellar mixture.

\[\text{Figure 3.31: Semilogarithmic plot of normalized STE- PFG NMR intensity decays of F68 incorporated at 0.4 mol\% into magnetically aligned bicelles with 5 mol\% cholesterol (q=4.5)}\]
3.3 Binding Assay

It is important to know the amount of F68 that gets incorporated in the lipid bilayer and the amount of F68 that is left in the solution. These two populations of F68 have different diffusion behaviours. F68 incorporated in the bilayer diffuses slower relative to F68 free in solution. Thus there will be two distinct diffusion coefficients obtained from these two populations and it could result in a biexponential or curved diffusion decay. In order to determine the fraction of F68 that can be incorporated in bicellar mixtures, a binding assay was conducted using lipid vesicles with F68 bound. The amount of POPC used in each vesicle sample was kept constant while the amount of F68 in each sample was varied. Based on the 1H NMR spectra of F68 bound MLV samples, the number of moles of F68 bound in the vesicle was determined relative to the known amount of DSS added (Table 3.4). Similarly the amounts of F68 present in the first supernatant samples were determined (Table 3.4).

<table>
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<tr>
<th>Mass F68 added (g)</th>
<th>Molar ratio F68 / POPC</th>
<th>Moles PO bound</th>
<th>Moles EO bound</th>
<th>EO / PO bound</th>
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<table>
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<th>Moles PO free</th>
<th>Moles EO free</th>
<th>EO / PO free</th>
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Table 3.4: The number of moles of F68 present as the bound and free in the binding assay based on the PPO and PEO peaks.

The number of moles of bound and unbound F68 based on the PPO resonance increases as the amount of F68 added to the vesicle samples (Figure 3.32). However this is not the same
for the 0.01 mol% F68 case. The same trend continues for the number of moles of bound and unbound F68 based on the PEO resonance except for the 0.01 mol% F68 case (Figure 3.32). The number of moles of F68 bound starts to plateau indicating that there may be a limit to the amount of F68 that can be bound in a vesicle (Figure 3.32). The number of moles of F68 unbound starts to increase exponentially indicating that there may be a lot more F68 that is left unbound in the solution as the amount of F68 that is added increases (Figure 3.33).

The mass of the bound and unbound F68 can be calculated based on the molecular weight of 8350 g/mol for F68. Given that F68 is highly polydisperse, there will be a range of molecular weights. Thus using 8350 g/mol as the molecular weight of F68 may not give the correct mass of F68 bound or unbound. In none of the cases, the amount of F68 bound and unbound based on PEO and PPO resonances do not match with the amount of F68 initially added to the vesicles. There can possibly be some fractions of F68 left in the second supernatant samples. However this binding assay demonstrates that there is a limit to how much F68 that can be incorporated inside the vesicles. It is also important to note that the amount of F68 bound is always less than the amount of F68 left in the supernatant. As the amount of F68 added in to each sample increases, the amount of F68 left in solution versus the amount of F68 bound increases from a factor of 4 to a factor of 79. This indicates that in bicellar mixtures where 0.4 mol% F68 is used relative to DMPC, there will be lower binding of F68 in the bilayer and most of the F68 will be in solution. It also indicates that F68 does not appear to have a good affinity to be incorporated inside the bilayer. The hydrophilic PEO chain may not be inclined to move through the bilayer in order to for the PPO block of the F68 to be incorporated inside the bilayer.
Figure 3.32: The plot of the number of moles of bound F68 in vesicles with respect to moles free in solution based on PEO and PPO peaks versus the molar ratio of F68 added relative to POPC.
Chapter 4
Conclusion

Pluronic tri-block copolymers consist of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) arranged in PEO-PPO-PEO structure. They are employed as drug delivery vehicles and are known to be incorporated readily into lipid bilayers. Here we describe stimulated echo pulsed field gradient (STE-PFG) $^1$H nuclear magnetic resonance (NMR) diffusion measurements of several polymers free in solution, and neutral and negatively charged magnetically aligned bicelles incorporating the Pluronic tri-block copolymer F68. Bicelles are model membrane systems composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC). They tend to align spontaneously in the magnetic field with their bilayer normals orthogonal to the direction of the applied magnetic field. This allows for diffusion measurements in the plane of the bilayer.

In this report, we have employed PFG NMR diffusion method to measure the F68 diffusion in the magnetically aligned bicelles. PFG NMR technique is used because it is rapid and non-invasive. In this report, we first determined the diffusion coefficient of a polymer composed of ethylene oxide monomers, PEG 20,000, using PFG NMR as a proof of principle. Then we looked at the lateral diffusion of a smaller polymer than PEG 20,000 (i.e. PEG 12,000) to compare the difference between the changes in the molecular weight. Next we looked at the lateral diffusion of F68 free in buffer. Pluronic F68 was investigated at different number of transients, concentrations, temperatures, and with and without salt. The range of self-diffusion coefficients of F68 that was observed in solution was not altered with the increase in the signal-to-noise level. As the concentration of F68 in solution increases the concentration-induced micellization causes the diffusion decays to become highly curved. Pluronic F68 goes from being molecularly dispersed mostly as a free polymer at 0.01 wt% to being micellized at 1.75 wt%. As the concentration decreases the mostly free polymer shows faster diffusion than the micellized F68 at higher concentrations. As the temperature increases, the diffusion of F68 becomes faster as the ratio of free polymer in solution become more hydrophilic while the amount of polymer micelles increases only slightly. The addition of salt does not appear to favour micellization and
causes similar diffusion decays compared to F68 free in solution. The persistent curvature in every case, even in the cases where there is no micellization at low concentration and low temperature is due to the polydispersity of the polymer.

It was demonstrated that Pluronic F68 incorporated into neutral bicellar mixtures ($q = [\text{DMPC}]/[\text{DHPC}] = 4.5$) exhibited non-Gaussian diffusion in that the F68 intensity decays were non-exponential and diffusion time dependent. Whereas Pluronic F68 incorporated in negatively charged bicellar mixtures, containing 1 mol\% (DMPG), exhibited Gaussian diffusion in that the F68 intensity decays were exponential and diffusion time independent. The implication may be that neutral bicellar mixtures incorporating Pluronic F68 consist of extended lamellae composed of meshed ribbon structures, while negatively charged bicellar mixtures incorporating Pluronic F68 consist of perforated lamellae. When the neutral bicellar mixtures contain salt the diffusion becomes slower and when salt is added to negatively charged bicellar mixtures, their diffusion decays resemble that of neutral bicellar mixtures. Addition of cholesterol can cause an increase in the lipid chain order and causes faster diffusion in the bilayer. Cholesterol does not dissolve well in water. The preparation of the cholesterol sample then should be done differently. The sample can be made by codissolving all the components in chloroform/methanol solution to obtain a molecular mixture, drying off the chlorofoam and then adding the water.

It was also concluded that there is a limit as to how much F68 that can be bound in a bilayer based on a binding assay conducted on lipid vesicles with various amounts of F68. The amount of F68 left behind in the aqueous solution is far more than that is trapped within the vesicles. This indicates that F68 does not appear to have a good affinity to be incorporated inside the vesicle. The hydrophilic PEO chain may not be inclined to move through the hydrophobic acyl chains of the bilayer in order to for the PPO block of the F68 to be incorporated inside the bilayer. Thus Pluronic F68 may then become confined within the lamellae of the bilayer.
Chapter 5

References


