ABSTRACT

Ou, Erkang. Effect of Silica Support on Electrostatics at Membrane-Water Interface and Lipid-Protein Interface by EPR-Active Molecular pH Probes. (Under the direction of Prof. Tatyana I. Smirnova).

Polarity, electrostatic potentials, and hydration are the major physico-chemical characteristics of lipid membranes that govern membrane-protein and protein-protein interactions as well as transport of small molecules through cellular membranes. At the membrane-water interface, the surface electrostatic potential of a lipid bilayer plays a fundamental role in such key processes of cellular functioning as endo- and exocytosis, membrane fusion and cellular division to name a few. At the lipid-protein interface, local dielectric constant of biomembranes determines stability, folding, and aggregation of membrane receptor proteins that are involved in a myriad of cellular functions. Some of the properties of cellular membranes could be mimicked by supported lipid bilayers (SLB) that serve as very useful model membrane platforms with the lipid bilayer providing a biocompatible interface and the solid support allowing for manipulation of lipid bilayer properties in a controllable manner. Such membrane-mimicking systems are considered to be the promising candidates for a number of biomedical and biotechnological applications. At present, little is understood about the influence of nanostructured support and the nanoconfinement on the properties of the membrane at membrane-water interface and protein-lipid interface. This PhD thesis project reports on employing EPR-active pH sensitive probes to assess the surface electrostatics and to profile a heterogeneous dielectric environment along a transmembrane peptide incorporated into both unsupported unilamellar lipid vesicles and the lipid membranes formed on the surface of silica beads.
EPR titration of spin-labeled pH-sensitive lipids allows us to experimentally determine the terms contributing to the interfacial of the lipid bilayers and calculate the magnitude of the surface electrostatic potential. By using this experimental approach we have investigated the effect of the lipid composition and silica support on the membrane surface potential. EPR titration experiments of spin labeled transmembrane peptide WALP with symmetric positions of the nitroxide-labeled sidechains with respect to the bilayer center revealed two sequential proton dissociations characterized by two $pK_a$ values that yielded local dielectric constant as a function of the label position, i.e., a profile of the dielectric constant along the protein-lipid interface using single and double labeled WALP mutants effects of the lipid composition and silica support on the effective $pK_a$ of the ionizable sidechain of the transmembrane peptide was examined. Supported lipid bilayer dynamics was also characterized by mobility and order parameters derived from spin label EPR spectra. It was observed that local rotational dynamics of spin-labeled lipids is affected by the silica support resulting in an increased rotational correlation time and a higher ordering of the lipids in the bilayer. Molecular accessibly of the bilayer surface and specific sites of the transmembrane WALP peptide was assessed by an EPR assay based on a reduction of EPR-active nitroxides to an EPR silent hydroxylamine upon reactions with hydrophilic ascorbate/ascorbic acid. In conclusion, the EPR-active pH-sensitive nitrooxide spin labels allow for an expansion of the existing arsenal of experimental EPR methods for assessing local electrostatics and dielectric properties of nanoscale heterogeneous systems and are fully suitable for studies of hybrid nanomaterials/nanosystems composed of inorganic nanoscale materials and lipids that are capable of self-assembly.
Effect of Silica Support on Electrostatics at Membrane-Water Interface and Lipid-Protein Interface by EPR-Active Molecular pH Probes

by

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DEDICATION

To my parents Kailiang Ou and Qiaoling Xia.
BIOGRAPHY

Erkang Ou was born in April 1988 and grew up in a lovely family, her father is an outstanding executive engineer, and her mother is an excellent analytical chemist, their wit, humor and integrity set a good example for her. During the years playing in her mother’s lab, Erkang found her passion about chemistry, haha, made her father a little down that he had hoped to help her discover the beauty of mechanical engineering.

In September 2007, she was accepted by one of the top universities in China, Sichuan University and majored in Chemistry. In 2009 she was luckily selected to be an exchange student studying chemistry in Pennsylvania for two semesters and made up her mind to pursue a graduate study in the United States.

In August 2011, Erkang decided to join Prof. Tatyana I. Smirnova’s group and began her doctoral studies at North Carolina State University. Her research interests range from investigating molecular mechanisms of nanomaterials toxicity, to studying biophysical aspects of interactions between model biological membranes and inorganic nanomaterials.
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TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................ viii

LIST OF FIGURES ...................................................................................................... x

Chapter 1: Introduction .................................................................................................1

1.1 Membrane Structures and Properties ....................................................................1

1.2 Lipid-Nano Hybrids Structure and Application .....................................................7

1.3 Preparation and Characterization of Silica Supported Lipid Bilayer ......................9

1.4 Interaction between Silica Beads and Lipid Bilayer .............................................12

1.5 Site Directed Spin Labeling Electron Paramagnetic Resonance ..........................16

Chapter 2: Effect of Silica on Surface Electrostatic Potential of Phospholipid Bilayer ..20

2.1 Introduction .............................................................................................................20

2.2 Materials and Methods ..........................................................................................31

2.2.1 Materials ..........................................................................................................31

2.2.2 Liposome Preparation .......................................................................................32

2.2.3 Liposome Characterization ...............................................................................33

2.2.4 Silica Beads Supported Lipid Bilayer Preparation ............................................34

2.2.5 Silica Beads Supported Lipid Bilayer Characterization ....................................35

2.2.6 Liposome pH Titration Experiments .................................................................36

2.2.7 Silica Beads Supported Lipid Bilayer pH Titration Experiments .......................36

2.2.8 CW-EPR Measurements ..................................................................................37

2.2.9 CW-EPR Characterization of Local Dynamics of 5-Doxyl-SA Incorporated into Liposomes and Silica Beads Supported Lipid Bilayers ..............................37
Chapter 3. Effect of Silica Support on pKₐ Of Ionizable Sidechain Located at Membrane-Protein Interface of Model Transmembrane Peptide Incorporated into Phospholipid Bilayer.................................85

3.1 Introduction.................................................................85

3.2 Materials and Methods..................................................95
3.2.1 Materials ..........................................................................................................................95
3.2.2 Peptide Spin Labeling ......................................................................................................96
3.2.3 Characterization of Double Labeled WALP Peptides ....................................................97
3.2.4 Phospholipid Liposome Preparation .............................................................................97
3.2.5 Silica Beads Supported Lipid Bilayer Preparation .......................................................97
3.2.6 Sample Characterization with DLS Size Measurement and Zeta Potential Measurement ..........................................................................................................................97
3.2.7 Liposome pH Titration Experiments .............................................................................98
3.2.8 Silica Beads Supported Lipid Bilayer pH Titration Experiment ....................................98
3.2.9 CW-EPR Measurements ...............................................................................................98
3.3 Results and Discussions ...................................................................................................99
  3.3.1 Control Experiments: Effect of pH on Dynamics of WALP Peptide
      Incorporated in Liposomes or Silica Supported Bilayers ..............................................99
  3.3.2 Determination of the Interfacial pK_a of Spin Labeled WALP (1Cys) Peptide
      Incorporated in LUVs .....................................................................................................100
  3.3.3 Determination of the interfacial pK_a of spin labeled WALP (2Cys) peptide
      incorporated in LUVs ....................................................................................................106
  3.3.4 Effect of silica support on dielectric environment and protonation of pH
      sensitive labels at protein-membrane interface .........................................................121
3.4 Conclusion ........................................................................................................................128
References ................................................................................................................................129
LIST OF TABLES

Table 2.01 Intrinsic pKₐ of PTE-IMTLS and PTE-IKMTSL [94, 99] ........................................27
Table 2.02 Titration data for spin label adducts in buffer/isopropyl alcohol solutions of various composition [94, 99] .................................................................28
Table 2.03 Interfacial pKₐ, intrinsic pKₐ, polarity induced shifts ΔpKₐ, and local dielectric constant ε measured and calculated for Triton X-100 with 1% PTE-IMTLS and PTE-IKMTSL [94, 99] .........................................................30
Table 2.04 Experimental ΔpKₐe calculated from the pH titration and the surface electrostatic potential, Ψ calculated for PTE-IMTLS liposome samples ........................................49
Table 2.05 Experimental ΔpKₐe calculated from the pH titration and the surface electrostatic potential, Ψ calculated for PTE-IKMTSL liposome samples ........................................50
Table 2.06 Experimental ΔpKₐe calculated from the pH titration using fit to Eq. 19 (top) and to Eq. 20 (bottom) and the surface electrostatic potential, Ψ calculated for silica bead supported IMTLS-PTE labeled bilayer samples .....................................................65
Table 2.07 Experimental ΔpKₐe calculated from the pH titration using fit to Eq. 19 (top) and to Eq. 20 (bottom) and the surface electrostatic potential, Ψ calculated for silica bead support IMTLS-PTE bilayer samples .....................................................66
Table 3.01 Estimates of the dielectric profile within different components of the lipid bilayer regions. Adapted from [144] ..........................................................87
Table 3.02 WALP peptides sequence ..........................................................91
Table 3.03 pKₐ values obtained from titration curves for WALP (1cys)-IKMTSL incorporated into DOPC LUVs using single dissociation model Eq.32 .....................................102
Table 3.04 pKₐ values obtained from titration curves for WALP (1cys)-IMTLS incorporated into DOPG LUVs using single dissociation model Eq.32 .....................................103
Table 3.05 pKₐ values obtained from titration curves for WALP (2cys)-IKMTSL incorporated into DOPC LUVs using independent double dissociation model Eq.34 .......................111
Table 3.06 pKₐ values obtained from titration curves for WALP (2cys)-IMTLS incorporated into DOPC LUVs using dependent double dissociation model Eq.42 .......................111
Table 3.07 pKₐ values obtained from titration curves for WALP (2cys)-IMTLS incorporated into DOPG LUVs using independent double dissociation model Eq.34 .......................112
Table 3.08 pKₐ values obtained from titration curves for WALP (2cys)-IMTLS incorporated into DOPG LUVs using dependent double dissociation model Eq.42 .......................112
Table 3.09 pKₐ values obtained from IKMTSL labeled WALP3, 21 incorporated into various lipid composition, experiments were carried out by Dr. Matthew Donohue, and data were analyzed by Erkang Ou ..........................................................115
Table 3.10 pKₐ values obtained from IMTLS labeled WALP3, 21 incorporated into various lipid composition, DOPG experiments was carried out by Dr. Matthew Donohue, and data were analyzed by Erkang Ou ..........................................................115
Table 3.11 Gradient in dielectric constant Δε is calculated from pKₐ calibration introduced in Table 2.02. For double labeled WALP-IMTSL in DOPG SUVs the absolute value of dielectric constant ε is calculated using Eq.43 ..........................................................120
Table 3.12 pKₐ values obtained from titration curves for WALP (2cys)-IKMTSL incorporated into DOPC bilayers and silica supported lipid bilayer using dependent double dissociation model Eq.42 ........................................................................................................................................127
LIST OF FIGURES

Figure 1.01 Schematic structure of a phospholipid and lipid bilayer. C, H, O N, P atoms are colored grey, pale blue, red, blue and yellow, respectively.
https://en.wikipedia.org/wiki/Phospholipid .......................................................... 2

Figure 1.02 Schematic structure of liposome.

Figure 1.03 Schematic structure of membrane protein.
https://commons.wikimedia.org/wiki/File:Membrane_protein.png .................................. 2

Figure 1.04 Common head groups found in phospholipids. The depicted ionization state corresponds to physiological pH levels ........................................................................... 3

Figure 1.05 Schematic profile of the electrostatic potential across typical lipid bilayer and its decomposition into three major components adapted from [3] ........................................... 4

Figure 1.06 Chemical structure of zwitterionic lipids DMPC (1,2-ditetradecanoyl-sn-glycero-3-phosphocholine) .............................................................................................................. 6

Figure 1.07 Cartoon illustrating supported lipid bilayer (SLB)
https://en.wikipedia.org/wiki/Model_lipid_bilayer ..................................................... 8

Figure 1.08 Formation of silica beads supported lipid bilayer, by cartoon (left) and by cryo-EM (right) adapted from [17] ........................................................................................................ 12

Figure 1.09 SLB on SiO₂ based on the electrostatic and van der Waals force and a thin layer of water separating the two surfaces. Adapted from [50] ...................................................... 13

Figure 1.10 Electronic spin energy levels in magnetic field .............................................. 17

Figure 1.11 Chemical structure of nitroxide IMTSL and its three EPR signal with three absorption peaks. The electron spin density of a nitroxide which primarily located on the nitrogen pₓ orbital is affected by enviromental and structural changes and determines the magnitude of hyperfine interaction. Adapted from [65] ...................................................... 18

Figure 1.12 Reversible protonation of nitroxides attached to the side chain of biopolymer .. 19

Figure 1.13 EPR line shape as a function of spin label motion. (A) Spin label in dilute solution – fast motion. (B) Spin label bond to a small peptide in solution. (C) Spin labeled peptide incorporated into a liposome – slow motion. (D) Spin labeled peptide in frozen sample. Adapted from [67] ........................................................................................................ 19

Figure 2.01 Chemical Structure of the non pH-sensitive nitroxide MTSL (S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate), pH-sensitive nitroxide IMTSL and IKMTSL, lipid PTE, synthetic spin-labeled lipid IMTSL-PTE and IKMTSL-PTE ........................................................................................................ 24

Figure 2.02 Schematics of four models to investigate silica beads effect on interfacial electrostatics ........................................................................................................ 26

Figure 2.03 Chemical structure of IMTSL-ME and IKMTSL-ME ........................................ 27

Figure 2.04 pKₐ of IMTSL-ME and IKMTSL-ME vs bulk dielectric constant ε of water–i-PrOH mixtures. The corresponding data for IMTSL-ME is shown as filled squares, for IKMTSL-ME is shown as filled triangle. The error bars are comparable with the size of symbols. The corresponding linear regressions are shown as solid lines with parameters given in table 2. Reproduced from [94, 99] ........................................ 29
Figure 2.05 Chemical structure of Triton X-100 detergent (left), cartoon illustrating spin labeled PTE lipids incorporated in Triton X-100 micelle ................................................................. 30
Figure 2.06 Cartoon illustrating vesicle formation mechanism, adapted from https://avantilipids.com/tech-support/liposome-preparation/ ................................................................. 33
Figure 2.07 Chemical structure of 5-doxyl stearic acid ......................................................................................... 38
Figure 2.08 Reduction of a nitroxide radical with sodium ascorbate produces an EPR-silent hydroxylamine .............................................................................................................. 38
Figure 2.09 Chemical Structure of PTE, POPC and POPG lipids ............................................................................. 40
Figure 2.10 Size distribution by intensity of POPC LUVs as measured by DLS ...................................................... 41
Figure 2.11 Representative intensity normalized CW X-band EPR spectra from pH titration control experiments of POPC 100nm LUVs doped with 1 mol % of non pH-sensitive PTE-MTSL (left), and silica supported lipid bilayer (right) ................................................................. 42
Figure 2.12 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of 1 mol % pH-sensitive PTE-IMTSL lipid incorporated into 100nm LUVs composed of POPC (left) and POPC 80% : POPG 20% (right) ................................................................. 44
Figure 2.13 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into 100nm LUVs composed of POPC (left) and POPC 80% : POPG 20% (right) ................................................................. 44
Figure 2.14 Decomposition of EPR spectrum from PTE-IKMTSL incorporated into POPC 80 mol %:POPG 20 mol % LUVs. Experimental spectra acquired at intermediate pH (black). Component of the experimental spectra corresponding to the neutral form of IKMTSL (blue). Component of the experimental spectra corresponding to the charged form of IKMTSL (red). The residual of fitting the experimental spectra to the sum of the simulated spectra (green). 46
Figure 2.15 Cartoon illustrating spin labeled PTE lipids incorporated into 100nm LUV ...... 47
Figure 2.16 pH titration curve of 1 mol % pH-sensitive PTE-IMTSL lipid incorporated into 100nm LUVs composed of POPC (blue) and POPC 80 mol % : POPG 20 mol% (red). Dashed lines are the best fit to single dissociation model Eq.11 ........................................................................ 49
Figure 2.17 pH titration curve of 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into 100nm LUVs composed of POPC (blue) and POPC 80% : POPG 20% (red). Dashed lines are the best fit to single dissociation model Eq.11 ........................................................................ 50
Figure 2.18 Cartoon illustrating POPC 100 mol % liposome doped with spin labeled lipid (left) and POPC 80 mol% : POPG 20 mol % POPG LUVs doped with spin labeled lipids (right) ........................................................................................................................................ 52
Figure 2.19 Compared to linker length of IMTSL (left), IKMTSL has longer and more “stiff” structure ........................................................................................................................................ 54
Figure 2.20 Zeta potential titration of silica beads in 0.5 x PBS buffer ................................................................. 59
Figure 2.21 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of silica supported samples doped with 1 mol % pH-sensitive PTE-IMTSL lipid incorporated into lipid vesicles composed of POPC (left) and POPC 80% : POPG 20% (right) ........................................................................................................................................ 61
Figure 2.22 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of silica supported samples doped with 1 mol % pH-sensitive PTE-IKMTSL lipid
incorporated into lipid vesicles composed of POPC (left) and POPC 80% : POPG 20% (right) ..............................................................61

**Figure 2.23** Cartoon illustrating spin labeled PTE lipids incorporated into vesicle that is coated on silica beads ........................................................................................................................................62

**Figure 2.24** Decomposition of EPR spectrum from PTE-IKMTSL incorporated into silica supported POPC bilayer. Experimental spectra acquired at intermediate pH (black). Component of the experimental spectra corresponding to the neutral form of IMTSL (blue). Component of the experimental spectra corresponding to the charged form of IMTSL (red). The residual of fitting the experimental spectra to the sum of the simulated spectra (green). 63

**Figure 2.25** pH titration curve for 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into silica supported bilayer POPC (blue) and POPC 80% : POPG 20% (red). Dashed lines are the best fit to single dissociation model Eq.11, solid lines are the best fit to double dissociation model Eq.20 .........................................................................................65

**Figure 2.26** pH titration curve for 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into vesicle and coated on silica beads POPC (blue) and POPC 80% : POPG 20% (red). Dashed lines are the best fit to single dissociation model Eq.11, solid lines are the best fit to double dissociation model Eq.20 ........................................................................................................66

**Figure 2.27** Surface charge density of a planer silica surface as a function of pH. The numerical result is from a multi-ion charge-regulation model [126], the theoretical result is from [127]. Figure is adapted from [126] ........................................................................................................70

**Figure 2.28** Schematic illustration of the double electrical layer structure, adapted from [125] ...............................................................................................................................................72

**Figure 2.29** EPR spectra of silica beads modified with IKMTSL precursor (red) and free spin labeling in same solvent (blue) .........................................................................................................................73

**Figure 2.30** Representative EPR spectra of 3-MPTS salinized silica beads modified with IKMTSL at various pH ........................................................................................................................................74

**Figure 2.31** EPR spectra from non-protonated forms of PTE-IKMTSL incorporated into liposome (black) and silica supported lipid bilayer (purple) ..........................................................................................75

**Figure 2.32** EPR spectra from non-protonated forms of PTE-IKMTSL incorporated into liposome (black) and silica supported lipid bilayer (purple) ..........................................................................................75

**Figure 2.33** EPR spectra from protonated forms of PTE-IKMTSL incorporated into liposome (black) and silica supported lipid bilayer (purple). The latter is decomposed as outer leaflet (blue) and silica supported inner leaflet (red) .........................................................................................76

**Figure 2.34** EPR spectra from protonated forms of PTE-IKMTSL incorporated into liposome (black) and silica supported lipid bilayer (purple). The latter is decomposed as outer leaflet (blue) and silica supported inner leaflet (red) .........................................................................................77

**Figure 2.35** Representative intensity-normalized room temperature EPR spectra of 5-doxyl-SL incorporated in LUVs (blue) and in silica supported bilayer (red) consisting of POPC/POPG mixture. Hyperfine splitting parameters $A_{\text{out}}$ and $A_{\text{in}}$ shown as measured for calculations of order parameter. Blue dotted lines are used to emphasize differences in spectra .........................................................................................79

**Figure 2.36** Mobility parameter of 5-Doxy-SL incorporated into liposomes composed of POPC 20 mol% : POPG 20 mol % (blue) and into silica supported lipid bilayer of the same composition (red) as function of temperature ......................................................................................................................80
Figure 2.37 Experimentally measured effective order parameters $S^{\text{eff}}$ as a function of temperature for 5-doxyl SL incorporated in LUVs (blue) or silica supported bilayers (red) prepared from POPC 20 mol% : POG 20 mol % mixture..................................................81

Figure 2.38 Nitroxide intensity decay for PTE-IKMTSL labeled POPC liposome and its corresponding silica beads supported sample induced by 80-fold molar excess of ascorbate at pH=7.4 under continuous nitrogen flow at room temperature..................................................82

Figure 3.01 Chemical structure of DOPC and DOPG lipids .................................................90

Figure 3.02 Schematic representation of the label’s positions on transmembrane WALP peptide. Adapted from [65]..................................................................................................................93

Figure 3.03 Representative intensity normalized CW X-band EPR spectra of DOPC 100 nm LUVs containing 1 mol % of WALP1-MTSL (left), and silica supported DOPC bilayer containing 1 mol % of WALP1-MTSL (right) obtained at various pH........................................100

Figure 3.04 Representative intensity normalized CW X-band EPR spectra of WALP1-IKMTSL incorporated into DOPC LUVs (left) and WALP2-MTSL incorporated into DOPG LUVs (right)...................................................................................................................101

Figure 3.05 Titration curves for WALP (1cys)-IKMTSL incorporated into DOPC LUVs. Labeling position WALP1 (green). Labeling position WALP3 (pink). Labeling position WALP5 (blue). Labeling position WALP6 (red). Labeling position WALP8 (cyan). Solid lines are the best fit to single dissociation model Eq.32..................................................................................................................102

Figure 3.06 Titration curves for WALP (1cys)-MTSL incorporated into DOPG LUVs. Labeling position WALP2 (green). Labeling position WALP3 (pink). Labeling position WALP4 (blue). Labeling position WALP6 (red). Labeling position WALP18 (cyan). Solid lines are the best fit to single dissociation model Eq.32..................................................................................................................102

Figure 3.07 Experimental pKa values as a function of spin label attachment site and its respective alpha carbon distance from the bilayer center. MTSL labeled WALPs in DOPC LUVs (open circle), IKMTSL labeled WALPs in DOPC LUVs (filled circle)..............................................103

Figure 3.08 Titration curves for WALP 1, 23 labeled with IKMTSL and incorporated into DOPG LUVs. Solid line is the best fit to dependent double dissociation model Eq.42, dashed line is the best fit to single dissociation model Eq.32..................................................................................................................107

Figure 3.09 Titration curves for WALP (2cys)-IKMTSL incorporated into DOPC LUVs. Labeling position WALP1,23 (green). Labeling position WALP3,21 (pink). Labeling position WALP5,19 (blue). Labeling position WALP6,18 (red). Labeling position WALP8,16 (cyan). Solid lines are the best fit to dependent double dissociation model Eq.42. Experimental data adapted from [65]..................................................................................................................111

Figure 3.10 Titration curves for WALP (2cys)-MTSL incorporated into DOPG LUVs. Labeling position WALP1,23 (green). Labeling position WALP3,21 (pink). Labeling position WALP5,19 (blue). Labeling position WALP6,18 (red). Experimental data adapted from [65]..................................................................................................................112

Figure 3.11 Titration curves for IKMTSL labeled WALP3, 21 incorporated into DOPG LUVs (red), DOPC/DOPG LUVs at a 1:1 molar ratio (purple), DOPC LUVs (blue), experimental data adapted from [65] (left). Titration curves for IMTSL labeled WALP3, 21 incorporated into DOPG LUVs (red), DOPC/DOPG LUVs at a 1:1 molar ratio (purple), experimental data

xiii
adapted from [65] (right). Solid lines are the best fit to dependent double dissociation model Eq.42...

**Figure 3.12** Calculated pK_{al} values corresponding to WALP (2cys) dependent double dissociation (filled circle) and calculated pK_{a} values corresponding to WALP (1cys) single dissociation (open circle) as a function of spin label attachment site’s respective alpha carbon distance from the bilayer center. IKMTSL labeled WALPs in DOPG (red) DOPC/DOPG (purple) and DOPC (blue) bilayers (left). IMTSL labeled WALPs in DOPG (red) and DOPC/DOPG (purple) bilayers (right)...

**Figure 3.13** Probability profiles of different molecular components of DOPC, adapted with modification from [152] The WALP peptide is drawn as a ribbon within the pink box...

**Figure 3.14** Representative intensity normalized CW X-band EPR spectra of WALP1-IKMTSL incorporated into DOPC LUVs (upper left), WALP1-IKMTSL incorporated into silica supported DOPC bilayer (upper right), WALP1,23-IKMTSL incorporated into DOPC LUVs (lower left), WALP1,23-IKMTSL incorporated into silica supported DOPC bilayer (lower right)...

**Figure 3.15** Titration curves for silica supported WALP1-IKMTSL incorporated into DOPC bilayer (dark red), WALP1-IKMTSL incorporated into DOPC LUVs (red), red solid line is the best fit to single dissociation model Eq.32, WALP1,23-IKMTSL incorporated into DOPC LUVs (blue), blue solid line is the best fit to dependent double dissociation model Eq.42, silica supported WALP1,23-IKMTSL incorporated into DOPC bilayer (dark blue), dark blue solid line is the best fit to dependent double dissociation model Eq.42...

**Figure 3.16** Nitroxide intensity decay for 0.074mM WALP1-IKMTSL incorporated into DOPC LUVs (red open circle), 0.078mM WALP1-IKMTSL incorporated into silica supported DOPC bilayer (dark red filled circle), 0.12mM WALP8-IKMTSL incorporated into silica supported DOPC bilayer (dark pink filled circle) induced by five-fold molar excess of ascorbic acid at pH=3.0 and under continuous N\textsubscript{2} flow at room temperature. The kinetic decay curves are the best fit to a single exponential equation...

**Figure 3.17** Titration curves for WALP3,21-IKMTSL (left) and WALP5,19-IKMTSL (right). spin labeled WALP incorporated into DOPC LUVs (blue open circle), silica supported spin labeled WALP incorporated into DOPC bilayer (blue filled circle). Solid lines are the best fit to dependent double dissociation model Eq.42...
Chapter 1 Introduction

1.1 Membrane structures and properties.

Membranes, approximately 4-6nm thick, are essential elements of all the eukaryotic cells, they form a sharp interface between the aqueous environment and cell interior and create a barrier that allow cells to build a gradient that can serve as a source of energy for chemical reactions and transport. The membranes are primarily composed of lipids and proteins that are responsible for many aspects of cellular function. The most basic and abundant component of membrane lipids is amphiphilic molecule phospholipid, which is composed of two fatty acids chains (i.e. hydrophobic tails) attached to a glycerol phosphate molecule (i.e. hydrophilic head). When exposed to an aqueous environment, most phospholipids self-assemble such that hydrophobic tails align against one another with the hydrophilic heads on either side facing the water, thus spontaneously form lipid bilayers (Figure 1.01). Depending on the appropriate molecular structure and environmental conditions, phospholipids are capable of organize into vesicles/liposome. In liposome bilayer encloses an aqueous volume, creating structure similar to the lipid portion of natural membranes (Figure 1.02). In this project we employed liposomes to mimic cell membrane. Membrane proteins can be divided into two general types: integral and peripheral. Integral proteins are subdivided into transmembrane proteins and monotopic proteins (Figure 1.03). In this project we employed transmembrane peptides that span the entire biological membrane and primarily adopt either a-helical or beta-barrel structures that favors lower Gibbs free energy.
Figure 1.01 Schematic structure of a phospholipid and lipid bilayer. C, H, O N, P atoms are colored grey, pale blue, red, blue and yellow, respectively.
https://en.wikipedia.org/wiki/Phospholipid

Figure 1.02 Schematic structure of liposome.

Figure 1.03 Schematic structure of membrane protein.
https://commons.wikimediawiki.org/wiki/File:Membrane_protein.png
Major physio-chemical characteristics of membranes include electrostatics, dielectric constant and hydration. Phospholipids hydrophilic head group at the membrane-water interface can possess a variety of polar or charged groups that create electrostatic potentials, while the interior of the bilayer is highly hydrophobic that favors primarily nonpolar and less polar residues of transmembrane proteins embedded in the lipid bilayer, (strongly polar amino acid only account for 8.5% and charged amino acid are only 1.2% of transmembrane sequences [1]), as a result, electrostatic environment within lipid bilayer and at the surface are very different. If we treat polar and charged head group as parallel plate and hydrocarbon interior as insulation system, when external field is applied to this system, charges which are localized at the membrane-water interface will respond by rearranging to partially cancel the applied field. The extent of such response is called dielectric constant.

![Figure 1.04](image) Common head groups found in phospholipids. The depicted ionization state corresponds to physiological pH levels.

As mentioned above, phospholipid is composed of hydrophobic tails and hydrophilic head. The hydrophilic head is composed of phosphate moiety and either anionic, zwitterionic or cationic head groups attached to it (Figure 1.04), the charges and oriented dipoles on the head creates the electric field potential. For example, phosphatidylcholines (PC) is a class of zwitterionic phospholipids that incorporate positively charged choline and negatively charged...
phosphate moiety and carries an electric dipole moment of about 10 D [2], and phosphatidylglycerol (PG) is a class of anionic phospholipids that incorporates neutral glycerol and negatively charged phosphate moiety.

**Figure 1.05** Schematic profile of the electrostatic potential across typical lipid bilayer and its decomposition into three major components adapted from [3].

The membrane electrostatic potential profiles across the membrane are shown in (Figure 1.05). Three major contributions of potential are distinguished: (1) the dipole potential, $\Psi_d$, formed between the aligned dipoles of hydrated lipid heads at the membrane surface and the low-polar interior of the bilayer. It is the strongest component of membrane electrostatics. Because the dipole potential formed by two monolayers of opposite sign, so it creates a strong positive charge in the lipid bilayer and a strong energy barrier for the penetration of ions. (2) The
transmembrane potential, \( \Psi_t \) results from the net separation of charges between aqueous phase and bilayer interior and ionic concentration gradient across the membrane since the membrane interior is hydrophobic in nature and poorly permeable to ions. This potential is negative and is one order of magnitude smaller than \( \Psi_d \). (3) The surface potential, \( \Psi_s \), originates from charged head groups and phosphate moiety, as well as adsorbed ions and the counter ions that reside closely in the vicinity of the head groups on both sides of the membrane. Thus it depends on the amount and type of the charged lipids in the bilayer and the concentration of protons and ions. This potential is negative and usually smaller than \( \Psi_d \) [3]. In this project we are interested in the surface potential \( \Psi_s \), details will be discussed in chapter 2.

As mentioned above, dielectric constant describes the local polarizability of the medium in response to an external electric field. Although lipid bilayers are frequently modeled as simple low-dielectric slabs, the transmembrane profile of dielectric constant \( \varepsilon \) is more complex. Along a phospholipid molecule, for example DMPC (1,2-ditetradecanoyl-sn-glycero-3-phosphocholine) shown in Figure 1.06, choline head group is positively charged and phosphate moiety is negatively charged sitting at the head group/interfacial hydration water region; due to different electronegativity of oxygen, carbon and hydrogen atoms, glycerol moiety is polar at the ester group region; hydrocarbon tails is much less polar at the interior region. Thus variations in polarity at these regions result in different local dielectric constant \( \varepsilon \).
Figure 1.06 Chemical structure of zwitterionic lipid DMPC (1,2-ditetradecanoyl-\textit{sn}-glycerol-3-phosphocholine).

For such a nonhomogeneous system, computation of the position-dependent dielectric constant \( \varepsilon \), which is referred to as the dielectric profile, represents a difficult problem. The head group dielectric gradient and its variations (from the ester region, to phosphate to choline serine or glycerol etc.) is very difficult to model. Besides, different lipid bilayers have different \( \varepsilon \) distributions along the bilayer axis, this is dependent on the nature of head group interactions (between lipids, lipid-solvent, lipid-ions and fluidity of the bilayer). Medium with higher dielectric constant has greater ability to stabilize charges, thus non polar hydrocarbon tails at the interior region with lower dielectric constant are highly impermeable to most ions and polar molecules, and thus the dielectric gradient significantly affects transportation of ions and nutrients across the lipid bilayer. In addition, dielectric gradient is also what drives membrane protein folding and assembly.

Electrostatic interactions and dielectric profile play the key roles in the myriad of cellular and molecular biology processes [4], affect the conformation and function of many molecules, intercellular and intracellular recognition [5], insertion of proteins, toxins and viruses into membrane [6], and lipid-protein association [7, 8]. Electrostatic interaction contribute to
membrane fusion [9] and regulate phase transition [10], govern transport of charged or polar molecules and nutrients through membrane during metabolic processes and signaling events [11], and directly involved in Protein-protein association [12] as well as structure and dynamics of DNA [13]. In conclusion, it is imperative to develop and measure lipid bilayer electrostatic potential and dielectric constant at well-defined positions with respect to the membrane-water interface and lipid-protein interface within such a heterogeneous system to further decipher the mechanisms of biological phenomena in cells.

1.2 Lipid-nano hybrids structure and applications.

Liposomes prepared from synthetic lipids allow learning about biological processes taking place in living organisms, thus are commonly used as models of complex cell membranes [14-17]. Despite their extraordinary usefulness, problems with size polydispersity as well as physical and chemical stability of liposomes often arise. Supported lipid bilayer (SLB) formed from liposomes, both on planar and spherical substrates, are better candidates for addressing some of these issues [18].

Because lipid bilayer formed on bulk planar surfaces are rather unstable, we focus on spherical supported lipid bilayer (SSLB) for our discussion. SSLB is composed of a particles core surrounded by a lipid shell, they are easier to handle and offer more freedom to work with different microscopy and spectroscopy techniques [17, 19, 20].
The robust inorganic core of SSLB enhances membrane stability of the wrapped lipid bilayer. The diameter of SSLB is determined by the diameter of the support. SSLB forms homogeneous membrane structures if the support core is monodispersed, thus solving the polydispersity and physicochemical stability issue. Lipid bilayer provides a biocompatible interface that allows for protein insertion/coupling and drug containment, support material possess optical, electrical, magnetic, and catalytic properties. SSLB preserve the key membrane functions, at the same time offering a wide range of opportunities to 1) systematically modulate pH and temperature, 2) vary the composition of the bilayer, and 3) allow to introduce specific receptors/membrane proteins into the supported membrane [21]. To summarize, SSLB offers opportunities as promising candidates for biomedical and biotechnological applications in materials science, drug delivery, imaging, and biosensor development.

Figure 1.07 Cartoon illustrating supported lipid bilayer (SLB).
https://en.wikipedia.org/wiki/Model_lipid_bilayer
Such a hybrid system combining all of the positive features of liposomes and inorganic material have been successfully formed on silica, polystyrene, and magnetic beads whose sizes range from tens of nanometers to several micrometers [22]. Because of spontaneous fusion of liposomes onto the naturally hydrophilic and biocompatible material, silica is most extensively studied for lipid interaction, and a large body of literature exists on this topic [23, 24]. It is evident that a thin water layer (~1 nm) separates the lipid bilayer from the silica surface to achieve a mobile bilayer [25, 26]. Thus supported lipid bilayers (SLB) retain many characteristics of natural cell membranes such as lateral fluidity, incorporation of proteins, phase separation, and impermeability to ionic species [27-29]. Although most experiments were performed on planar silica surfaces, silica beads were also studied [17, 30-38]. Lipid-coated silica beads have been used for important delivery systems and analytical assays [18, 39, 40], employed for detection of anti-phospholipid antibodies in human body. Silica supported lipid bilayer have been used to measure the affinity of biologically active substances to biological membranes, and found applications in chromatography for the separation of proteins. In this project we focus on silica beads supported lipid bilayer for our discussion.

1.3 Preparation and characterization of silica supported lipid bilayer.

Both simple liposome adsorption and direct formation of supported lipid bilayers (SLBs) on silica are possible [18, 41], and depends on the lipid-silica interaction. The existing protocols for preparing silica beads supported lipid bilayer usually rely on electrostatic attraction between the silica beads (often negatively charged) and small unilamellar vesicles (SUVs) Parameters that affect lipid-silica interaction are listed below.
1. SSLB surface charge. Lipid bilayer affinity to silica beads surface varies in accordance with the sequence: anionic lipids < neutral lipids < cationic lipids [22]. Silica supported lipid bilayer are generally restricted to cationic and neutral lipids and only allow a limited fraction (no more than 20%) of anionic lipids. Additional charge or specific chemical interactions have been harnessed to facilitate SLB formation using a broad range of lipids [42].

2. Particle size and curvature. Simulations of lipid–colloid interactions suggest that the liposome curves away from the small particles and encapsulates the large particles [43]. Another early theoretical work indicates the balance of liposome adhesion energy and the curvature energy is responsible for driving liposome deformation and fusion onto the silica surface [44]. Later, Richter and co-workers reported that liposomes are adsorbed only at highly curved particles, whereas fusion is facilitated at low curvature [45]. There is some evidence that local curvature might mechanically control the spatial organization of membrane composition and properties such as phase transition [46].

3. Bilayer fluidity, temperature, size and concentration are also found to be important for liposome fusion with silica [23, 24].

4. Solution ionic strength and pH attribute to an extra electrostatic contribution on top of the attractive van der Waals force [25, 47]. As observed for planar surfaces, increased ionic strength of the solution favors vesicle fusion other than adsorption [47], Moura and Carmona-Ribeiro studied phosphatidylcholine (PC) vesicle adsorption onto silica and calculated corresponding affinity constants, they observed that in the absence of
salt, little/no adsorption or fusion of vesicles onto silica occurs while an increase in the solution ionic strength promote neutral lipid fusion [48]. Full wrapping was found to occur only for intermediate salt concentrations [49] because high salt concentrations (>90 mM NaCl, pH 7.4) can lead to silica precipitation. Divalent metals such as Ca$^{2+}$ have also been proved to be able to modulate SLB formation.

Liposome formed on planar surfaces is often studied by quartz crystal microbalance with dissipation (QCM-D), atomic force microscopy (AFM), and fluorescence microscopy. These methods probe liposome adsorption, fusion with a surface, lipid organization on a surface, and fluidity [50]. For silica beads solution-phase, available measurements are listed below.

1. The calcein leakage test is a commonly used assay for probing membrane integrity in biochemistry [51].

2. Fusion amount can be measured using rhodamine (Rh), nitrobenzoxadiazol (NBD), or other fluorophore-labeled liposomes [52]. But this can’t visualize hybrid structure. Most inorganic NPs can be precipitated using a common benchtop centrifuge, but free liposomes cannot. By quantifying the fluorescence in the supernatant, the amount of liposome wrapping silica can be measured as a function of buffer composition. Typically, NBD is labeled in a lipid tail and thus it does not interfere with liposome adsorption, but NBD has a low fluorescence quantum yield and is easily bleached. Rh and Texas red are much brighter dyes that are typically used to label the lipid head group, and control experiments are needed to ensure that adsorption is not through the dyes [50].
3. Cryo-TEM is a powerful technique for measuring the morphology of liposomes after adsorption [53]. Samples are prepared by quick freezing in liquid ethane, and both intact liposomes and NP-supported bilayers can be well resolved [17].

![Figure 1.08](image) Formation of silica beads supported lipid bilayer, by cartoon (left) and by cryo-EM (right) adapted from [17].

4. Cell uptake studies help confirm NP adsorption reaction [54]. Free PC liposomes are not internalized by cells because of their antifouling property, whereas liposome/NP complexes are often taken by cells.

5. Dynamic light scattering (DLS) is used to study the size and zeta potential of liposomes, NPs, and their complexes.

6. Differential scanning calorimetry (DSC) is powerful thermal chemistry methods for probing the lipid phase transition [42].

1.4 Interaction between silica beads and lipid bilayer

Liposomes can be attached to the support surface via covalent linkages, specific bio interactions (e.g., via DNA hybridization or biotin–avidin interactions), or simple
physiosorption (Figure 1.09). We are interested in studying physisorption because it is cost-effective and readily available to most researchers [50].

![Physisorption](image)

**Figure 1.09** SLB on SiO$_2$ based on the electrostatic and van der Waals force and a thin layer of water separating the two surfaces. Adapted from [50].

Depending on the composition, size, and surface chemistry of both liposomes and silica surfaces, various interaction mechanisms are proposed. The kind and strength of interactions between membrane and solid surface are very important for stabilization of the supported phospholipid layers on the surface, but they are still not well understood.

Lipid bilayer can interact with silica beads surfaces through a number of forces. Electrostatic interaction is probably the most dominant, where oppositely charged phospholipid moieties and silica beads are mixed. Silica beads have natural hydroxyl surface groups ($\approx 4$–$6$ –OH/nm$^2$ [55]) making them very hydrophilic. Two-dimensional NMR data suggest strong hydrogen bonding of silanol groups with O–P group of the polar head group of DPPC bilayer [56]. Van
The good stability of silica beads supported lipid bilayer is achieved through balanced interactions between the silica beads surface and the bilayer. A question therefore arises whether and how silica beads surface and these above interactions affect lipid bilayer properties listed below.

1. Transition temperature. This is characteristic of structural properties of lipid bilayers. Silica beads surface might cause perturbations to supported lipid phase transition.

2. Interleaflet distribution. Each leaflet in a SLB is in a different environment: the outer one is exposed to the bulk solution, while the inner one faces the solid surface. Though it is commonly assumed to be symmetrical, the interleaflet distribution induced membrane asymmetry has also been extensively investigated [59, 60].

3. Mobility. Lipid assemblies as a whole can be laterally mobile and undergo collective shape changes, to what extend they are immobile on silica surface need to be fully studied.

4. Lipid packing. It plays a critical role in various biological events. For instance, the functions of transmembrane proteins are affected by the lateral pressure exerted by the surrounding lipids, which is related to lipid packing [61]. Another example is that the binding of amphipathic helical peptides to membranes is sensitive to lipid packing [62]. Silica beads surface might have significant effect on the packing density.
5. Surface defects and its patterns on the SLB is a very important consideration for current and prospective applications. For example, the activity of lipid digesting enzymes was proposed to be triggered by much less than one percent of point-defects in membranes, membranes that contains a few percent of surface defects may be acceptable for protein adsorption studies [63].

6. Ion permeability. Silanol groups (Si-OH) are believed to induce electrostatic interactions with membrane proteins or the quaternary ammonium groups of phosphatidylcholine leading to membrane destabilization and thus changing its permeability [64].

In this project we mainly focus on the influence of silica support on electrostatics and local dielectric properties of biological membranes as well as effects on the trans-membrane protein-lipid interface studied by EPR-Active Molecular pH Probes. Although we have relatively rough understanding of gradients in polarity, electric potentials, and hydration in model biological membranes, very little is known about these properties at the membrane-water interface and protein-membrane interface and even less is known about these characteristics of lipid bilayers once they are incorporated in lipid-nano-structured hybrid materials. The solid support may alter dynamic behavior of the supported lipid bilayer, as well as change water penetration into bilayer thus altering local polarity and dielectric environment. Such changes would modify energetics of the lipid-protein interactions within the membrane and potentially affect protein insertion. Understanding of electrostatics in supported lipid bilayers is critical
for designing reliable and practical lipid-nanostructured hybrids for materials and biological applications.

1.5 SDSL EPR

EPR (electron paramagnetic resonance) is the most selective and highly sensitive spectroscopic technique to characterize molecular species that have at least one unpaired electron. Each electron possess a magnetic moment $\mu$ and has spin $S = 1/2$ with projection quantum number $m_s = \pm 1/2$. In the absence of the magnetic field these two energy states are degenerate. In the presence of the magnetic field the state with $m_s = +1/2$ moves up in energy $E_\alpha = (+1/2) g_e \mu B_0$ and the other state with $m_s = -1/2$ moves down in energy $E_\beta = (-1/2) g_e \mu B_0$ (Zeeman Effect). The two energy levels are separated by $\Delta E = g_e \mu B_0$ (Figure 1.10) where $g_e$ is the electronic g-factor, $\mu_B$ is the Bohr magneton and is $B_0$ the external magnetic field. Resonant absorption of radiation between these two energy states occurs when an electromagnetic field of the appropriate frequency matches the energy-level separation, $\Delta E = \hbar \nu = g_e \mu B_0$. At the resonance condition the rate of transitions from the lower to higher spin states are higher than the reverse, thus there is a net absorption energy which can be detected electrically and converted into the EPR spectra.
Many biological systems are EPR silent so SDSL (site-directed spin labeling) is developed and widely applied so that membrane lipids and proteins are amenable to study by EPR. In this project we employed stable free radicals nitroxide with small molecular volume that can be covalently attached via disulfide bonds to the to the sulfhydryl groups of lipids or peptides. The nitroxide possess an electronic spin that is delocalized primarily over N-O moiety. The most abundant $^{16}$O has no nuclear spin while the abundant $^{14}$N has nuclear spin $I=1$ (with projection quantum number $M_I= -1, 0, 1$) and can interact with the unpaired electron (hyperfine interaction) leading to splitting of unpaired electron EPR energy levels into three sublevels and resonance frequency gives rise to three lines in X-band (9.5 GHz) EPR (Figure 1.11).
Imidazoline and imidazolidine nitroxides are known for high sensitivity of EPR spectra to pH changes, tunability of the pKₐ range through introduction of various substituents into the nitroxides’ side chains, and the reversibility of pH effects [66]. They contains basic nitrogen functionalities in the heterocyclic ring and its protonation will result in a localized positive charge. Then a component of the positive internal electric field directed along the N-O bond will partially stabilize the nitrogen p orbital and destabilizing the oxygen p orbital, leading to a partial shift of the spin density from N to O, such spin density change will be reflected as magnetic parameters (such as hyperfine coupling constant) of the EPR spectra [4].
Another useful information that can be obtained from EPR parameters is the rotational correlation time $\tau_c$, the time necessary for the spin label to rotate through an angle of a radian. It is a mobility parameter in the time scale of 0.1 to 100 nsec that can be detected by EPR spectroscopy. Generally the slower rotational motion in radical, the broader EPR spectrum.

Figure 1.12 Reversible protonation of nitroxides attached to the side chain of biopolymer.

Figure 1.13 EPR line shape as a function of spin label motion. (A) Spin label in dilute solution – fast motion. (B) Spin label bond to a small peptide in solution. (C) Spin labeled peptide incorporated into a liposome – slow motion. (D) Spin labeled peptide in frozen sample. Adapted from [67]
Chapter 2 Effect of silica on surface electrostatic potential of phospholipid bilayer.

2.1 Introduction

Surface electrostatic potential has long been recognized to play a fundamental role in key processes of cell functioning. Membrane stability, flexibility and fusion, membrane transport, protein insertion and folding, energy generation and its utilization, depend strongly on the surface interactions.

A macroscopic description of the surface electrostatic potential is given by the Gouy-Chapman theory [68], which assumes that the phospholipid membrane is a featureless continuous medium and the lipid bilayer interface is a perfect impenetrable planar surface, and states that ion double layer is formed at a charged surface. Thus the interfacial potential at the charged surface is attributed to the presence of ions attached to the bilayer surface and to an equal number of ions of opposite charge in solution. Surface electrostatic potential is defined as a difference between the potential at the bilayer surface and the bulk. This theory gives the relationship between $\Psi_s$, the electrostatic potential at the surface, the bilayer surface charge density and the ionic concentration in the aqueous phase. The limitation of the theory is in treatment of the membrane as a homogeneous system.
A atomic-scale description of the surface electrostatic potential is given by the molecular dynamics (MD) simulations [69], which assumes each atom is the source of an electrostatic field and it “feels” the electrostatic field of all other atoms. MD simulations compute the forces between all atoms in the system and to study their motion at times up to hundreds of nanoseconds. However, this approach usually gives little insight into the fundamental physical processes in the membranes, since membrane is an integral assembly that possess integrated functional properties, thus numerous atomic details mask the general picture.

A middle level description of electrostatic properties is highly needed, but it is not easily achievable due to technical limitations associated with each method and problems arising in the comparison of results obtained by different methods. There are only limited analytical methods for assessing electrostatic properties of the biological interface. NMR [70, 71], electron-electron double resonance [72] atomic force [73, 74], interaction force measurements [75], fluorescent spectroscopy [76-79], , and spin probe [80-84]. All of these methods have some advantages as well as some limitations.

The first group of EPR method employs charged amphiphilic probe molecule that partition between the membrane and the aqueous phase. The partition coefficient is directly related to the Gibbs energy of transferring the probe from aqueous to membrane phase thus is dependent on the surface potential and can be determined by analyzing continuous wave (CW) EPR spectra of the probe and then the surface potential is derived through a calibration [85-87]. This method gives an accurate estimation of the relative populations of the probe bound to the
charged bilayer and free in the bulk solution. However the exact location of the measured potential with respect to the bilayer surface is unknown, and lipid composition may affect probe’s Gibbs energy and cause additional errors [4].

The second group of EPR and NMR methods is based on measurements of collision frequency between a freely diffusing charged nitroxide with another nitroxide attached to bilayer interface or a biomolecule such as protein and DNA [72, 88]. In NMR experiment the site-specific proton relaxation enhancement upon collisions of exposed residues with charged paramagnetic relaxers it measured [70, 89] This method gives an accurate estimation of electrostatic potential for small molecules [90], however the exact location is still uncertain for larger membrane system [4], and data sets obtained by NMR method do not yield consistent value [91].

The third group of NMR, fluorescence and EPR methods is based on observing reversible ionization of specific moieties [4]. NMR is mainly applied to protein and RNA samples [92, 93]. Fluorescence technique was utilized to measure the lipid bilayer electrostatic surface potential, this approach is based on observation of reversible ionization of molecular probes located at the bilayer interface upon pH titration, with spectral readout of the proton-exchange equilibrium directly assigned to the location of the probe. Fluorescence have very low concentration requirement [94], making this method highly popular, however, all the fluorescent probes for lipid bilayer investigated to this date are relatively bulky with easily polarized aromatic fragments [71] and are likely to cause some local perturbations to the
membrane interface. In addition, one of the most common fluorescent probe 4-alkyl-7-hydroxycoumarin was found to be located approximately within the ester group region of the lipid bilayer that the ionizable OH-group of coumarin is unavoidably positioned below the lipid phosphate moiety, such a location is deeper than one would wish to have for studying the bilayer surface potential [95].

Site directed spin-labeling EPR is a good alternative to fluorescence labeling of membrane system. Nitroxide are relatively small molecules and possess lesser dipolar moment than the fluorescent tags, thus introducing a minimal perturbation in the vicinity of the probe [96]. Besides, EPR spin labeling method provides complementary data on local structure and dynamics of biomolecules [97]. Lastly, it can be applied to nontransparent/opaque samples that are difficult to study with optical method.

In this project we employ two ionizable lipid-mimicking EPR probes [4, 94, 98, 99]. Small pH sensitive nitrooxide, either S-(1-oxy-2,2,3,5,5-pentamethylimidazolidin-4-yl)methyl methanesulfonothioate (IMTSL), or S-4-(4-(dimethylamino)-2-ethyl-5,5-dimethyl-1-oxy-2,5-dihydro-1H-imidazol-2-yl)benzyl methanethiosulfonate (IKMTSL) is covalently attached to the polar head of a synthetic phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (PTE) (Figure 2.01). In such pH sensitive lipid probe the acyl chain and the polar head regions mimic the phospholipid structure as close as possible. Due to its lipid like nature, the probe becomes an integral part of the lipid bilayer positioning within the bilayer in the same way as lipid molecules. Due to the compact volume of the tethered nitrooxide moiety, it causes minimal
perturbation to the lipid bilayer. Last but not least, the nitroxide moiety is positioned exactly at the membrane-water interface for surface electrostatic potential measurement.

**Figure 2.01** Chemical Structure of the non pH-sensitive nitroxide MTSL (S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate), pH-sensitive nitroxide IMTSL and IKMTSL, lipid PTE, synthetic spin-labeled lipid IMTSL-PTE and IKMTSL-PTE.
PTE-IMTSL and PTE-IKMTSL undergo reversible protonation at the tertiary amine during acid-base equilibrium at the membrane–water interface. Protonated and non-protonated forms of the nitrooxide moiety have different magnetic parameters resulting in different EPR spectra. Once positioned at the membrane-water interface, protonated and non-protonated forms of PTE-IMTSL and PTE-IKMTSL show different rotational dynamics that is directly detected by EPR spectra. Electrostatic potential of the membrane affects the concentration of ions in the immediate vicinity to the membrane surface and equilibrium of charged and uncharged species, thus the observed pKₐ of the probes is ultimately a function of the bilayer electrostatic surface potential.

For liposome/vesicle the observed pKₐ of the spin probe at membrane-water interface is different from pKₐ of the probe in aqueous phase, the observed interfacial pKₐᵢ contains contributions arising from the change in the Gibbs free energy ΔG upon transferring the probe from the bulk water into a media with a different electric permittivity E contributing to ΔG^{pol}, and a different local electric potential Ψ contributing to ΔG^{el}. The observed pKₐ of the probe is determined by both local electrostatic potential and the effective interfacial dielectric constant. Because pKₐ = −log₁₀ (Kₐ), where Kₐ is the equilibrium constant of protonation of the nitrooxide moiety tertiary amino group, the probe interfacial pKₐᵢ is given by [4, 76, 94, 95, 98, 99].

\[
pKₐᵢ = pKₐ^0 + ΔpKₐ^{pol} + ΔpKₐ^{el}
\]

Eq. 1
Where $pK_a^0$ is the intrinsic $pK_a$ of the spin probe in aqueous phase, $\Delta pK_a^{\text{pol}}$ and $\Delta pK_a^{\text{el}}$ are the polarity and electrostatic contributions, respectively. The electrostatic shift, $\Delta pK_a^{\text{el}}$, is related to the electrostatic surface potential, $\Psi$, as

$$\Delta pK_a^{\text{el}} = -\frac{e\Psi}{\ln(10) kT} \quad \text{Eq. 2}$$

Where $e$ is the elementary charge, $k$ is the Boltzmann’s constant, and $T$ is absolute temperature.

If the lipid bilayer is positioned on silica surface, another additive component is $\Delta pK_a^{\text{silica}}$ from silica surface charge electrostatic contribution. Thus the interfacial $pK_a$ of the probe can be calculated as

$$pK_a^i = pK_a^0 + \Delta pK_a^{\text{pol}} + \Delta pK_a^{\text{el}} \text{ or } \Delta pK_a^{\text{silica}} \quad \text{Eq. 3}$$

**Figure 2.02** Schematics of four models to investigate the effect of silica support on interfacial electrostatics of a phospholipid bilayer.

In this chapter, we employ CW EPR spectroscopy to monitor the change in protonation of the lipid-mimicking spin probes. By pH-titration, we experimentally determined the magnitudes
of intrinsic $pK_a^0$, interfacial polarity shift $\Delta pK_a^{\text{pol}}$, the shift $\Delta pK_a^{\text{el}}$ due to phospholipid membrane surface charge, and silica beads surface charge electrostatic contribution, $\Delta pK_a^{\text{silica}}$.

Since PTE-IMTSL and PTE-IKMTSL are not soluble in water, IMTSL and IKMTSL molecules were first modified with a mercaptoethanol (ME) to create IMTSL – 2-mercaptoethanol adduct and IKMTSL – 2- mercaptoethanol (Figure 2.03) to increase the molecule’s solubility in aqueous medium. Note that the PTE-IMTSL and PTE-IKMTSL has longer hydrophobic tails and ionizable phosphate moiety compared to the IMTSL-ME and IKMTSL-ME adduct. However, it was found that the tertiary amino functionality of spin labels are not affected by other substitutions or other ionizable groups, thus it is expected that the hydroxyethyl moiety of ME is mimicking the inductive effects of the PTE head group attachment rather well and the $pK_a$ value of IMTSL-ME and IKMTSL-ME in water provide a close estimate of the intrinsic $pK_a^0$ of PTE-IMTSL and PTE-IKMTSL [94]. Experiments were conducted and analyzed by Prof. M. Voinov and Dr. C. Scheid in our group and the results were published in [94, 98, 99].

![Chemical structure of IMTSL-ME and IKMTSL-ME.](image)

**Figure 2.03** Chemical structure of IMTSL-ME and IKMTSL-ME.

**Table 2.01** Intrinsic $pK_a^0$ of PTE-IMTSL and PTE-IKMTSL [94, 99].

<table>
<thead>
<tr>
<th>Spin labeling</th>
<th>Temperature °C</th>
<th>$pK_a^0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMTSL</td>
<td>20</td>
<td>3.33 ± 0.03</td>
</tr>
<tr>
<td>IKMTSL</td>
<td>21</td>
<td>5.98 ± 0.04</td>
</tr>
</tbody>
</table>
The IMTSL-ME and IKMTSL-ME adducts were then dissolved into a series of water-isopropyl alcohol solutions with different volume ratio so that the dielectric constants $\varepsilon$ of the homogeneous solutions were known, to build a calibration between dielectric constant $\varepsilon$ and the interfacial $pK_a$. Experiments carried out and analyzed by Prof. M. Voinov and Dr. C. Scheid to examine dependence of $pK_a$ versus bulk $\varepsilon$, and the results were published in [94, 98, 99].

<table>
<thead>
<tr>
<th><strong>Table 2.02</strong> Titration data for spin label adducts in buffer/isopropyl alcohol solutions of various composition [94, 99].</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IMTSL-ME</strong></td>
</tr>
<tr>
<td><strong>T = 20°C</strong></td>
</tr>
<tr>
<td>Bulk $\varepsilon$</td>
</tr>
<tr>
<td>$pK_a$</td>
</tr>
<tr>
<td>$pK_a = 0.2461(±0.1104) + 0.0383(±0.0019) x \varepsilon$</td>
</tr>
</tbody>
</table>

| **IKMTSL-ME** | [i-PrOH], v/v % | |
| **T = 17°C** | 0 | 20 | 30 | 40 | 50 | 60 |
| Bulk $\varepsilon$ | 81.52 | 69.31 | 62.80 | 55.98 | 48.84 | 41.34 |
| $pK_a$ | 6.16 ± 0.03 | 5.67 ± 0.03 | 5.21 ± 0.02 | 4.95 ± 0.02 | 4.62 ± 0.03 | 4.28 ± 0.03 |
| $pK_a = 2.2996(±0.1033) + 0.0475(±0.0017) x \varepsilon$ | $R^2 = 0.995$ |

It is indicated that the experimental $pK_a$ is primarily affected by the changes in the bulk dielectric permittivity, $\varepsilon$, of the solvent and that the linear relationship between the probe $pK_a$ and the bulk $\varepsilon$ is temperature-independent within the biologically relevant 17– 48 °C interval [99]. Thus, it is proposed that in the absence of external electric fields IMTSL-ME and IKMTSL-ME could be used as an EPR molecular probe of local effective dielectric permittivity $\varepsilon$. Later we employ these two calibration curve for the calculation of interfacial $\varepsilon$ of PTE-IMTSL and PTE-IKMTSL at 21°C.
Figure 2.04 $pK_a$ of IMTSL-ME and IKMTSL-ME vs bulk dielectric constant $\varepsilon$ of water–i-PrOH mixtures. The corresponding data for IMTSL-ME is shown as filled squares, for IKMTSL-ME is shown as filled triangle. The error bars are comparable with the size of symbols. The corresponding linear regressions are shown as solid lines with parameters given in table 2. Reproduced from [94, 99].

The spin labels were then covalently attached to the head groups of a phospholipid mimetic, 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (PTE). The spin labeled PTE molecules were incorporated into Triton X micelles, the nonionic surfactant Triton X-100 lacks any charged moieties, forming micelles that can be employed as a nonpolar electrically neutral reference interface between aqueous phase and lipidlike phase, thereby eliminating any shift in the observed $pK_a$ due to electrostatic effects, left $\Delta pK_a^{\text{pol}} = pK_a^i - pK_a^0$, allowing for calculations of $\Delta pK_a^{\text{pol}}$ and estimation of local dielectric constant $\varepsilon$ at the lipid-buffer interface from the empirical calibration curve given in Table 2.02. The PTE-IKMTSL reports on slightly
higher effective dielectric constants at the surface of the micelles than PTE-IMTSL, this is because IKMTSL structure is more bulky compared to IMTSL and its linker head protrudes further into the aqueous phase where local dielectric constant $\varepsilon$ increases closer to polar bulk water dielectric constant $\varepsilon$ [99]. Notably, $\varepsilon = 60$ of Riske et al. [84] is the same as $\varepsilon \approx 60$ estimated for the interfacial location of the reporter nitroxide of IMTSL-PTE incorporated into Triton X-100 micelles.

![Figure 2.05](image)

**Figure 2.05** Chemical structure of Triton X-100 detergent (left), cartoon illustrating spin labeled PTE lipids incorporated in Triton X-100 micelle.

**Table 2.03** Interfacial $pK_a^{i}$, intrinsic $pK_a^{0}$, polarity induced shifts $\Delta pK_a^\text{pol}$, and local dielectric constant $\varepsilon$ measured and calculated for Triton X-100 with 1% PTE-IMTSL and PTE-IKMTSL [94, 99].

<table>
<thead>
<tr>
<th>Spin labeling</th>
<th>$pK_a^{i}$</th>
<th>$pK_a^{0}$</th>
<th>$\Delta pK_a^\text{pol}$</th>
<th>$\varepsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMTSL</td>
<td>2.52 ± 0.01</td>
<td>3.33 ± 0.03</td>
<td>-0.81 ± 0.03</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>IKMTSL</td>
<td>5.40 ± 0.03</td>
<td>5.98 ± 0.04</td>
<td>-0.58 ± 0.04</td>
<td>65 ± 3</td>
</tr>
</tbody>
</table>

The spin labeled PTE molecules were then incorporated into POPC or POPC/POPG liposomes. Using previously determined intrinsic $pK_a$ of the probes and experimentally determined shift in $pK_a$ due to polarity effects, $\Delta pK_a^\text{el}$ was determined using Eq. 3.
Finally, POPC or mixture of POPC and POPG liposome with spin labeled PTE were coated onto silica beads, and using the previously determined contributions to observed pK$_a$, $\Delta$pK$_a$$_{\text{silica}}$ from Eq.3 was determined.

These titration experiments lead to a wealth of information pertaining to the surface electrostatic properties. In this chapter, we report on: 1) the effect of the linker and the nature of the nitroxide probe (IKMTSL vs IMTSL) on the measurements of the membrane surface potential of large unilamellar vesicles (LUVs), 2) the effect of lipid composition (the fraction of negatively charged lipids) and silica support on the membrane surface potential of LUVs, and 3) effect of silica support on the structure and mobility of silica supported phospholipid bilayer.

2.2 Materials and methods

2.2.1 Materials.

PTE (phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol), zwitterionic lipid POPC (1-hexadecanoyl-2--(9Z-octadecenoyl)-sn-glycero-3-phosphocholine) and anionic lipid POPG (1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)) were purchased from Avanti polar lipids (Alabaster, AL) as chloroform solutions (>99% pure) and stored at -80°C freezer before use without further purification. All solvents were reagent grade and used as received. MTS, IMTSL, IKMTSL, PTE-IMTSL and PTE-IKMTSL were synthesized by Prof. M. Voinov as previously reported [4, 94, 98, 99]. A
non pH-sensitive analog PTE-MTSL were synthesized from PTE and MTSL and crude product was purified similar to previous published procedure [4, 94, 98, 99]. Non-porous and non-functionalized silica beads (310nm, \( \sim 3.407 \times 10^{12} \) beads/ml) were purchased from Bangs Laboratories (Fishers, IN) in 10 wt. % solids in DI water and used as received without further surface cleaning. Silica beads have a size coefficient of variation (CVs) of 10-15%. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Water used for preparation of the liposome and silica supported lipid bilayer suspensions and buffer solution was from Milli-Q system (resistivity 18.2 M\( \Omega \)cm).

2.2.2 Liposome preparation.

The liposomes composition was 5 w/v % desired lipids (POPC, or POPC80:POPG20 mixture) doped with 1 mole % of desired spin labeled lipids (PTE-MTSL, PTE-IMTSL or PTE-IKMTSL) and an average of 10 gA channels per 100nm vesicles. The stoichiometrically calculated amount of lipids mixture were added into a 5-ml glass conical vial, and organic solvent chloroform was removed by evaporating the sample under a nitrogen flow while rotating the vial, leading to the formation of a thin, even film on the inner walls of the vial. The sample was then placed in a vacuum desiccator under low pressure and left overnight. The lipid film was rehydrated the following day by adding appropriate amount of 0.5 x PBS buffer solution at pH=7.5 (composition is NaCl 137mM, KCl 2.7mM, Na\(_2\)HPO\(_4\) 10mM, KH\(_2\)PO\(_4\) 1.8mM). The resulting lipid suspension was mixed on a vortex mixture for 1min and subjected to 10 freeze-thaw cycles between quick freezing by liquid nitrogen and melting in a water bath at 37°C to disrupt large aggregates of lipids and form 5% w/v suspension of multilamellar
vesicles (MLVs). MLVs were stored at -80°C freezer for later experiments. Unilamellar vesicles (LUVs) were prepared by extruding the MLVs sample using polycarbonate membranes with 100nm pores and mini extruder (Avanti polar lipids, Alabaster, AL) at room temperature. The resulted clear ULVs solutions were transferred to a clean 1.5mL Eppendorf plastic vial and stored at 4°C fridge at neutral pH no more than 3 days to guarantee sample freshness.

Figure 2.06 Cartoon illustrating vesicle formation mechanism, adapted from https://avantilipids.com/tech-support/liposome-preparation/.

2.2.3 Liposome characterization.

Liposome size was characterized by dynamic light scattering (DLS) size measurement. Diluted LUVs and SUVs suspension (0.25 w/v %) were placed into a polystyrol/polystyrene cuvette
(10*10*45 mm) and DLS measurements were performed on a Zeta Sizer Nano ZS (Malvern Instruments Inc., Westborough, MA) instrument. Dynamic light scattering is a non-invasive technique for measuring the size of particles and molecules in suspension. The maximum count rate for the measurement was kept at 500-600 kcps, thus the LUVs and SUVs concentration was controlled at 0.25 w/v %.

2.2.4 Silica beads supported lipid bilayer preparation.

The amount of lipid required to achieve single bilayer coverage was calculated using the surface area occupied by the lipid head group (0.64 nm$^2$ for POPC and POPG [100]) and the total surface area of the nanobeads, with the assumption that the latter was a planar surface. MLV were first diluted by Trizma buffer at pH=7.4 (composition is Tris 10mM, NaCl 200mM) to 10mM/mL concentration in a small glass conical vial (because glass transfer sonication efficiently than plastic container). Then small unilamellar vesicles (SUV) were prepared with titanium tip sonicator (Misonix, Ultrasonic Processors, S-4000, USA) at 30% power amplitude, utilizing 30sec sonication/ 30 sec resting time sequence till the sample achieved optical clarity. To prevent sample overheating during sonication, the glass conical vial was placed into an ice-water bath in a small beaker. The lipid suspension was transferred to a centrifuge vial and was spun down for 30min at 25000 x g to remove the titanium particles from sonication tip if any. Next, the resulting clear SUVs were transferred to a 1.5mL Eppendorf plastic vial, combined with equal volume of silica beads solution and placed on a rotating shaker for 3h. Using this approach, the SUV are reported to spontaneously collapse into a continuous bilayer surrounding the silica beads, besides, it has been observed that after
formation of single-supported lipid bilayer wrapping silica beads, further engulfment by another vesicle did not occur [41]. It was found that mixing of approximately equal volumes of the two materials was preferable to the addition of small quantities of one into the other; the latter sometimes resulted in precipitation of the nanoparticles, possibly due to initial inhomogeneity upon mixing [34]. Following reassembly, the mixture was centrifuged for 10min at 1000xg. After discarding the supernatant, the lipid bilayer coated silica beads were re-suspended in 0.5 x PBS buffer (composition is Na$_2$HPO$_4$ 5mM, KH$_2$PO$_4$ 0.9mM, NaCl 68mM, KCl 1.3mM) at pH=7.4. Centrifugation and re-suspension steps were repeated 6 times to remove all remaining unbound SUVs, resulting in single lipid bilayer covered silica beads. The top supernatant after each wash was tested by CW EPR, showing a nitroxide signal intensity decay to zero after the last wash, indicating that all SUVs were removed from the prep. After each washing, the fraction containing silica beads supported lipid bilayer was tested by CW EPR. The nitroxide signal intensity was determined to be approximately constant after resuspension in a few last steps, confirming stable SLB formation. Samples were then stored at 4 °C at neutral pH for no more than 3 days to guarantee freshness.

2.2.5 Silica beads supported lipid bilayer characterization.

For DLS zeta potential measurement, the silica beads suspension in 0.5 x PBS buffer at various pH and silica supported lipid bilayer suspension were placed into folded capillary cell (DTS1070) and performed on a Zeta Sizer Nano ZS (Malvern Instruments Inc., Westborough, MA) instrument.
2.2.6 Liposome pH titration experiments.

In all of the experiments, the pH values were measured at room temperature with an Orion microcombination pH electrode 98 series (Thermo Electron Corporation, Beverly, MA) with four points calibrated using standard VWR (VWR International) solutions: at pH=1.68, 4.00, 7.00, 10.00. Approximately 80uL of lipid suspension (5 w/v %) was placed into a 1.5mL Eppendorf tube and pH of the solution was adjusted by titration with aliquots of 0.25M, 0.10M, 0.05M, 0.025M HCl solution or NaOH solution to reach the desired pH. All of the titration experiments pH of lipid samples was proceeded from high-to-low, and samples were measured at increments of about 0.3pH units. Samples were lightly vortexed after addition of the abovementioned titrant and sit at room temperature for approximately 25min to assure complete equilibration of the inner vesicle space and outer water bulk solution.

2.2.7 Silica beads supported lipid bilayer pH titration experiments.

Unlike liposome pH titration experiment by directly adding aliquot of HCl or NaOH titrant, pH of silica beads supported lipid bilayer was controlled by incubation with a set of 0.5 x PBS buffers at pH of increments of 0.3 pH units. Silica beads covered by single lipid bilayer in 0.5 x PBS buffer at pH=7.4 were centrifuged for 10min at 1000 x g. After discarding the supernatant, sample were re-suspended in 0.5 x PBS buffer at desired pH and sit at room temperature for 8min and this step was repeated 3 times to assure complete pH equilibrium. The final pH value was recorded by measuring the supernatant of the last washing. All of the titration experiments pH was proceeded from high-to-low, and samples were measured at increments of about 0.3 pH units.
2.2.8 CW-EPR measurements.

Liposome samples or concentrated silica supported lipid bilayer samples were drawn into polytetrafluoroethylene capillary (i.d. = 0.81 mm, o.d. = 1.12 mm, NewAge Industries, Inc., Southampton, PA) subsequent to pH equilibration. The capillaries were then folded and inserted into a 3 × 4 mm clear fused quartz tube open from both ends (VWR International, Radnor, PA). Continuous wave (CV) measurements were conducted on a Bruker (Billerica, MA) ELEXSYS E500 spectrometer at approximately 9.5 GHz (X-band) at room temperature. 2048 point spectra were collected over a 100 G range with center around 3500 G. The modulation amplitude was 1 G and the modulation frequency was 100 kHz. The time constant was set at 20.48 ms with a 30.65 ms conversion time and a 30 s sweep time. The incident microwave power was 2 mW. Normally, about 30-100 scans were averaged for each sample.

2.2.9 CW-EPR characterization of local dynamics of 5-doxyl-SA incorporated into liposomes and silica beads supported lipid bilayers.

Samples were prepared with 1% 5-doxyl stearic acid as abovementioned. Continuous wave (CW) X-band (9.5 GHz) EPR spectra were acquired with a Varian (Palo Alto, CA) Century Series E-109 spectrometer. Typical spectrometer settings were similar to the EPR titration measurement above. Variable temperature were obtained using a temperature controller described previously [101]. Sample temperature was measured with a VWR International (West Chester, PA) digital thermometer equipped with a stainless steel microprobe positioned in the cavity just above the sample. The VWR thermometer has a resolution of 0.001 °C and accuracy of ±0.05 °C.
2.2.10 Measurements of accessibility to nitroxide probe by monitoring the kinetics of nitroxide reduction by ascorbic acid or sodium ascorbate.

The freshly prepared samples were placed in a 1.5mL Eppendorf vial and mixed with sodium ascorbate at pH=7.4 or sodium ascorbic acid pH=3.0 solution in an appropriate ratio and immediately transferred to a micro capillary tube and inserted straightly into a 3 × 4 mm clear fused quartz tube open from one ends (VWR International, Radnor, PA). Continuous wave (CV) measurements were conducted on a Bruker (Billerica, MA) ELEXSYS E500 spectrometer at approximately 9.5 GHz (X-band) at room temperature and 2D spectra were collected versus time. Spectrometer settings were same to the EPR titration measurement above.

Figure 2.08 Reduction of a nitroxide radical with sodium ascorbate produces an EPR-silent hydroxylamine.

Figure 2.07 Chemical structure of 5-doxyl stearic acid.
2.3 Results and discussions

2.3.1 Choice of lipids for model bilayers.

Phosphocholine (PC) lipids deposited on silica is the most commonly used system because PC lipids represent a major component of the eukaryotic cell outer membrane [102]. Small unilamellar liposomes prepared from PC lipids readily fuse with silica under physiological conditions [31, 42, 103-106]. The PC head group contains a choline and a phosphate. Choline is a quaternary ammonium cation and is always positively charged. Phosphate has a pK$_a$ of less than 2, in the pH range used in most of the experiments, the phosphate is deprotonated and negatively charged. Therefore, the PC head group has a net charge of zero. To investigate effect of lipid composition on electrostatics we also utilized anionic lipid phosphatidylglycerol (PG), the most abundant negatively charged lipid in prokaryotic membranes, to a maximum of 20 mol % content, since exceeding this amount of anionic lipid may perturb SLB stability [17]. It should be noted that the anionic lipid contents of cell plasma membranes often exceed 20%.

In this project we are interested in biologically relevant fluid phase lipid system under a wide range of pH and ionic strength conditions at room temperature, thus we favor lipids with low phase transition temperature. We also ensure that bilayer remains in fluid phase when lipid bilayer are coated onto silica support. For a fixed head group structure, the hydrophobic tails of lipids can take many different forms and govern the phase of the lipid bilayer. Lowering T$_m$ can be achieved by decreasing the chain length or increasing the number of unsaturated bonds in the chains. PTE lipids have two saturated C16 carbon tails, same as DPPC (1,2-dipalmitoyl-
sn-glycero-3-phosphocholine) and DPPG (1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)), but $T_m$ of these two lipids are as high as 41°C. DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)) with shorter chain length C14 still have $T_m = 23^\circ$C. Thus we selected POPC ((1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine)) and POPG (1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)) as our model lipid bilayer. POPC and POPG mimic mammalian phospholipid composition since it has one saturated chain in the sn-1 position and one unsaturated chain in the sn-2 position, here POPC and POPG bilayers can serve as good model for membranes and silica supported phospholipid membranes.

**Figure 2.09** Chemical structure of PTE, POPC and POPG lipids.
2.3.2 Size distribution and stability of liposomes.

The Z-Average diameter of liposomes was obtained is an intensity-weighted mean diameter derived from the cumulants analysis. For liposomes prepared by probe sonication and measured shortly after preparation, the size was determined to be about 50nm. For liposomes prepared by extrusion through 100nm membrane and measured shortly after, the homogenous size was consistent and was determined to be around 135nm. 135 nm result is somewhat larger than 100nm membrane pore size, because DLS measures the hydrodynamic diameter which includes the hydration sphere (surrounding water and salt). Thus DLS size, in general, depends on the size of the liposome core, the surface structure, and the type and concentration of ions in the medium. The width of size distribution reported here is the intensity-weighted size distribution. Though some researchers use volume or number size distribution converted from intensity, DLS tends to overestimate the width of the peaks in the distribution and this effect can be further magnified by the transformations to volume and number. Previously, Dr. A. Koolivand and Dr. M. Donohue in our group had demonstrated that no significant changes of vesicle size was observed when the pH of the samples changed from 7.3 to 2.8, and the size of the liposomes remained constant over the time period of 16-18 hours for samples kept at room temperature, thus it can be concluded that the liposome samples are stable under the experimental conditions.

Figure 2.10 Size distribution by intensity of POPC LUVs as measured by DLS
2.3.3 Control experiments.

Before incorporating PTE-IMTSL and PTE-IKMTSL spin labeled lipids into charged lipid vesicles and coating on silica, experiment of PTE-MTSL nonionizable nitroxide labeled lipids were first used as control to check for the following effects: (1) effect of lipid phosphatidyl group protonation below pH = 2.0 on lipid packing and nitroxide tumbling is negligible; (2) the immediate environment (i.e. microviscosity and polarity) of nitroxide tethered to the lipid polar head is not affected by pH, thus the ionizable nitroxide mobility change is only affected by protonation upon pH titration. In addition it can be noticed that spectra of silica supported lipid bilayer is broader than spectra of liposome, details will be discussed later in section 2.3.8.

![Figure 2.11 Representative intensity normalized CW X-band EPR spectra from pH titration control experiments of POPC 100nm LUVs doped with 1 mol % of non pH-sensitive PTE-MTSL (left), and silica supported lipid bilayer (right).](image-url)
2.3.4 Determination of the electrostatic shift $\Delta pK_a^{el}$ at the liposome surface.

For both PTE-IMTSL and PTE-IKMTSL, spin labeled lipids were incorporated into (A) zwitterionic lipid POPC and (B) mixed 80 mol % POPC with 20 mol % anionic lipid POPG to investigate effect of lipid surface charge on interfacial electrostatics. Representative spectra (normalized by intensity) for each spin labeled lipid are shown in Figure 2.12 and 2.13.

The EPR spectra displayed in Figures 2.12-2.13 demonstrate that the spectral line shape is altered upon adjustment of the bulk pH of the sample. As the pH of the sample is lowered, the most apparent change in the EPR spectra is the progressive rise of the low-field shoulder around 3490 G. This feature is characteristic of slower and more restricted nitroxide tumbling. Lowering pH results in protonation of nitroxide probe. Electrostatic interactions (charge-charge and charge-dipole) between the positive protonated nitroxide and lipid bilayer surface, results in decreases of the nitroxide tumbling rate.
Figure 2.12 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of 1 mol % pH-sensitive PTE-IMTSL lipid incorporated into 100nm LUVs composed of POPC (left) and POPC 80% : POPG 20% (right).

Figure 2.13 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into 100nm LUVs composed of POPC (left) and POPC 80% : POPG 20% (right).
At the higher pH all nitroxides exist as non-protonated form and at the lower pH all nitroxides exist as protonated form, while at the intermediate pH spectra are a superposition of both forms. The intensities of each form can be obtained by simulating EPR spectra at different pH levels using reference spectra corresponding to protonated and non-protonated forms by EWVoigt program developed by Dr. Smirnov. This simplified slow chemical exchange model assumes a linear superposition of the spectra from the non-protonated and protonated specie, \(F_N(B)\) and \(F_{NH^+}(B)\) respectively.

\[
E(B) = a \cdot F_N(B) + b \cdot F_{NH^+}(B) \quad Eq. 4
\]

For the decomposition procedure, spectra \(F_N(B)\) and \(F_{NH^+}(B)\) are measured experimentally and then coefficients \(a\) and \(b\) are derived in a course of least-squares procedure involving continuous adjustment of the spectral positions due to a shift in the resonator frequency. In addition, the phases of \(F_N(B)\) and \(F_{NH^+}(B)\) were allowed to vary to account for some admixture of an out-of-phase dispersion component that could be present in EPR spectra from liquid aqueous samples [123]. Examples of a spectral decomposition is shown in Figure 2. 14. The fit residuals, difference between the experimental and simulated spectra show only negligible deviations, thus demonstrating the validity of this model for liposome samples.

The fraction of the non-protonated form of the nitroxide \(f\) is then calculated from the double integrals \(D_N\) and \(D_{NH^+}\) of the corresponding \(F_N(B)\) and \(F_{NH^+}(B)\) spectra, and was plotted against pH to yield the titration data points.

\[
f = \frac{D_N}{D_N + D_{NH^+}} \quad Eq. 5
\]
Figure 2.14 Decomposition of EPR spectrum from PTE-IKMTSL incorporated into POPC 80 mol %:POPG 20 mol % vesicles. Experimental spectra acquired at intermediate pH (black). Component of the experimental spectra corresponding to the neutral form of IKMTSL (blue). Component of the experimental spectra corresponding to the charged form of IKMTSL (red). The residual of fitting the experimental spectra to the sum of the simulated spectra (green).
Liposome with diameter larger than 100nm can be treated as planner samples and the curvature effect on lipids packing density can be neglected. Lipid bilayer is highly impermeable to most ions and polar molecules, so gramicidin A (gA) was incorporated into liposomes to assure a complete pH equilibration between bulk and inner aqueous volume of liposomes. gA consists of 15 amino acids and forms β-helix. In lipid bilayers gA form a dimer with another gramicidin molecule to form an ion channel. One half of the dimer situates itself on one side of the membrane bilayer while the other half of the dimer is joined to it while sitting in the other half of the membrane bilayer. A single channel opening event may allow flux of ions approximately $10^{6-7}$ [107]. Thus we expect evenly distributed spin labeled PTE-IMTSL or PTE-IKMTSL in both inner and outer leaflets to experience the same pH and exactly the same surface electrostatics environment and have equal pK$_{a}$ values.

**Figure 2.15** Cartoon illustrating spin labeled PTE lipids incorporated into 100nm LUVs.
The titration data were plotted as fraction of neutral spin labels vs. pH and were fitted to the Henderson-Hasselbach equation (for derivation, see equations 6 through 11) the fitting results are shown in Figures 2.16-2.17.

\[
pH = pK_a + \log \frac{[N]}{[NH^+]} \quad \text{Eq. 6}
\]

\[
\frac{[N]}{[NH^+]} = 10^{pH-pK_a} \quad \text{Eq. 7}
\]

\[
f = \frac{[N]}{[NH^+] + [N]} \quad \text{Eq. 8}
\]

\[
\frac{[N]}{[NH^+]} = \frac{f}{1-f} \quad \text{Eq. 9}
\]

Thus

\[
\frac{[N]}{[NH^+]} = \frac{f}{1-f} = 10^{pH-pK_a} \quad \text{Eq. 10}
\]

\[
f = \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad \text{Eq. 11}
\]

where \([N]\) represents the concentration of non-protonated form of nitroxide, \([NH^+]\) represents the concentration of protonated form of nitroxide, and \(f\) represents the fraction of the non-protonated form of the nitroxide.

The interfacial \(pK_a\)'s of the spin labels were determined from the fits and are summarized in the Table 2.04-2.05.
Figure 2.16 pH titration curve of 1 mol % pH-sensitive PTE-IMTSL lipid incorporated into 100nm LUVs composed of POPC (blue) and POPC 80 mol % : POPG 20 mol% (red). Dashed lines are the best fit to single dissociation model Eq.11.

Table 2.04 Experimental $\Delta pK_a^{el}$ determined from the pH titration and the surface electrostatic potential, $\Psi$ calculated for IMTSL-PTE liposome samples.

| Lipids composition mol % | $pK_a^i$ | $\Delta pK_a^i$ | $\Delta pK_a^{pol}$ | $\Delta pK_a^{el}$ | $\Psi_{exp}$, mV | $\Psi_{GC}$, mV $A_L=0.53\AA^2$ | $\Psi_{GC}$, mV $A_L=0.70\AA^2$
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>3.53</td>
<td>±0.01</td>
<td>3.33</td>
<td>3.33</td>
<td>-0.81</td>
<td>1.01 ± 0.04</td>
<td>1.30 ± 0.04</td>
</tr>
<tr>
<td>POPC80:POPG20</td>
<td>3.82</td>
<td>±0.03</td>
<td>3.03</td>
<td>3.03</td>
<td>0.03</td>
<td>1.30 ± 0.04</td>
<td>1.75 ± 0.04</td>
</tr>
</tbody>
</table>

$\Psi_{GC}$ values are taken from [94, 99]

$* \Psi_{GC}$, $\Psi_{GC}$ surface electrostatic potential predicted by the GC theory using areas per polar head group as indicated.
Figure 2.17 pH titration curve of 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into 100nm LUVs composed of POPC (blue) and POPC 80% : POPG 20% (red). Dashed lines are the best fit to single dissociation model Eq. 11.

<table>
<thead>
<tr>
<th>Liposome composition mol</th>
<th>pKₐ</th>
<th>†pKₐ₀</th>
<th>‡ΔpKₐpol</th>
<th>ΔpKₐₑl</th>
<th>Ψₑₓp</th>
<th>*ΨGC, mV</th>
<th>#ΨGC, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>5.73±0.02</td>
<td>5.98±0.04</td>
<td>-0.58 ±0.04</td>
<td>0.33 ±0.06</td>
<td>-19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>POPC80:POPG20</td>
<td>6.23±0.02</td>
<td>6.43±0.04</td>
<td>0.83 ±0.06</td>
<td>-48</td>
<td>-79</td>
<td>-66</td>
<td></td>
</tr>
</tbody>
</table>

†pKₐ₀, ‡ΔpKₐpol values are taken from [94, 99]
*ΨGC, †ΨGC surface electrostatic potential predicted by the GC theory using areas per polar head group as indicated.
The electrostatic terms, $\Delta pK_a^{el}$ of the vesicles doped with either PTE-IMTSL or PTE-IKMTSL and composed of either 100 mol % POPC or POPC 80 mol % : POPG 20 mol % lipids were readily calculated using the values for $pK_a^i$, $pK_a^0$ and $\Delta pK_a^{pol}$ and Eq. 1. The surface electrostatic potentials, $\Psi_{exp}$ were calculated using the value for $\Delta pK_a^{el}$ and Eq. 2, and are summarized in Table 2.04 and Table 2.05.

With the same nitroxide spin labeled lipids either PTE-IMTSL or PTE-IKMTSL, vesicles with 20% negatively charged POPG mixture show higher interfacial $pK_a^i$ and more negative surface electrostatic potentials $\Psi_{exp}$. For spin labeled PTE-IMTSL lipids, the calculated surface electrostatic potential for POPC 80 mol % : POPG 20 mol % is $\Psi_{exp} = -75 \pm 2$ mV is lower than that calculated for 100 mol % POPC $\Psi_{exp} = -58 \pm 2$ mV by the difference of $-17 \pm 3$ V. Similarly, for spin labeled PTE-IKMTSL lipids, the calculated surface electrostatic potential reported for POPC 80 mol % : POPG 20 mol % $\Psi_{exp} = -48 \pm 3$ mV is lower than that for 100 mol % POPC $\Psi_{exp} = -19 \pm 3$ mV by the difference of $-29 \pm 4$ mV. This is expected as addition of negatively charged lipids increases surface charge density of the vesicles.

Zwitterionic lipids POPC has polar head groups contain spatially separated charges of opposite signs, the protonated nitroxides attached to PTE lipids are expected to be firstly influenced by the positively charged trimethylammonium groups of the choline moiety closer to nitroxides and secondly attracted by the negatively charged phosphate moiety, together leading to charge-dipole interactions that can be observed from the spectral line shape changes. Assuming that the phosphorus and nitrogen atoms are the centers of scattering of each of these roughly
spherical fragments and a phosphate-nitrogen distance of 4.5 Å is obtained from the crystal structure of POPC, the dipole is calculated to be canted with an angle of 22 ± 4° with respect to the bilayer surface [108]. Besides, the positively charged trimethylammonium groups attract negatively charged ions and the negatively charged phosphate moiety attract positively charged ions from the bulk buffer solution, thus establishing a diffuse double layer which provides an electrostatic screening for the protonated nitroxide, the measured interfacial pK$_a$ is controlled by interplay of charge-dipole interaction and electrostatic screening. For 20% anionic lipids POPG mixture, extra Coulombic potential between protonated nitroxide and negatively charged phosphate moiety of POPG arises, in addition to charge-dipole potential. Thus the negative electric potential of 20 mol % POPG mixture is greater resulting in lower surface potential. Liposome model with different lipids composition are shown in Figure 2.18

**Figure 2.18** Cartoon illustrating POPC 100 mol % liposome doped with spin labeled lipid (left) and POPC 80 mol% : POPG 20 mol % POPG liposome doped with spin labeled lipids (right).

The measurements of the surface electrostatic potential of same lipid bilayers by PTE-IMTSL and PTE-IKMTSL yield different values. For 100 mol % POPC liposome, the calculated
interfacial potential reported by IMTSL $\Psi_{\text{exp}} = -58 \pm 2 \text{ mV}$ is lower than that reported by IKMTSL $\Psi_{\text{exp}} = -19 \pm 3 \text{ mV}$ by difference of $-39 \pm 4 \text{ mV}$. Similarly, for POPC 80 mol % : POPG 20 mol % liposome, the calculated interfacial potential reported by IMTSL $\Psi_{\text{exp}} = -75 \pm 2 \text{ mV}$ is lower than that reported by IKMTSL $\Psi_{\text{exp}} = -48 \pm 3 \text{ mV}$ by difference of $-27 \pm 4 \text{ mV}$. It is hypothesized that the main factors contributing to this difference are the chemical structures of the linker and the reporter nitroxide. Approximate sizes of the nitroxide molecules were determined by the molecular mechanics method using Hyper Chem Pro 6.3 (Hypercube Inc., Gainesville, FL, USA), the molecular volume for IMTSL moiety is approximately 0.62nm (length) x 0.54nm (height) x 4.4nm (width), for IKMTSL moiety is approximately 0.71nm (length) x 0.67nm (height) x 7.1nm (width) [109]. The linker length of IMTSL is approximately 0.9nm while for IKMTSL it is approximately 1.1nm as shown in figure 2. PTE-IKMTSL is expected to be more “stiff” and less prone to bending toward the lipid bilayer interface than PTE-IMTSL, thus PTE-IKMTSL will protrude further into the bulk aqueous solution away from the charged lipid bilayer interface. Such a location of the ionizable moiety with respect to the bilayer interface is expected to diminish the stabilizing effect of the negative electrostatic potential on the protonated nitroxide species, reduce the observed interfacial $pK_a^i$, and therefore result in a smaller electrostatic term, $\Delta pK_a^{el}$. In other words, the nitroxide of IKMTSL-PTE reports on a local electrostatic potential within a plane that is further away from the bilayer surface and where the electrical charges of lipids bilayer are better screened by counter ions [99]. This hypothesis is further supported by higher values of the effective dielectric permittivity constant reported by IKMTSL-PTE incorporated into nonionic Triton X-100 micelles versus those reported by IMTSL-PTE. This results also agree with spin labeled
lipids incorporated into MLV DMPG previously reported in our group. The magnitudes of the negative surface electrostatic potentials, $\Psi$, for MLV DMPG doped with PTE-IKMTSL $\Psi(17 \degree C) = -128 \pm 3 \text{ mV}, \Psi(48 \degree C) = -120 \pm 3 \text{ mV}$) were found to be measurably lower than those obtained from the EPR titrations of the PTE-IMTSL $\Psi(17 \degree C) = -183 \pm 5 \text{ mV}, \Psi(48 \degree C) = -161 \pm 3 \text{ mV}$) [99].

![Chemical structure](image)

**Figure 2.19** Compared to IMTSL (left), IKMTSL has longer linker and more “stiff” structure.

For 100 nm POPC LUVs, the $pK_a$ values reported by PTE-IMTSL and the magnitude of the calculated corresponding surface electrostatic potentials $\Psi$ were found to be the same within error range compared to POPC MLVs reported previously [98]. The calculated surface electrostatic potential reported for 100 mol % POPC LUVs $\Psi_{\text{exp}} = -58 \pm 2 \text{ mV}$, for MLVs POPC $\Psi_{\text{exp}} = -56 \pm 3 \text{ mV}$. However, for 100nm POPC 80 mol % : POPG 20 mol % LUVs, the $pK_a$ values reported by PTE-IMTSL and the magnitude of the calculated corresponding surface electrostatic potentials $\Psi$ were found to be about 30% higher than those for POPC 80 mol % : POPG 20 mol % MLVs reported previously [98]. The calculated surface electrostatic potential reported for POPC 80 mol % : POPG 20 mol % LUVs is $\Psi_{\text{exp}} = -75 \pm 2 \text{ mV}$, for
MLVs is $\Psi_{exp} = -107 \pm 3$ mV. This could be related to the difference in electrolyte concentration difference, LUVs samples has electrolyte concentration of 75mM and MLVs has electrolyte concentration of 50mM. Addition of electrolyte (NaCl) to LUVs results in a strong electrostatic screening effect for negatively charged lipid bilayer, this is supported by a set of experiments for DMPG MLVs doped with PTE-IMTSL conducted in our group, it was found that experimentally observed $pK_a^i$ had a gradual decrease and determined $\Psi_{exp}$ changes lineally with the salt concentration from 50mM to 150mM [98]. In addition it was found that under same electrolyte concentration, experiments for 100 nm DMPG ULVs, the $pK_a^i$ values reported by IKMTSL-PTE were slightly higher than those for MLVs. The calculated surface electrostatic potential reported for 100 mol % DMPG LUVs $\Psi_{exp} = -142 \pm 3$ mV, for MLVs $\Psi_{exp} = -120 \pm 3$ mV at 48°C. It was suggested that such result is related to a somewhat higher average packing density of lipids in the ULVs of ca. 100 nm diameter versus that in MLVs that are composed of many lamellae spanning a rather broad range of local curvature [99].

The Gouy-Chapman (GC) theory has long been used to predict the surface electrostatic potential. In brief, the Gouy-Chapman theory combines the Poisson equations with the Boltzmann relation. Gouy-Chapman theory suggests that the interfacial potential at them charge surface is attributed to the presence of ions attached to the surface and to an equal number of ions of opposite charge in solution. Therefore, the counter ions are able to diffuse into the liquid phase. In this theory, the change in concentration of the counter ions near a charged surface follows the Boltzmann distribution. We have compared our experimental results with the surface electrostatic potential calculated using the GC theory [110].
\[ \Psi_{GC} = \frac{2kT}{e} \text{asinh}\left( \frac{\lambda_D e\sigma}{2\varepsilon_0 \varepsilon kT} \right) \quad \text{Eq. 12} \]

where \( \sigma \) is the lipid surface charge density, \( \lambda_D \) is the Debye screening length, \( \varepsilon_0 \) is the permittivity of vacuum, and \( \varepsilon \) is the dielectric constant of the medium.

The Debye screening length is given by

\[ \lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon kT}{2000 e^2 N_A C_{el}}} \quad \text{Eq. 13} \]

where \( N_A \) is Avogadro’s number and \( C_{el} \) is the bulk molar electrolyte concentration.

The surface charge density was estimated as

\[ \sigma = \frac{e\alpha}{A_L} \quad \text{Eq. 14} \]

where \( A_L \) is the surface area per ionizable group of a lipid and \( \alpha \) is the degree of dissociation of the phosphatidyl group.

For POPC the pK\(_a\) of ionizable group is 1.0 pH units [5] and for POPG the pK\(_a\) of ionizable group is 3.1 pH units, therefore, within the pH used in out titration experiments \( \alpha \approx 1 \). The reported experimental values of area per lipid in POPC bilayers are in a range from 54 nm\(^2\) to 68.8 nm\(^2\) [111-115]. The estimates for POPG surface area do vary widely in the literature (e.g., from \( A_L = 0.53 \text{ nm}^2 \) [116] to 0.65 nm\(^2\) [117] and even 0.70 nm\(^2\) [118]) Here we propose to use minimum \( A_L = 0.53 \text{ nm}^2 \) and maximum \( A_L = 0.70 \text{ nm}^2 \) for the POPC and POPG bilayer surface area per ionizable group. Then the corresponding surface charge densities were calculated to be \( \sigma = -0.061 \text{C/m}^2 \) and \( -0.046 \text{C/m}^2 \) for POPC 80 mol % : POPG 20 mol % mixture samples respectively. For \( C_{el} = 0.075 \text{ M} \), the Debye screening length at 21°C is \( \lambda_D = 1.1 \text{ nm} \). For PTE-
IMTSL, using \( \varepsilon = 59 \) for interfacial location, the GC theory predicts the surface potential value of \( \Psi_{GC} = -83 \text{ mV} \) using minimum \( A_L = 0.53 \text{ nm}^2 \) and \( \Psi_{GC} = -71 \text{ mV} \) using maximum \( A_L = 0.70 \text{ nm}^2 \). This range \(-71 \text{ mV} \) to \(-83 \text{ mV} \) agrees well with the experimentally observed \( \Psi_{exp} = -75 \pm 2 \text{ mV} \). For PTE-IKMTSL, using \( \varepsilon = 65 \) for interfacial location of the reporter nitrooxide the GC theory predicts the surface potential value of \( \Psi_{GC} = -79 \text{ mV} \) using minimum \( A_L = 0.53 \text{ nm}^2 \) and \( \Psi_{GC} = -66 \text{ mV} \) using maximum \( A_L = 0.70 \text{ nm}^2 \). The surface electrostatic potential measured in this work by IKMTSL-PTE (\( \Psi_{exp} = -48 \pm 3 \text{ mV} \)) deviates by \( \approx 30\% \) from the \( \Psi_{GC} \) range calculated \(-66 \text{ mV} \) to \(-79 \text{ mV} \). Again this is because the nitrooxide of IKMTSL-PTE reports on a local electrostatic potential within a plane that is further away from the bilayer surface and where the electrical charges of lipids bilayer are better screened by counter ions. The GC theory underestimates considerably the bilayer surface potential for purely POPC lipid since it assumes the POPC surface charge is electrically neutral. Our work reaffirm that POPC bilayer has a large permanent electric dipole formed by the choline (net charge \(+e\)) and phosphate (net charge \(-e\)) groups, and Triton X-100 micelles but not zwitterionic lipids should be employ as a neutral reference interface in measurement of bilayer surface potentials.

POPC bilayer surface charge density, \( \sigma \), was estimated from experimental surface potentials using the GC theory. If we accept a monovalent electrolyte as an approximation of our experimental conditions, then \( \sigma \) is given by

\[
\sigma = \sinh\left(\frac{e\Psi_{exp}}{2kT}\right) \cdot \sqrt{8000 \ kT \varepsilon_0 \varepsilon C_{el} N_A} \quad \text{Eq. 15}
\]
For $C_{el} = 0.075$ M, $\varepsilon = 59$ for interfacial location of the reporter nitroxide PTE-IMTSL incorporated into Triton X-100 micelles, and $\Psi_{exp} = -58$ mV for 100 mol % POPC liposome, the calculated surface charge density is $\sigma_{exp} = -0.039$ C/m$^2$. Similarly, $\Psi_{exp} = -75$ mV for 80 mol % POPC : 20 mol % POPG liposome, the calculated surface charge density is $\sigma_{exp} = -0.058$ C/m$^2$, agrees with the range $\sigma = -0.061$C/m$^2$ to $-0.046$C/m$^2$ predicted by the GC theory from different areas per polar head group.

2.3.5 Choice of silica support.

Silica beads supported lipid bilayer can be formed in the range of sizes from $\sim 50$ nm to $\sim 80$ $\mu$m. In chapter one we have discussed the effect of support particle size and curvature on formation of supported lipid bilayer and reported that liposome fusion on larger particle occurs much easier. In this project we utilized non-porous silica beads with diameter greater than 100nm because they form stable supported lipid bilayer. In addition, for silica beads with sizes higher that 100nm, the curvature effects on physical properties, especially on surface charge density, are negligible. Although silica beads density (1.96 g/cm$^3$) allows for easy separation of beads from solution by centrifugation, this technique is difficult to use with beads smaller than 300nm in diameter because the speeds required to separate small beads is very high, and the resulting pellet is very dense and hard to re-suspend. Taking in account the above considerations, we selected silica beads with 300 nm diameter for our experiments.
2.3.6 Determination of the zeta potential of bare silica beads and silica supported bilayer

The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other, if the particles have low zeta potential values then there is no force to prevent the particles coming together and coagulation.

At pH=7.4, the zeta potential of silica beads suspension was measured to be -23.6mV (a previous study for 50nm silica beads zeta potential = -20mV at pH=7.4 [119]), and zeta potential of silica supported lipid bilayer suspension was measured to be -7.3mM. This indicates that lipid bilayer wrapping around silica beads partially screens the surface charge of the silica. Besides, POPC molecule is a zwitterion, its positive charge belonging to the ammonium group and negative charge belonging to the phosphate group are favorable for electrostatic interaction with negatively charged silica surface and causes decrease in the negative value of the silica particle zeta potential. Silica beads zeta potential titration is shown in Figure 2.20, the isoelectric point $\text{pH}_{\text{iso}} \approx 3.2$ where the net surface charge is zero can be determined from this plot.

![Figure 2.20](image)

**Figure 2.20** Zeta potential titration of silica beads in 0.5 x PBS buffer.
2.3.7 Effect of silica support on lipid bilayer electrostatics. Determination of $\Delta pK_a^\text{ silica}$.

For both PTE-IMTSL and PTE-IKMTSL, spin labeled lipids were incorporated into (A) zwitterionic lipid POPC and (B) mixed 80 mol % POPC with 20 mol % anionic lipid POPG and then SUVs were coated on non-porous silica beads surface. Representative spectra (normalized by intensity) for each silica supported lipid bilayer samples are shown in Figures 2.21-2.22.

Similar to liposomes, the EPR spectra displayed in Figures 2.21-2.22 demonstrate that the spectral line shape is altered upon adjustment of the bulk pH value of the sample. As the pH of the sample is lowered, the most apparent change in the EPR spectra is the progressive rise of the low-field shoulder around 3490 G. This feature is characteristic of slower and more restricted nitroxide tumbling. Lowering pH induces charge-charge or charge-dipole electrostatic interaction between the positive protonated nitroxide and lipid bilayer surface, as well as electrostatic interaction between the positive protonated nitroxide and silica support surface, leading to a decrease of the nitroxide tumbling rate. Note that upon lowering the pH an additional splitting in the central component of the spectra from the silica supported lipid bilayer doped with PTE-IKMTSL has appeared close to protonated forms. We speculate that this splitting resulted from the lipid bilayer distortion caused by pH change and illustrates a lower rigidity of the supported lipid bilayer.
Figure 2.21 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of silica supported samples doped with 1 mol % pH-sensitive PTE-IMTSL lipid incorporated into lipid vesicles composed of POPC (left) and POPC 80% : POPG 20% (right).

Figure 2.22 Representative intensity normalized CW X-band EPR spectra from pH titration experiments of silica supported samples doped with 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into lipid vesicles composed of POPC (left) and POPC 80% : POPG 20% (right).
EPR spectra of silica supported lipid bilayer doped with spin labeled lipids is a combination of contribution from nitroxides in the outer leaflet facing the bulk solution and nitroxides in the inner leaflet supported by silica beads. Figure 2.23 shows a cartoon illustrating spin labeled PTE lipids incorporated into vesicle that is coated on silica beads.

When pH is approaching pK$_a$ of the probes, EPR spectra of outer leaflet nitroxides reveals two components of protonated and non-protonated forms as those in liposome, EPR spectra of inner leaflet nitroxides also reveals two components of protonated and non-protonated forms that are supported on silica beads. Specifically an experimental EPR spectrum at an intermediate pH, E(B), is assumed to be a superposition of the spectra from the protonated F$_{1N}$ (B) and non-protonated F$_{1NH^+}$ (B) species of outer leaflet, plus a superposition of the spectra from the protonated F$_{2N}$ (B) and non-protonated F$_{2NH^+}$ (B) species of inner leaflet.

$$E (B) = m \cdot [a_1 \cdot F_{1N} (B) + b_1 \cdot F_{1NH^+} (B)] + n \cdot [a_2 \cdot F_{2N} (B) + b_2 \cdot F_{2NH^+} (B)] \quad Eq.16$$

Assuming the spin labeled lipids are evenly distributed in the outer and inner leaflets, then m=n=0.5. If we can experimentally measure F$_{1N}$ (B), F$_{1NH^+}$ (B), F$_{2N}$ (B) and F$_{2NH^+}$ (B), EPR
spectra can be analyzed with least-squares decomposition to derive the coefficient $a_1$, $b_1$, $a_2$, and $b_2$. However what we obtained from EPR spectrometer scanning is a combination of inner and outer leaflets, i.e. the spectrum at highest pH as non-protonated form is a superposition of inner and outer species, the spectra at the lowest pH as protonated form is also a superposition of inner and outer species. The intensities of each inner and outer species cannot be differentiated without further advanced analysis model. At present we just follow simplified slow chemical exchange model to treat the inner and outer leaflets combination as one entity.

\[ E(B) = a \cdot F_N(B) + b \cdot F_{NH^+}(B) \]

Eq.17

Examples of a spectral decomposition is shown in Figure 2.24. The fit residuals, difference between the experimental and simulated spectra show only negligible deviations, thus demonstrating the validity of this model for silica supported bilayer samples.

**Figure 2.24** Decomposition of EPR spectrum from PTE-IMTSL incorporated into POPC vesicles. Experimental spectrum acquired at intermediate pH is shown in (black). Component of the experimental spectra corresponding to the neutral form of PTE-IMTSL (blue). Component of the experimental spectra corresponding to the charged form of PTE-IMTSL (red). The residual of fitting the experimental spectra to the sum of the simulated spectra (green).
The fraction of the non-protonated form of the nitroxide $f$ is then calculated from the double integrals $D_N$ and $D_{NH^+}$ of the corresponding $F_N$ ($B$) and $F_{NH^+}$ ($B$) spectra, and was plotted against pH to yield the titration data points.

$$f = \frac{D_N}{D_N + D_{NH^+}} \quad \text{Eq. 18}$$

Treating the inner and outer leaflet combination as entity, the titration data points of fraction of neutral spin labels $f$ vs. pH can be fitted to the single dissociation Henderson-Hasselbach equation shown as dashed lines in Figure 2.1-2.2 from which the interfacial $pK_a^i$ of the spin labels were determined and summarized in the Table 2.

$$f = \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad \text{Eq. 19}$$

Deposition on silica beads may perturb the inner leaflet differently from the outer leaflet, i.e. outer leaflet facing bulk solution and inner leaflet facing more negative surface charge of silica beads. During titration process from higher to lower pH, the negative surface charge density of silica beads decreases as shown in zeta potential section, the inner leaflet undergoes dynamic interaction with silica support, because of lipid diffusion and transbilayer movement, the outer leaflet undergoes dynamic interaction with inner leaflet as well, thus fitting titration points to single dissociation may overlook the dynamics. Assuming each inner and outer leaflet possess even distribution of spin labeled lipids, the above Eq.19 can be modified as double dissociation shown as solid lines in Figure 2.25-2.26 from which the interfacial $pK_a^i$ of two dissociation were determined and summarized in Table 2.06-2.07

$$f = 0.5 \frac{10^{(pH-pK_a1)}}{1 + 10^{(pH-pK_a1)}} + 0.5 \frac{10^{(pH-pK_a2)}}{1 + 10^{(pH-pK_a2)}} \quad \text{Eq. 20}$$
Figure 2.25 pH titration curve for 1 mol % pH-sensitive PTE-IMTLS lipid incorporated into silica supported bilayer POPC (blue) and POPC 80% : POPG 20% (red). Dashed lines are the best fit to single dissociation model Eq.11, solid lines are the best fit to double dissociation model Eq.20.

Table 2.06 Experimental $\Delta pK_a$ determined from the pH titration using fit to Eq. 19 (top) and to Eq. 20 (bottom) and the surface electrostatic potential, $\Psi$ calculated for silica supported PTE-IMTLS labeled bilayer samples.

<table>
<thead>
<tr>
<th>Lipids composition</th>
<th>$pK_a^i$</th>
<th>$pK_a^0$</th>
<th>$\Delta pK_a^{pol}$</th>
<th>$\Delta pK_a^{el}$</th>
<th>$\Psi_{exp}$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>$pK_a=3.65 \pm 0.03$</td>
<td>3.33 $\pm 0.03$</td>
<td>-0.81 $\pm 0.03$</td>
<td>1.13 $\pm 0.05$</td>
<td>-65 $\pm 3$</td>
</tr>
<tr>
<td>POPC20:POPG80</td>
<td>$pK_a=4.68 \pm 0.02$</td>
<td></td>
<td></td>
<td>2.16 $\pm 0.05$</td>
<td>-124 $\pm 3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipids composition</th>
<th>$pK_a^i$</th>
<th>$pK_a^0$</th>
<th>$\Delta pK_a^{pol}$</th>
<th>$\Delta pK_a^{el}$</th>
<th>$\Psi_{exp}$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>$pK_{a1}=3.25 \pm 0.05$</td>
<td>3.33 $\pm 0.03$</td>
<td>-0.81 $\pm 0.03$</td>
<td>$\Delta pK_{a1}^{el}=0.73 \pm 0.07$</td>
<td>-42 $\pm 4$</td>
</tr>
<tr>
<td></td>
<td>$pK_{a2}=4.06 \pm 0.05$</td>
<td></td>
<td></td>
<td>$\Delta pK_{a2}^{el}=1.54 \pm 0.07$</td>
<td>-89 $\pm 4$</td>
</tr>
<tr>
<td>POPC20:POPG80</td>
<td>$pK_{a1}=4.40 \pm 0.06$</td>
<td></td>
<td></td>
<td>$\Delta pK_{a1}^{el}=1.88 \pm 0.07$</td>
<td>-108 $\pm 4$</td>
</tr>
<tr>
<td></td>
<td>$pK_{a2}=4.96 \pm 0.06$</td>
<td></td>
<td></td>
<td>$\Delta pK_{a2}^{el}=2.44 \pm 0.07$</td>
<td>-140 $\pm 4$</td>
</tr>
</tbody>
</table>
Figure 2.26 pH titration curve for 1 mol % pH-sensitive PTE-IKMTSL lipid incorporated into vesicle and coated on silica beads POPC (blue) and POPC 80% : POPG 20% (red). Dashed lines are the best fit to single dissociation model Eq.11, solid lines are the best fit to double dissociation model Eq.20.

Table 2.07 Experimental $\Delta pK_a^{el}$ determined from the pH titration using fit to Eq. 19 (top) and to Eq. 20 (bottom) and the surface electrostatic potential, $\Psi$ calculated for silica bead support PTE-IKMTSL bilayer samples.

<table>
<thead>
<tr>
<th>Lipids composition</th>
<th>$pK_a^i$</th>
<th>$pK_a^0$</th>
<th>$\Delta pK_a^{pol}$</th>
<th>$\Delta pK_a^{el}$</th>
<th>$\Psi_{exp}$ mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>$pK_a^i$=6.11 ± 0.02</td>
<td>5.98 ± 0.04</td>
<td>-0.58 ± 0.04</td>
<td>0.71 ± 0.06</td>
<td>-41 ± 3</td>
</tr>
<tr>
<td>POPC20:POPG80</td>
<td>$pK_a^i$=7.34 ± 0.03</td>
<td></td>
<td></td>
<td>1.94 ± 0.06</td>
<td>-112 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipids composition</th>
<th>$pK_a^i$</th>
<th>$pK_a^0$</th>
<th>$\Delta pK_a^{pol}$</th>
<th>$\Delta pK_a^{el}$</th>
<th>$\Psi_{exp}$ mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>$pK_{a1}$=6.11 ± 0.02</td>
<td>5.98 ± 0.04</td>
<td>-0.58 ± 0.04</td>
<td>$\Delta pK_{a1}^{el}$=0.71 ± 0.06</td>
<td>-41 ± 3</td>
</tr>
<tr>
<td>POPC</td>
<td>$pK_{a2}$=6.11 ± 0.02</td>
<td>5.98 ± 0.04</td>
<td>-0.58 ± 0.04</td>
<td>$\Delta pK_{a2}^{el}$=0.71 ± 0.06</td>
<td>-41 ± 3</td>
</tr>
<tr>
<td>POPC20:POPG80</td>
<td>$pK_{a1}$=6.87 ± 0.05</td>
<td>5.98 ± 0.04</td>
<td>-0.58 ± 0.04</td>
<td>$\Delta pK_{a1}^{el}$=1.47 ± 0.08</td>
<td>-85 ± 4</td>
</tr>
<tr>
<td>POPC20:POPG80</td>
<td>$pK_{a2}$=7.81 ± 0.05</td>
<td>5.98 ± 0.04</td>
<td>-0.58 ± 0.04</td>
<td>$\Delta pK_{a2}^{el}$=2.41 ± 0.08</td>
<td>-139 ± 4</td>
</tr>
</tbody>
</table>
If one compared the results obtained for surface electrostatic potential on liposome with the one for silica supported lipid bilayer, it can be seen that the negative charge density measured for silica supported lipid bilayer is higher than corresponding liposome samples. For spin labeled PTE-IMTSL lipids, the calculated surface electrostatic potential reported for silica supported lipid bilayer composed of POPC $\Psi_{\text{exp}} = -65 \pm 3$ mV is lower than that reported for POPC liposome $\Psi_{\text{exp}} = -58 \pm 2$ mV by difference of $-7 \pm 4$ mV; the calculated surface electrostatic potential reported for silica supported lipid bilayer composed of POPC 80 mol % : POPG 20 mol % $\Psi_{\text{exp}} = -124 \pm 3$ mV is lower than that reported for POPC 80 mol % : POPG 20 mol % liposome $\Psi_{\text{exp}} = -75 \pm 2$ mV by difference of $-49 \pm 4$ mV. For spin labeled PTE-IKMTSL lipids, the calculated surface electrostatic potential reported for silica supported lipid bilayer composed of POPC $\Psi_{\text{exp}} = -41 \pm 3$ mV is lower than that reported for POPC liposome $\Psi_{\text{exp}} = -19 \pm 3$ mV by difference of $-22 \pm 4$ mV; the calculated surface electrostatic potential reported for silica supported lipid bilayer composed of POPC 80 mol % : POPG 20 mol % $\Psi_{\text{exp}} = -112 \pm 3$ mV is lower than that reported for POPC 80 mol % : POPG 20 mol % liposome $\Psi_{\text{exp}} = -48 \pm 3$ mV by difference of $-64 \pm 4$ mV.

The lowering of the membrane surface electrostatic potential is attributed to negatively charged functional silanol groups on silica beads surface and a thin water layer of $\sim 1$ nm that separates the lipid head groups from the silica surface. Silica beads surface reveals a charge-regulated nature due to the protonation/deprotonation reactions of the dissociable silanol groups. The surface charge density of the silica beads is modeled by the following two protonation reactions with equilibrium constants $K_A$ and $K_B$, respectively.
\[ SiOH \leftrightarrow SiO^- + H^+ \]

\[ SiOH + H^+ \leftrightarrow SiOH_2^+ \]

Literature value of \( pK_A = 7.6, pK_B = 1.9 \) [120]. The equilibrium constants are calculated as

\[ K_A = \frac{[H^+] \cdot N_{SiO^-}}{N_{SiOH}} \]

\[ K_B = \frac{N_{SiOH_2^+}}{[H^+] \cdot N_{SiOH}} \]

The charged silica beads surface gives rise to a Coulomb potential between the solid surface and lipid head group moieties. Previous NMR studies have shown that the water layer in the narrow gap between two hydrophilic surfaces is partly structured and differs significantly in its dynamics from that of free water [121]. Thus the supported lipid bilayer can behave asymmetrically as one side of the bilayer is facing the bulk solution while the other leaflet is facing the solid surface.

The protonation state of a spin label in inner leaflet facing silica beads surface can be affected by two factors, one factor is the local proton concentration that could be altered by a possible distortion of water structure within a thin water layer of \( \sim 1 \) nm, another factor is the silica surface charge that cause formation of the surface double electrical layer [109]. However since the surface charge density undergoes dynamic changes, we cannot deconvolve the individual contributions, thus the double dissociation provides a range of how surface electrostatic potential is affected by silica support.
The calculated surface electrostatic potentials reported for silica beads support may give rise to higher lipids packing and facile transbilayer movement of supported lipid bilayer thus affecting the surface charge density of the bilayer is measured by spin probes. Numbers of studies employing NMR, fluorescence, and capacitance measurements have shown that the lipid flip-flop is possible in the absence of protein-mediated process, and recent label-free direct measurement of the transbilayer movement by sum-frequency vibrational spectroscopy found lipid flip-flop to be considerably faster (on the order of minutes) for lipid bilayer in the fluid phase [123]. We speculate that supported lipid bilayer undergoes dynamic relocation and reorganization that the negative charged lipids flip-flop to accommodate silica surface charge created by protonation/deprotonation reactions of the silanol groups. As a results both outer and silica beads supported inner leaflets exhibit lower surface electrostatic potential than the one in liposome. Induced membrane asymmetry has been previously reported [23] for interaction between the supported negatively charged lipids, and asymmetric partitioning of charged lipids between inner and outer leaflets was rationalized by an electrostatic interactions with charged silica surface [23, 59, 122, 123].

As a heterogeneous system tends to reduce its surface energy, the development of a net charge at the silica beads surface affects the distribution of ions in the surrounding interfacial region, leading to an increased concentration of counter ions close to the surface. The liquid layer surrounding the particle exists as two parts; an inner region, called the Stern layer, where the ions are strongly bound, and an outer, diffusive region where they are less firmly attached, thus an electrical double layer (EDL) formed by interaction of silica beads surface charge and buffer
ions exists around each particle [124]. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move with it and induce a near surface Stern potential. But any ions beyond the boundary do not travel with the particle, this boundary is called the surface of hydrodynamic shear or slipping plane, the potential that exists at this boundary is known as the Zeta potential [125]. Previously we’ve shown the zeta potential titration of bear silica beads versus pH, the absolute value of zeta potential drops from -29.4mV at pH=8.9 to -0.7mV at pH=3.4 with a rough estimate of isoelectric point $\text{pH}_{\text{iso}} \approx 3.2$. This means if more alkali is added to this suspension above 3.2 pH unit the silica particles will tend to acquire a more negative charge. If acid is then added to this suspension below 3.2 pH unit the negative charge is neutralized and start to build positive surface charge.

![Figure 2.27](image)

**Figure 2.27** Surface charge density of a planner silica surface as a function of pH. The numerical result is from a multi-ion charge-regulation model [126], the theoretical result is from [127]. Figure is adapted from [126].
A mathematic model called a multi-ion surface charge-regulation model (Figure 2.27) that doesn’t consider Stern layer predicts that pH$_{iso}$ ≈ 4.0, higher than zeta potential pH$_{iso}$ ≈ 3.2 for about 0.8 pH unit. This discrepancy could be explained by taking in consideration that multi-ion surface charge regulation model calculated potential at a plain that is further away from silica beads surface.

In addition to the surface electric potential indicating the surface charge density, another fundamental parameter of DEL is effective thickness Debye lengths, a distance from the surface affected by the double layer ion interaction. The Debye lengths reported in the literature varies from ~3.0 nm to ~0.95 nm for a salt concentration between 10 to 100 mM NaCl [120] effect explained by compression of the double layer thickness with the ionic strength increase [128]. Our silica supported lipid bilayer system with a thin water layer of ~1 nm that separates the lipid head groups from the silica surface has a electrolyte concentration of 75mM, thus in addition to diffuse layer zeta potential, the near surface electrostatic potential within Stern layer may also play a key role in affecting silica supported lipid bilayer surface electrostatics.
Figure 2.28 Schematic illustration of the double electrical layer structure, adapted from [125].

Ideally, spin labeling approach should be applicable for measuring surface electrostatic potential for biological system as well as solid system. Bare silica beads modified with spin labels at the surface can be employed as control experiment for further elucidate effect of pH on surface charge density of silica beads. The reported local pH value for investigation of proton-related process is supposed to reflect pK_a shift dependent on the surface electrostatic potential of silica beads.

2.3.8 Spin-labeling of silica nanoparticles.

We’ve test two different approaches for spin-labeling of the silica beads. One is direct covalent attachment of silica beads with IKMTSL precursor, in which the silanol groups were modified
through an ester bond with the IKMTSL precursor. The second approach consisted of the hydrolysis of 3-MPTS (3-mercaptopropyltrimethoxysilane) with water in acetonitrile followed by the silica beads salinization with 3-MPTS. Silanized silica beads then were further treated with either IKMTSL or IMTSL to attach the probes through formation of disulfide bonds. To preserve silanol group charges and to avoid signal broadening due to dipole-dipole interaction between neighboring spin labels, nitroxide to silica weight ratio was selected to ensure average spin-spin distance of 3nm or more. The preliminary results show that silica beads surface indeed can be modified with EPR pH sensitive spin labels, however, the first approach suffered from instability of the EPR signal and the second approach suffer from complicated superposition of isotropic and anisotropic signals in addition to nitroxide stability issue. Further experiments will be carried out to investigate acid-base properties of bare silica beads by EPR of molecular pH labels.

**Figure 2.29.** EPR spectra of silica beads modified with IKMTSL precursor (red) and free spin labeling in same solvent (blue).
Figure 2.30 Representative CW X-band EPR spectra from 3-MPTS salinized silica beads modified with IKMTSL at various pH

2.3.9 Effect of silica support on dynamics of phospholipid bilayer.

Comparing EPR spectra of liposome and its corresponding silica supported lipid bilayer doped with non pH-sensitive PTE-MTSL spin labeled lipids, it can be noticed that EPR spectra of PTE-MTSL in silica supported lipid bilayer are broader than the corresponding spectra of PTE-MTSL in liposomes, indicating that silica supported lipid bilayer possesses less mobility. Comparing EPR spectra of liposome and its corresponding silica supported lipid bilayer doped with pH-sensitive either IMTSL or IKMTSL spin labeled lipids, the non-protonated forms of
silica supported lipid bilayer show slightly broadened peaks indicating slower motion of the spin label.

Figure 2.31. EPR spectra from non-protonated forms of PTE-IMTSL incorporated into LUVs (black) and silica supported lipid bilayer (purple).

Figure 2.32 EPR spectra from non-protonated forms of PTE-IKMTSL incorporated into LUVs (black) and silica supported lipid bilayer (purple).

Comparing EPR spectra of liposome and its corresponding silica supported lipid bilayer doped with pH-sensitive either IMTSL or IKMTSL spin labeled lipids, the protonated forms of silica supported lipid bilayer show exceptional broadened shoulder in the high-field part of the EPR spectra shown in Figure 2.33-2.34. We propose that the protonated forms of silica supported lipid bilayer reveal two components, corresponding to probe in the outer leaflet (blue) facing the bulk solution, and another corresponding to probe in the inner leaflet (red) supported by
silica beads. The latter spectrum is characteristic of a probe with lower mobility and higher anisotropy of motion.

Figure 2.33 EPR spectra from protonated forms of PTE-IMTSL incorporated into LUVs (black) and silica supported lipid bilayer (purple). The latter is decomposed as outer leaflet (blue) and silica supported inner leaflet (red).

Note that sometimes there is a very small amount (<1%, based on the double-integral intensity) of a free spin label IMTSL (might be leached from spin labeled PTE-IMTSL) in solution appearing in EPR spectra as three sharp peaks (for an example of a spectrum from free label sees Figure 2.33).
Figure 2.34 EPR spectra from protonated forms of PTE-IKMTSL incorporated into LUVs (black) and silica supported lipid bilayer (purple). The latter is decomposed as outer leaflet (blue) and silica supported inner leaflet (red).

Mobility and order parameter of nitroxide are used to characterize silica supported lipid bilayer dynamics. Here we employ 5-doxyl-SA incorporated into liposome and silica beads supported lipid bilayer and to characterize dynamics of phospholipid bilayer as a function of temperature. Representative spectra are shown in Figure 2.35.
A particularly informative parameter of EPR spectra is peak-to-peak width \( \Delta H_{p-p} (m_I = 0) \) of the central nitrogen hyperfine component (where \( m_I = 0 \) is the nitrogen spin quantum number). When tumbling of a spin label falls into an intermediate to slow motional regime, this width is approximately proportional to the rotational correlation time \( \tau_c \). In studies of local dynamics of protein side chains, an inverse of the linewidth of the central component, \( 1/\Delta H_{p-p}^{-1} \), is typically reported as a “mobility” parameter [129]. A larger value of the mobility parameter corresponds to faster motion of the label. Mobility parameter of the 5-doxyll-SA is shown as a function of temperature in Figure 2.35. Mobility parameter of 5-doxyll-SA in silica supported bilayer is measurably smaller than that of the probe in liposomes at the same temperature. The temperature dependence of the mobility parameter in Figure 2.36 was fitted to a linear dependence in \( \log (1/\Delta H_{p-p}^{-1}) \) vs \( 1/T \) coordinate system to estimate the activation energy associated with motion of the spin-label. Results of linear fits show small, but measurable difference: \( E_a = 7.7 \text{ kJ/mol} \) for the probe in LUVs and \( 8.1 \text{ kJ/mol} \) for the probe in silica supported lipid bilayer, indicating tighter packing of lipids in silica-supported bilayer.
**Figure 2.35** Representative intensity-normalized room temperature EPR spectra of 5-doxyl-SA incorporated in LUVs (blue) and in silica supported bilayer (red) consisting of POPC/POPG mixture. Hyperfine splitting parameters $A_{out}$ and $A_{in}$ shown as measured for calculations of order parameter. Blue dotted lines are used to emphasize differences in spectra.
Figure 2.36 Mobility parameter of 5-doxySL incorporated into LUVs (blue) composed of POPC 20 mol% : POPG 20 mol % and into silica supported lipid bilayer of the same composition (red) as function of temperature.

Another empirical parameter commonly used for characterizing rotational motion of a nitroxide is the effective order parameter ($S^{\text{eff}}$) [129]. We used the definition of McConnell and Hubbell to calculate the effective order parameter ($S^{\text{eff}}$) to describe the anisotropy of spin-label motion [130, 131].

$$S^{\text{eff}} = \frac{(A_{||} - A_{\perp}) A_0}{\frac{1}{3} (A_{||} + 2A_{\perp}) \Delta A} \quad \text{Eq. 21}$$

where $A_{||} = A_{\text{out}}$, (i.e., a half of the outer hyperfine splitting), and $A_{\perp}$ is calculated from $A_{\text{in}}$, a half of the inner hyperfine splitting expressed in Gauss.

$$A_{\perp} = (A_{\text{in}} + 0.85) \quad \text{for} \quad S^{\text{app}} < 0.45 \quad \text{Eq. 22}$$

$$A_{\perp} = A_{\text{in}} + 1.32 + 1.86 \log(1 - S^{\text{app}}) \quad \text{for} \quad S^{\text{app}} > 0.45 \quad \text{Eq. 23}$$
\[ S^{app} = \frac{(A_{out} - A_{in})}{\Delta A} \quad Eq. 24 \]

where \( A_0 \) is the isotropic nitrogen hyperfine coupling constant and \( \Delta A \) is the maximum extent of the axial nitrogen hyperfine anisotropy [132, 133].

\[ A_0 = \frac{1}{3} \left( A_{XX} + A_{YY} + A_{ZZ} \right) \quad Eq. 25 \]

\[ \Delta A = A_{ZZ} - \frac{1}{2} \left( A_{XX} + A_{YY} \right) \quad Eq. 26 \]

In our case the following parameters were used: \( A_{XX} = 5.9 \text{G}, A_{YY} = 5.4 \text{G}, A_{ZZ} = 32.9 \text{G} \), resulting in \( S^{app} > 0.45 \), we used Eq. 23 to calculate \( A_\perp \).

![Graph showing order parameter vs. temperature for 5-doxyl SL incorporated in LUVs (blue) or silica supported bilayers (red) prepared from POPC 20 mol% : POPG 20 mol % mixture.](image)

**Figure 2.37** Experimentally measured effective order parameters \( S^{eff} \) as a function of temperature for 5-doxyl SL incorporated in LUVs (blue) or silica supported bilayers (red) prepared from POPC 20 mol% : POPG 20 mol % mixture.

Results show silica beads supported bilayer has higher order parameter indicating more anisotropic motion of lipids in the supported bilayer compared with LUVs. Combined with
results for mobility parameter, these data confirm that silica support does affect dynamics and packaging of lipid bilayer.

2.3.10 Effect of silica support on accessibly of membrane surface to water soluble reducing agents.

Organization of the lipid bilayer on the silica surface was probed by observing the kinetics of surface associated EPR probe, IKMTSL-PTE, incorporated into POPC liposomes and compared to that of the probe incorporated into silica supported POPC bilayer. Reduction of the EPR signal intensity was monitored as function of time upon addition of 87 mM of sodium ascorbate to the corresponding sample at pH 7.4. At this pH sodium ascorbate is completely dissociated in water to form ascorbate anion. The intensity of the EPR signal as function of time upon addition of the reducing agent is shown in Figure 2.38.

![Figure 2.38 Nitrooxide intensity decay for PTE-IKMTSL labeled POPC liposome and its corresponding silica beads supported sample induced by 80-fold molar excess of ascorbate at pH=7.4 under continuous nitrogen flow at room temperature.](image-url)
Sodium ascorbate reduction experiment shows that for IKMTSL-PTE labeled POPC liposomes, 45% lipids are readily reduced, corresponding to reduction of nitroxide in the outer leaflet of liposomes, followed by slow reduction due to probe flip-flop from inner to outer leaflet. The “fast” component is characterized by effective rate constant \( k = 0.08 \pm 0.01 \text{ min}^{-1} \). For silica supported IKMTSL-PTE labeled POPC, all lipids are readily reduced with \( k = 0.16 \pm 0.04 \text{ min}^{-1} \) indicating that inner leaflet is easily accessible. Effective rate of reduction increases by a factor of 2, as compared with LUVs. This increase is attributed to increase in ascorbate accessibility of inner layer, as well as more “loose” packing of lipids on silica support. These result also indicates that under our method of sample preparation, POPC lipids do not form continuous uninterrupted “shell” around the silica particles, but form “patches” of lipid bilayer on the silica surface.
2.4 Conclusions.

Two lipid like electrostatic spin probes, IMTSL-PTE and IKMTSL-PTE were employed in this project to investigate lipid bilayer surface electrostatic potential and the effect of silica support on the surface potential. The $\Delta pK_a^{el}$, the contribution to the experimental $pK_a$ of the probe due to electrostatic potential at the membrane surface was assessed by pH titration of probes incorporated into LUVs composed of POPC or mixture of POPC/POPG lipids. The shift in $\Delta pK_a$ caused by silica support was evaluated by titrating silica bead supported bilayer. For LUVs composed of negatively charged POPG mixture IMTSL-PTE reports a local electrostatic potential that is in a good agreement with values predicted by the Gouy-Chapman (GC) theory for POPC/POPG mixture. The surface electrostatic potential of silica supported bilayers was measured to be lower as compared with LUVs, indicating interaction of silica surface silanol groups with lipids. Titration curves also indicate potential rearrangement of lipids resulting in very similar surface potentials for inner and outer leaflets of silica supported lipid bilayer. In addition, we have shown that lipids in silica beads supported bilayer exhibit slower mobility with higher order parameter and higher heterogeneity of lipid dynamics as compared with lipids in LUV. Last but not least, we have shown that the inner leaflet that directly interacts with silica beads surface is easily accessible to small molecules in bulk solution.
Chapter 3. Effect of silica support on pKₐ of ionizable sidechain located at membrane-protein interface of model transmembrane peptide incorporated into phospholipid bilayer.

3.1 Introduction.

The ionization states of individual amino acid residues of membrane proteins are difficult to decipher or assign directly in the lipid-bilayer membrane environment. The effective pKₐ values of protein groups are determined by a complex interplay between local polarity, Coulomb interactions, and a structural reorganization. The analysis is further complicated by the dearth of information about gradients in polarity, electric potentials, and hydration at the protein-membrane interface. The goal of our work is to expand the existing arsenal of spin-labeling EPR methods for assessing effects of membrane surface potential, local environment at the protein-membrane interface on effective pKₐ of membrane-burred ionisable groups.

The dielectric constant of biomembranes is an important parameter in cell electrophysiology [134], because it ultimately determines phenomena such as protein sidechain ionization, the membrane permeability to ions and membrane potential formation [135-137]. The dielectric constant also determines the cell’s response to externally applied electrical fields employed by bioelectrical techniques, such as dielectrophoresis [138], impedance spectroscopy [139], or electroporation [140]. The value of effective dielectric constant at the membrane surface gives information about the membrane-liquid interfacial properties, particularly those relating to the
hydration of the membrane surface, which plays an important role in phenomena such as lipid bilayer fusion [141] and ensures the correct insertion, folding, and function of membrane proteins.

The dielectric constant of bulk materials is typically determined using the Fröhlich-Kirkwood theory [142]. This method uses the fact that spontaneous polarization fluctuations of a material induce a dielectric response or reaction field from the surrounding medium, which then acts to further polarize the material. The bulk dielectric constant of a material can then be assessed from the correlation of spontaneous polarization with its own reaction field [143]. For homogeneous aqueous solutions, the dielectric constant $\varepsilon$ is a bulk fluid property, there’s plenty of data for liquids such as water with dielectric constant $\varepsilon = 80$ and hydrocarbons with dielectric constant $\varepsilon = 2$, and these values are widely accepted. In heterogeneous systems, the response of a system to its own reaction field is complex and a direct application of the Fröhlich-Kirkwood theory is not possible.

An atomic-scale description of dielectric constant can be obtained by the molecular dynamics (MD) simulations, a theoretical and computational technique. Previous membrane simulations have revealed the basic features of the dielectric profile [143-146]. A widely accepted dielectric profile in lipid bilayer study is presented by Stern and Feller [144]. They calculated the permittivity profile of a dipalmitoylphosphatidylcholine (DPPC) lipid bilayer in water from an all-atom 20-ns molecular dynamics simulation and related the static dielectric permittivity profile to the net system dipole moment and the local polarization density, results
are summarized in Table 3.01. The component of the permittivity parallel to the bilayer shows a nonmonotonic decrease from the value in bulk water to the value in the membrane interior [144]; the interfacial region itself has a very large permittivity, greater than that of bulk water that can be attributed to the relatively high dipole moment of the DPPC head group. The most serious limitation of the current calculation is that it is based on an atomistic model that does not include electronic polarization [143]. The dielectric constant in the bilayer core has a dielectric constant of 1 in atomistic simulation, when in reality electronic polarization should result in a dielectric constant closer to that of bulk alkanes, which is approximate 2. This factor of 2 is very significant difference as it would result in a factor of 2 difference in the value of the electrostatic component of the transfer free energy from bulk water to the bilayer interior [143]. In addition, true description of the dielectric profile in membrane cannot have such defined boundaries as in the assumed model.

Table 3.01 Estimates of the dielectric profile within different components of the lipid bilayer regions. Adapted from [144].

| Distance from bilayer center | Environment                  | $\epsilon_{||}$ |
|-----------------------------|------------------------------|-----------------|
| 0–10 Å                      | Hydrocarbon interior         | 1               |
| 10–15 Å                     | Hydrocarbon, ester           | 4 ± 3           |
| 15–20 Å                     | All groups present           | 180 ± 30        |
| 20–25 Å                     | Choline, phosphate, water    | 210 ± 30        |
| 25–30 Å                     | Mostly water                 | 89 ± 2          |

Experimentally local electrostatic interactions and local polarity remain to be elusive parameters because of the limited spectroscopic methods (namely microscopy, fluorescence
spectroscopy and EPR) capable of measuring these effects unambiguously in the highly heterogeneous environment of membranes.

Scanning probe microscopy approach with atomic force microscopy (AFM) measurements can probe the electrostatic forces at the nanoscale, however both charges and membrane dielectric polarization influence the result thus making a precise quantification of the membrane dielectric constant difficult [147]. Scanning capacitance microscopy and electrostatic force microscopy can precisely measure and quantify the dielectric constant of biomembranes, however these measurements were performed in air on dried biomembranes due to difficulties in applying these techniques in a liquid environment [134]. Dynamic electrostatic force microscopy can quantify the dielectric constant of supported lipid bilayers in electrolyte liquid solutions, measurements of submicrometric dipalmitoylphosphatidylcholine lipid bilayer patches gave dielectric constants of \( \varepsilon \sim 3 \), which are higher than the values typically reported for the hydrophobic part of lipid membranes (\( \varepsilon \sim 2 \)) and suggest a large contribution of the polar head group region to the dielectric response of the lipid bilayer [134]. However this value is only a rough estimate because 1) the overlapping of surface double-layers or other interfacial electric phenomena were not taken into account, and 2) the calculated result is highly sensitive to the thicknesses assigned to the hydrophobic and polar regions, while the assignment is just an estimation. Last but not least, this method didn’t differentiate dielectric constant at different region along lipid bilayer axis and the model is based on a planner substrate rather than cell mimic shaped liposome.
Fluorescence is widely used spectroscopic technique thanks to its high sensitivity, it involves polarity sensitive probes, pH indicator dyes such as hydroxycoumarin (HC) and aminocoumarin (AC), and fluorophore-labeled phospholipids Dansylphosphatidylethanolamine (DPE), 1-4-nitrobenzo-2-oxa-1,3-diazole-PE (NBD-PE), and lissamine rhodamine B sulfonyl-PE (Rh-PE) for measurements of surface dielectric constant [76, 141]. A micellar surface dielectric constant of $\varepsilon \approx 32$ was interpolated for pH indicator dyes HC and AC [76]. A membrane surface dielectric constant $\varepsilon$ varies from 11 to 30 was examined upon the addition of various cations and poly-(ethylene glycol) on surface dielectric properties [141]. However fluorescence indicators that contain bulky easily polarized aromatic fragments are likely to cause some local perturbations to the membrane-water interface and molecular interaction at the lipid-protein interface. In many cases, the size of the reporter group is large and the exact location of the reporter is not well defined.

Nitroxide probes PTE-IMTSL and PTE-IKMTSL described in Chapter 2 are a good alternative to fluorescence pH indicator dyes and were used to measure micellar surface dielectric constant. The dependence of IMTSL and IKMTSL $pK_a$ upon the dielectric constant of the solvent was measured in a series of buffer/isopropyl mixtures for which the dielectric constant is known. By comparing aqueous $pK_a^0$ of a probe with the $pK_a^0$ of the probe incorporated into neutral micelles Triton X-100 $pK_a$, utilizing the experimental calibration, the micellar surface dielectric constant of $\varepsilon \approx 69$ was calculated for the location of PTE-IKMTSL and $\varepsilon \approx 60$ for the location of PTE-IMTSL [94, 99]. Nitroxide labels that are typically employed in spin labeling studies are relatively small molecules and possess smaller dipolar moment than the
fluorescent tags, thus nitroxides typically introduce a minimal perturbation in the vicinity of the probe [96]. Besides, EPR method simultaneously with $\varepsilon$ determination provides complementary data on local structure and dynamics of the labeled site [97].

The abovementioned fluorescence and EPR method opens apparently great potentials for lipid bilayer surface dielectric constant measurement. Our group developed a novel spin labeling approach to address the gradient of the dielectric permittivity at a variety depths within the lipid bilayer at the membrane-peptide interface.

![Chemical Structure of DOPC and DOPG Lipids](image)

**Figure 3.01** Chemical structure of DOPC and DOPG lipids.

A series of WALP peptides shown in Table 3.02 are known to adopt a $\alpha$-helical transmembrane conformation within DOPC, DOPG or mixed DOPC/DOPG liposomes. Cysteine residues are displayed in red font in order to highlight the point of attachment of the pH sensitive, thiol specific spin labels. WALP peptides consist leucine (L) - alanine (A) repeats capped by terminal tryptophan (W). The hydrophobic L-A repeats are located in the hydrophobic interior
of the lipid bilayer and the capping W partitioned into the polar head group region serve as the anchors ensuring the peptide consistent registration in the lipid bilayer [148]. The termini of all peptides were capped to prevent unwanted electrostatic effects from free amine and carboxyl groups. The membrane arrangement of WALP peptides in DOPC bilayers is well characterized by several spectroscopic techniques such as circular dichroism (CD) and IR spectroscopy and was demonstrated that the hydrophobic length of WALP peptides (34.5 Å) match well with the hydrophobic thickness of the DOPC/DOPG bilayers (27 Å) and total thickness of the DOPC/DOPG bilayer (50 Å) [149].

<table>
<thead>
<tr>
<th>Peptide label position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walp1</td>
<td>Ac-(C(sl))WWLALALALALALALALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp2</td>
<td>Ac-(GC(sl))WLALALALALALALALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp3</td>
<td>Ac-(GW(C(sl))LALALALALALALALWWG-NH(_2)</td>
</tr>
<tr>
<td>Walp4</td>
<td>Ac-(GWWC(sl))ALALALALALALALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp5</td>
<td>Ac-(GWWLC(sl))LALALALALALALWWG-NH(_2)</td>
</tr>
<tr>
<td>Walp6</td>
<td>Ac-(GWWLAC(sl))ALALALALALALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp8</td>
<td>Ac-(GWWLALAC(sl))ALALALALALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp16</td>
<td>Ac-(GWWLALALALALAC(sl))ALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp18</td>
<td>Ac-(GWWLALALALALALAC(sl))ALALWWA-NH(_2)</td>
</tr>
<tr>
<td>Walp1,23</td>
<td>Ac-(C(sl))GWWLALALALALALWWGC(sl)-NH(_2)</td>
</tr>
<tr>
<td>Walp3,21</td>
<td>Ac-(GWC(sl))LALALALALALALAC(sl)WG-NH(_2)</td>
</tr>
<tr>
<td>Walp5,19</td>
<td>Ac-(GWWLC(sl))LALALALALALAC(sl)LWWG-NH(_2)</td>
</tr>
<tr>
<td>Walp6,18</td>
<td>Ac-(GWWLAC(sl))ALALALALALAC(sl)LWWG-NH(_2)</td>
</tr>
<tr>
<td>Walp8,16</td>
<td>Ac-(GWWLALAC(sl))ALALALALALAC(sl)LALWWG-NH(_2)</td>
</tr>
</tbody>
</table>

A series of WALP23 peptides each containing either one Cys residues, or two Cys residues located symmetrically from the termini of the peptide, were labeled with non pH-sensitive probe MTSL and pH-sensitive probes IMTSL and IKMTSL. The depth of the MTSL probe
attached to WALP peptide incorporated into phospholipid bilayer was previously determined by paramagnetic relaxers accessibility measurements [150]. WALP peptides have been proposed as a ruler to determine the location of the labeled sites within the lipid bilayer. In case of double labeled WALP peptide, the distance between labels can be determined experimentally, and the depth of the label within the bilayer can be calculated. Knowing the location of the probe within the bilayer, the gradient of the dielectric constant within the lipid bilayer at the membrane-peptide interface can be investigated.

For transmembrane WALP peptides containing one Cys residues labeled with pH sensitive probe (IMTSL or IKMTSL), it is expected that the spin labels experience lower and lower dielectric permittivity as the corresponding Cys residues position deeper into the hydrophobic core of the lipid bilayer, thus the spin labels experiencing lower dielectric permittivity would lead to lower pKₐ. In such experiment, the relative difference between the local dielectric constants could be determined using the calibration plot introduced in chapter 2 resulting in an estimation of the dielectric gradient as a function of the position of Cys residues.
Figure 3.02 Schematic representation of the label’s positions on transmembrane WALP peptides, adapted from [65].

For WALP peptides containing two Cys residues labeled with pH sensitive probe (IMTSL or IKMTSL), it is expected that the two spin labels are located symmetrically within the bilayer at the same depth from the center of the bilayer (Figure 3.03) Symmetric location of the labels ensures that both nitroxide reporter groups are located in areas of the same dielectric environment, with the same local dipole and electrostatic field from the surface charges (if present). To ensure that “outer” and “inner” pH are always fully equilibrated, gramicidin (gA) proton channels were incorporated into the lipid bilayer.

\[ pK_a^{\text{observed}} = pK_a^{\text{intrinsic}} + \Delta pK_a^{\text{polarity}} + \Delta pK_a^{\text{electrostatic}} \]  
\text{Eq. 27}
Upon changing pH, one of the two labels of the peptide protonates in a statistical manner - the protonated label could be located in either the inner or outer leaflet. Thus, the first $pK_{a1}$ is determined by the intrinsic $pK_a$ of the probe, the $pK_a$ shift due to the electric field from the surface charges, and the shift due to local dielectric constant as in Eq. 27. Once the first label is protonated, the second protonated label experiences an additional Columbic interaction with the now protonated first label that results in $pK_{a2}$ of the second label being lower. Sample EPR spectra of a series of liposome with double labeled WALP peptides transmembrane are analyzed with least-squares decomposition and is fitted into “protonated” and “non-protonated” components, resulting a titration curve of nonprotonated form fraction $f$ vs. pH. Then the titration curves are fitted to a model assuming two transitions with $pK_{a1}$ and $pK_{a2}$ as adjustable parameters. Absolute value of $\Delta pK_a = |pK_{a1} - pK_{a2}|$ is calculated.

$\Delta pK_a = |pK_{a1} - pK_{a2}|$ is determined solely by $\Delta G_{Columb}$ defined by the distance, $r$, between the labels and the effective dielectric constant, $\epsilon$, for the medium between the two interacting labels as all other contributions to $pK_a$s are the same for both labels:

$$\Delta pK_a = \frac{-e\Psi}{ln(10)kT} \quad \text{Eq. 28}$$

$$\Delta G = ln(10)kT \Delta pK_a \quad \text{Eq. 29}$$

$$\Delta G_{Columb} = \frac{q^2}{4\pi \epsilon_0 \epsilon_r r} \quad \text{Eq. 30}$$

$$\Delta G = kTln(10)\Delta pK_a = \frac{q^2}{4\pi \epsilon_0 \epsilon_r r} \quad \text{Eq. 31}$$
The distance between the two labels on the same WALP peptide can be measured experimentally by DEER at 35GHz. Thus with known $\Delta pK_a$ and $r$, the effective dielectric constant $\varepsilon$ can be calculated from Eq.31.

In this chapter we used single and double labeled WALP to examine the effect of lipid composition and silica support on effective $pK_a^i$ of ionizable sidechain of transmembrane peptide.

3.2 Materials and methods.

3.2.1 Materials.

Zwitterionic lipid DOPC (1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine) and anionic lipid DOPG (1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)) were purchased from Avanti polar lipids (Alabaster, AL) as chloroform solutions (>99% pure) and stored at -80°C freezer before use without further purification. All solvents were reagent grade and used as received. MTSL, IMTSL and IKMTSL were synthesized by Prof. M. Voinov as previously reported. WALP peptides were synthesized by RS Synthesis (Louisville, KY) by solid-phase synthesis. Non-porous and non-functionalized silica beads (310nm, $\sim 3.407e^{12}$ beads/ml) were purchased from Bangs Laboratories (Fishers, IN) in 10 wt. % solids in DI water and used as received without further surface cleaning. Silica beads have a size coefficient of variation (CVs) of 10-15%. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).
3.2.2 Peptide spin labeling.

For the spin labeling reaction, approximately 6 mg of crude peptide was dissolved in 300 μL of 2,2,2-trifluoroethanol (TFE) and 2 molar equivalents of triethylamine (TEA) and 4 molar equivalents of the spin label were added to the peptide solution. Nitrogen was bubbled through the reaction mixture for approximately 5 minutes. The solution was left overnight in closed container at room temperature.

Spin labeled peptides were purified on a C4 reversed phase HPLC column (Vydac 214TP1010). For double labeled peptides, the solvent gradient was 77-88% methanol with 0.1% trifluoroacetic acid (TFA) over 10 minutes, followed by 88-96% methanol with 0.1% TFA over 50 minutes and finally 96-80% methanol with 0.1% TFA over the final 5 minutes with a flow rate of 4 mL/minute. For single labeled peptides, the solvent gradient was 77-88% methanol with 0.1% trifluoroacetic acid (TFA) over 10 minutes, followed by 88-96% methanol with 0.1% TFA over 35 minutes and finally 96-80% methanol with 0.1% TFA over the final 5 minutes with a flow rate of 4 mL/minute. The spin labeled peptides eluted at approximately 30-40 minutes. The peak at 3 minutes contains unreacted free label.

After separating the spin labeled peptide from unreacted label, the sample was then lyophilized overnight to remove the solvent. Following lyophilization, the solid spin labeled peptide was dissolved in 1 mL of TFE. Aliquots of this peptide solution were then diluted for use in UV-Vis and mass spectrometry measurements [65].
3.2.3 Characterization of double labeled WALP peptides.

Double labeled WALP peptides were analyzed by mass spectrometry (MS) based on chromatographic fractions of spin labeled peptides for positive identification. (2) Nitroxide concentration in WALP samples was calculated the double integral of the EPR spectra with calibration curve built using standard Tempol solutions of known concentration in same solvent. Peptide concentrations were calculated using Beer-Lambert Law based on the ultraviolet absorbance of tryptophan residues at 280 nm measured with A Varian Cary® 50 UV-Vis spectrometer.

3.2.4 Phospholipid liposomes preparation.

Phospholipids sample composition was 5 w/v % desired lipids (DOPC, DOPG or DOPC50:DOPG50 mixture) with 1 mole % of desired spin labeled WALP peptides and an average of 10 gA channels per 100nm vesicles.

3.2.5 Silica beads supported lipid bilayer preparation.

Refer to chapter 2 section 2.2.4.

3.2.6 Sample characterization with DLS size measurement and zeta potential measurement.

Refer to chapter 2 section 2.2.3 and section 2.2.5.
3.2.7 Liposome pH titration experiments.
Refer to chapter 2 section 2.2.6.

3.2.8 Silica beads supported lipid bilayer pH titration experiments.
Refer to chapter 2 section 2.2.7.

3.2.9 CW-EPR measurements.
Refer to chapter 2 section 2.2.8.
3.3 Results and discussion

3.3.1 Control experiments: effect of pH on dynamics of WALP peptide incorporated in liposomes or silica supported bilayers.

Similar to spin labeled lipids, control experiments of liposome and silica supported lipid bilayer using non pH-sensitive analog WALP1-MTSL were carried out to reaffirm that the changes in EPR spectra of WALP peptide labeled with pH-sensitive nitroxides IMTSL and IKMTSL are indeed arising from reversible protonation of pH-sensitive nitrooxide and not from changes in the dynamics of the peptide upon changes in pH. Spectra from WALP1-MTSL incorporated into 100 nm DOPC LUVs collected as function of pH are shown in Figure 3.04 (left). Simple comparison of the spectra indicates no changes in the EPR line shape as a function of pH. It can be noticed that the spectra obtained from WALP1-MTSL incorporated into DOPC bilayer on silica supported are broader than spectra from liposome, and contain low-field shoulder around 3490 G and high field features around 3550 G that are characteristic of slower and more restricted nitroxide tumbling. The latter indicate that positioning of WALP containing bilayer on silica support affect the peptide dynamics. More details will be discussed later in 3.3.4 session. Figure 3.03 (right panel) shows intensity normalized EPR spectra from silica supported DOPC bilayer containing WALP1-MTSL as function of pH. No changes in the spectra were detected over the range of pH used in this work, indicating no changes in dynamics of the peptide with varying the pH of the samples.
Figure 3.03 Representative intensity normalized CW X-band EPR spectra from DOPC 100 nm LUVs containing 1 mol % of WALP1-MTSL (left), and silica supported DOPC bilayer containing 1 mol % of WALP1-MTSL (right) obtained at various pH.

3.3.2 Determination of the interfacial $pK_a$ of spin labeled WALP (1Cys) peptide incorporated in LUVs.

WALP peptides containing one Cys residues, WALP1, WALP3, WALP5, WALP6, WALP8 were labeled with IKMTSL and incorporated into DOPC LUVs; WALP2, WALP3, WALP4, WALP6, WALP18 were labeled with IMTSL and incorporated into DOPG LUVs. These two set of WALP peptides containing one Cys residues samples were titrated to compare $pK_a$ as a function of the label depth within the bilayer. Representative spectra from WALP1-IKMTSL incorporated into DOPC LUVs and from WALP1-IMTSL incorporated into DOPG LUVs are shown in Figure 3.04.
Figure 3.04 Representative intensity normalized CW X-band EPR spectra from WALP1-IKMTSL incorporated into DOPC LUVs (left) and WALP2-IMTSL incorporated into DOPG LUVs (right) as function of pH.

EPR spectra taken at intermediate pH values were treated as a superposition of two components (protonated and nonprotonated states of the spin label). The fraction $f$ of the non-pronotonated form was calculated and plotted as a function of pH to obtain titration curves. The experimental data points were fitted to the Henderson-Hasselbach equation Eq.32 using model of single protonation, from which the single pK$_a$ of the spin labels were determined.

$$f = \frac{10^{(pH-pK_a)}}{[1 + 10^{(pH-pK_a)}]} \quad Eq.32$$
Figure 3.05 Titration curves for WALP (1cys)-IKMTSL incorporated into DOPC LUVs. Labeling position WALP1 (green). Labeling position WALP3 (pink). Labeling position WALP5 (blue). Labeling position WALP6 (red). Labeling position WALP8 (cyan). Solid lines are the best fit to single dissociation model Eq.32.

Table 3.03 pKₘ values obtained from titration curves for WALP (1cys)-IKMTSL incorporated into DOPC LUVs using single dissociation model Eq.32.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>Single pKₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP1</td>
<td>5.99 ± 0.02</td>
</tr>
<tr>
<td>WALP3</td>
<td>6.02 ± 0.03</td>
</tr>
<tr>
<td>WALP5</td>
<td>5.72 ± 0.01</td>
</tr>
<tr>
<td>WALP6</td>
<td>5.59 ± 0.01</td>
</tr>
<tr>
<td>WALP8</td>
<td>5.17 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 3.06 Titration curves for WALP (1cys)-IMTSL incorporated into DOPG LUVs. Labeling position WALP2 (green). Labeling position WALP3 (pink). Labeling position WALP4 (blue). Labeling position WALP6 (red). Labeling position WALP18 (cyan). Solid lines are the best fit to single dissociation model Eq.32.

Table 3.04 pK\textsubscript{a} values obtained from titration curves for WALP (1cys)-IMTSL incorporated into DOPG LUVs using single dissociation model Eq.32.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>Single pK\textsubscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP2</td>
<td>4.59 ± 0.03</td>
</tr>
<tr>
<td>WALP3</td>
<td>3.93 ± 0.02</td>
</tr>
<tr>
<td>WALP4</td>
<td>3.62 ± 0.04</td>
</tr>
<tr>
<td>WALP6</td>
<td>3.10 ± 0.05</td>
</tr>
<tr>
<td>WALP18</td>
<td>3.15 ± 0.04</td>
</tr>
</tbody>
</table>
As the pH of the sample is lowered, the most apparent change in the EPR spectra is the progressive rise of the low-field shoulder around 3490 G. This feature is characteristic of slower and more restricted nitroxide tumbling. The line shape change is more sensitive to the peptide labeled with IMTSL and incorporated into DOPG samples compared to the peptide labeled with IKMTSL and incorporated into DOPC samples. The change in motion of the label upon protonation is attributed to the electrostatic interactions of the label with either electric dipoles of lipid head groups or local dipoles of α-helix.

For WALP (1cys) labeled with IKMTSL and incorporated into DOPC, WALP1 and WALP3 show essentially same pK\textsubscript{a} values, 5.99 ± 0.02 and 6.02 ± 0.03, respectively. WALP1 and WALP3 are supposed to sit within the lipids carbonyls region [145, 150], where protonated nitroxides are more likely to be affected by the lipid head group charges. IKMTSL moiety with a volume of approximate 0.71nm (length) x 0.67nm (height) x 7.1nm (width) [109] and a linker length of approximate 1.1nm has more flexibility and rotational mode. Upon titration it might be located within an area of lowest free Gibbs energy thus we suspect WALP1 and WALP3 with α-carbon distance difference of 3Å are within this area. From WALP 3 to WALP 8, as attached nitroxides are getting closer to the hydrophobic bilayer center, pK\textsubscript{a} values drops by 0.82 pH unit. This drop is attributed to lower local dielectric permittivity at position WALP8.

For WALP (1cys) labeled with IMTSL and incorporated into DOPG, WALP6 and WALP18 shows essentially same pK\textsubscript{a} values, 3.10 ± 0.05 and 3.15 ± 0.05 respectively. Oxygen accessibility measurements performed on these spin labeled samples show roughly same depth
of spin label within the membrane [65, 151]. These two results shows good agreement with the fact that spin labeling positions 6 and 18 are symmetric with respect to the bilayer center. From WALP 2 to WALP 6, as attached nitroxides are getting closer to the hydrophobic bilayer center, pK\(_a\) values drop by about 1.44 pH unit. This drop is also attributed to lower local dielectric permittivity at position WALP6 as compared to WALP2.

Additional observation is that the single pK\(_a\) values for IMTSL labeled peptides incorporated into DOPG are more sensitive to the position of their respective \(\alpha\)-carbon attachment than those for IKMTSL labeled peptides incorporated into DOPC, i.e. scanning the peptide backbone with IMTSL from the termini to the reports on a steeper gradient of the spin label’s dissociation constant [65] (Figure 3.07). This is attributed to the electrostatic nature of the phospholipids and possibly more rotational flexibility of IKMTSL that can relocate to higher dielectric permittivity region. Fitting the experimental pK\(_a\) values with the distance of the spin label’s alpha carbon attachment from the bilayer center yielded the following linear relationships:

\[
pK_a = 0.0796d + 4.81 \quad \text{IKMTSL in DOPC}
\]

\[
pK_a = 0.2379d + 0.86 \quad \text{IMTSL in DOPG}
\]
Figure 3.07 Experimental pK$_a$ values as a function of spin label attachment site and its respective alpha carbon distance from the bilayer center. IMTSL labeled WALPs in DOPG LUVs (open circle), IKMTSL labeled WALPs in DOPC LUVs (filled circle).

3.3.3 Determination of the interfacial pK$_a$ of spin labeled WALP (2Cys) peptide incorporated in LUVs.

For WALP peptides containing two Cys residues, WALP1, 23, WALP3, 21, WALP5, 19, WALP6, 18, WALP8, 16 were labeled with IKMTSL and incorporated into DOPC liposome. WALP1, 23, WALP3, 21, WALP5, 19, WALP6, 18, WALP8, 16 were labeled with IMTSL and incorporated into DOPG liposome. These two set of double labeled WALP samples were titrated to compare pK$_a$ of the probe ionization as a function of the label depth. Experiments were carried out by Dr. M. Donohue and data were analyzed by Erkang Ou. EPR spectra taken at intermediate pH values were treated as a superposition of two components (protonated and
nonprotonated states of the spin label). The fraction $f$ of the non-pronotated form was calculated and plotted as a function of pH to form titration plots.

If we assume that the two nitroaxes covalently attached to the WALP peptides undergo protonation independently, these two probes should have the same pK$_a$ since the two nitroaxes are located symmetrically at the same depth from the center of the lipid bilayer in areas of the same dielectric environment and same electric field In this situation, we should be able to fit the titration curves to the Henderson-Hasselbach equation corresponding to single dissociation. However the fitting shows WALP (2cys) samples should be fit with equation corresponding to double dissociation. This is to say that protonation of the first nitroxide affects the pK$_a$ of the second nitroxide attached to the same WALP peptide because of the positive charge created on the first nitroxide upon protonation.

![Titration curves for WALP 1, 23 labeled with IKMTSL and incorporated into DOPG LUVs. Solid line is the best fit to dependent double dissociation model Eq.42, dashed line is the best fit to single dissociation model Eq.32. Experimental data adapted from [65].](image)

**Figure 3.08** Titration curves for WALP 1, 23 labeled with IKMTSL and incorporated into DOPG LUVs. Solid line is the best fit to dependent double dissociation model Eq.42, dashed line is the best fit to single dissociation model Eq.32. Experimental data adapted from [65].
If we treat each of the two nitroxides covalently attached to the WALP peptides separately and look at a single nitroxide as a unit, that each nitroxide gives a single dissociation. If two labels, despite symmetric location of the labeled residues, are experiencing different environments, than they would have different pKₐ values. If protonation of two labels occurs independently, meaning protonation of the first label does not affect pKₐ of the second label, the titration curve should be described by a summation of two transitions:

\[
f = a \frac{10^{pH-pK_{a1}}}{(1 + 10^{pH-pK_{a1}})} + b \frac{10^{pH-pK_{a2}}}{(1 + 10^{pH-pK_{a2}})} \quad Eq. 33
\]

Since the amount of spin labels is equal on each side of the peptides, thus a=b=0.5

\[
f = 0.5 \frac{10^{pH-pK_{a1}}}{(1 + 10^{pH-pK_{a1}})} + 0.5 \frac{10^{pH-pK_{a2}}}{(1 + 10^{pH-pK_{a2}})} \quad Eq. 34
\]

Another model to consider is one where two labels are experiencing the same local environment, and, as result, should have the same pKₐ values, however, once the first label is protonated, the second one is experiencing an additional Coulombic interaction with positively charged first label that makes protonation of the second label more difficult by destabilizing second positive charge on WALP peptide. WALP (2cys) peptide undergoes two sequential protonation during pH titration with pKₐ₁ for first nitroxide and pKₐ₂ for second nitroxide. Since the second protonated nitroxide experience the destabilization of the protonated form due to interaction with the first protonate nitroxide, we expect pKₐ₁>pKₐ₂.

\[
NN + H^+ \leftrightarrow NNH^+
\]

\[
NNH^+ + H^+ \leftrightarrow NH^+ NH^+
\]
Where NN represent WALP (2cys) with two neutral nitroxide, NNH\(^+\) represent one of the nitroxide is charged and the other nitroxide is neutral, NH\(^+\)NH\(^+\) represent WALP (2cys) with both nitroxides in charged form. At the highest pH of titration curve, all WALP peptides exist as NN neutral form, at the lowest pH of titration curve, all WALP peptides can exist as NH\(^+\)NH\(^+\) charge form, at intermediate pH, WALP peptides can potentially exist as a mixture of NN, NH\(^+\)NH\(^+\) and NNH\(^+\) forms. Though NNH\(^+\) form has two orientations with the protonated nitroxide in either inner leaflet or outer leaflet of lipid bilayer, since bilayer curvature is negligible and pH of the inner volume is always equilibrated with bulk solution, these two are identical.

Henderson-Hasselbach equation states that

\[
pH = pK_{a_1} + \log \frac{[NN]}{[NNH^+]} \quad \text{Eq. 35}
\]

\[
pH = pK_{a_2} + \log \frac{[NNH^+]}{[NH^+NH^+]} \quad \text{Eq. 36}
\]

We can express [NN] in terms of [NNH\(^+\)]:

\[
[NN] = [NH^+NH^+] \cdot 10^{(pH-pK_{a_1})} \quad \text{Eq. 37}
\]

We can express [NH\(^+\)NH\(^+\)] in terms of [NNH\(^+\)]:

\[
[NH^+NH^+] = \frac{[NNH^+]}{10^{(pH-pK_{a_2})}} \quad \text{Eq. 38}
\]

The fraction of protonated form \(f_{\text{charged}}\) is equal to

\[
f_{\text{charged}} = \frac{1 \cdot [NNH^+] + 2 \cdot [NH^+NH^+]}{2 \cdot [NNH^+] + 2 \cdot [NH^+NH^+] + 2 \cdot [NN]} \quad \text{Eq. 39}
\]
The numerator represents the total protonated nitroxide concentration, the coefficient 1 before \([\text{NNH}^+]\) means NNH\(^+\) form of peptide has one protonated nitroxide, the coefficient 2 before \([\text{NH}^+\text{NH}^+]\) means NH\(^+\)NH\(^+\) form of peptide has two protonated nitroxides. The denominator represents the total nitroxide concentration, since NN, NNH\(^+\) and NH\(^+\)NH\(^+\) forms of peptide, each contribute two to the total number of nitroxides. Then we can express the fraction of protonated form \(f_{\text{charged}}\) in terms of \([\text{NH}^+]\),

\[
f_{\text{charged}} = \frac{1 \cdot [\text{NNH}^+] + 2 \cdot \frac{[\text{NNH}^+]}{10^{(\text{pH}-pK_{a2})}}}{2 \cdot [\text{NNH}^+] + 2 \cdot \frac{[\text{NNH}^+]}{10^{(\text{pH}-pK_{a2})}} + 2 \cdot [\text{NNH}^+] \cdot 10^{(\text{pH}-pK_{a1})}} \quad \text{Eq. 40}
\]

This leads to

\[
f_{\text{charged}} = \frac{1 + 2 \cdot \frac{1}{10^{(\text{pH}-pK_{a2})}}}{2 + 2 \cdot \frac{1}{10^{(\text{pH}-pK_{a2})}} + 2 \cdot 10^{(\text{pH}-pK_{a1})}} \quad \text{Eq. 41}
\]

Thus the fraction of non-protonated form \(f_{\text{neutral}}\) is equal to

\[
f_{\text{neutral}} = \frac{1 + 2 \cdot 10^{(\text{pH}-pK_{a1})}}{2 + 2 \cdot \frac{1}{10^{(\text{pH}-pK_{a2})}} + 2 \cdot 10^{(\text{pH}-pK_{a1})}} \quad \text{Eq. 42}
\]

Now we assess the spin labeled WALP (2cys) incorporated into liposome samples and fit the data to double dissociation equation and this will be employed as the control experiments for investigation of effect of silica support on lipid-peptide interface. The pK\(_a\) values calculated using independent double dissociation and dependent double dissociation models are displayed in Table 3.05-3.08, which are positioned below their respective titration curves.
Figure 3.09 Titration curves for WALP (2cys)-IKMTSL incorporated into DOPC LUVs. Labeling position WALP1,23 (green). Labeling position WALP3,21 (pink). Labeling position WALP5,19 (blue). Labeling position WALP6,18 (red). Labeling position WALP8,16 (cyan). Solid lines are the best fit to dependent double dissociation model Eq.42. Experimental data adapted from [65].

Table 3.05 $pK_a$ values obtained from titration curves for WALP (2cys)-IKMTSL incorporated into DOPC LUVs using independent double dissociation model Eq.34.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>$pK_{a2}$</th>
<th>$pK_{a1}$</th>
<th>$\Delta pK_a$</th>
<th>Single $pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP1,23</td>
<td>5.13 ± 0.05</td>
<td>6.25 ± 0.05</td>
<td>1.12</td>
<td>5.70 ± 0.05</td>
</tr>
<tr>
<td>WALP3,21</td>
<td>5.36 ± 0.15</td>
<td>5.96 ± 0.15</td>
<td>0.60</td>
<td>5.67 ± 0.04</td>
</tr>
<tr>
<td>WALP5,19</td>
<td>5.04 ± 0.06</td>
<td>5.95 ± 0.09</td>
<td>0.91</td>
<td>5.45 ± 0.03</td>
</tr>
<tr>
<td>WALP6,18</td>
<td>5.42 ± 0.16</td>
<td>5.42 ± 0.16</td>
<td>0.00</td>
<td>5.42 ± 0.02</td>
</tr>
<tr>
<td>WALP8,16</td>
<td>4.79 ± 0.12</td>
<td>5.33 ± 0.14</td>
<td>0.54</td>
<td>5.05 ± 0.03</td>
</tr>
</tbody>
</table>

Table 3.06 $pK_a$ values obtained from titration curves for WALP (2cys)-IKMTSL incorporated into DOPC LUVs using dependent double dissociation model Eq.42.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>$pK_{a2}$</th>
<th>$pK_{a1}$</th>
<th>$\Delta pK_a$</th>
<th>Single $pK_a$</th>
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</thead>
<tbody>
<tr>
<td>WALP1,23</td>
<td>5.10 ± 0.04</td>
<td>6.28 ± 0.04</td>
<td>1.18</td>
<td>5.70 ± 0.05</td>
</tr>
<tr>
<td>WALP3,21</td>
<td>5.27 ± 0.09</td>
<td>6.06 ± 0.09</td>
<td>0.79</td>
<td>5.67 ± 0.04</td>
</tr>
<tr>
<td>WALP5,19</td>
<td>4.99 ± 0.05</td>
<td>6.00 ± 0.07</td>
<td>1.01</td>
<td>5.45 ± 0.03</td>
</tr>
<tr>
<td>WALP6,18</td>
<td>5.13 ± 0.06</td>
<td>5.71 ± 0.05</td>
<td>0.58</td>
<td>5.42 ± 0.02</td>
</tr>
<tr>
<td>WALP8,16</td>
<td>4.68 ± 0.07</td>
<td>5.44 ± 0.09</td>
<td>0.76</td>
<td>5.05 ± 0.03</td>
</tr>
</tbody>
</table>
Figure 3.10 Titration curves for WALP (2cys)-IMTSL incorporated into DOPG LUVs. Labeling position WALP1,23 (green). Labeling position WALP3,21 (pink). Labeling position WALP5,19 (blue). Labeling position WALP6,18 (red). Experimental data adapted from [65].

Table 3.07 pKₐ values obtained from titration curves for WALP (2cys)-IMTSL incorporated into DOPG LUVs using independent double dissociation model Eq.34.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>pKₐ₂</th>
<th>pKₐ₁</th>
<th>ΔpKₐ</th>
<th>Single pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP1,23</td>
<td>5.08 ± 0.19</td>
<td>5.08 ± 0.19</td>
<td>0.00</td>
<td>5.08 ± 0.04</td>
</tr>
<tr>
<td>WALP3,21</td>
<td>4.77 ± 0.14</td>
<td>4.77 ± 0.14</td>
<td>0.00</td>
<td>4.77 ± 0.01</td>
</tr>
<tr>
<td>WALP5,19</td>
<td>3.48 ± 0.10</td>
<td>4.53 ± 0.08</td>
<td>1.05</td>
<td>4.03 ± 0.04</td>
</tr>
<tr>
<td>WALP6,18</td>
<td>3.04 ± 0.08</td>
<td>4.42 ± 0.07</td>
<td>1.38</td>
<td>3.71 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3.08 pKₐ values obtained from titration curves for WALP (2cys)-IMTSL incorporated into DOPG LUVs using dependent double dissociation model Eq.42.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>pKₐ₂</th>
<th>pKₐ₁</th>
<th>ΔpKₐ</th>
<th>Single pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP1,23</td>
<td>5.08 ± 0.08</td>
<td>5.08 ± 0.08</td>
<td>0.00</td>
<td>5.08 ± 0.04</td>
</tr>
<tr>
<td>WALP3,21</td>
<td>4.58 ± 0.03</td>
<td>4.95 ± 0.03</td>
<td>0.37</td>
<td>4.77 ± 0.01</td>
</tr>
<tr>
<td>WALP5,19</td>
<td>3.44 ± 0.09</td>
<td>4.57 ± 0.07</td>
<td>1.13</td>
<td>4.03 ± 0.04</td>
</tr>
<tr>
<td>WALP6,18</td>
<td>3.02 ± 0.08</td>
<td>4.44 ± 0.07</td>
<td>1.42</td>
<td>3.71 ± 0.05</td>
</tr>
</tbody>
</table>
Among the above 10 titration experiments, WALP1,23-IMTSL incorporated into DOPG liposome can only be fitted to single dissociation. We speculate that the Coulombic interaction between two labels on the same WALP peptide is screened because of very high value of the local dielectric constant and resulting short Debye length of the charge at that location. The dielectric permittivity in the region close to the phosphate charges was predicted to be exceptionally high, 230 [144]. However, titration curve for WALP1,23-IKMTSL incorporated into DOPC LUVs actually can be fitted to double dissociation model. We speculate that because DOPC is a zwitterionic lipid, the effect of the DOPC dipole on screening the charge of the first protonated label is weaker compared to the screening by negatively charged DOPG. Data for WALP8,16-IMTSL incorporated into DOPG were excluded because the titration curve indicated the labels remained partially deprotonated until a pH of 1.8 was reached.

Dr. M. Donohue carried out another two sets of experiment of WALP (2cys)-IKMTSL incorporated into DOPC/DOPG liposome at a 1:1 molar ratio and WALP (2cys)-IKMTSL incorporated into DOPG liposome, data were analyzed by Erkang Ou. With independent double dissociation fitting model, WALP6,18-IKMTSL-DOPC, WALP1,23-IKMTSL-DOPC/DOPG, WALP6,18-IKMTSL-DOPC/DOPG, WALP8,16-IKMTSL-DOPC/DOPG, WALP3,21-IMTSL-DOPG titrations give identical pK_{a1} and pK_{a2}, indicate that the two nitroxides are experiencing exactly same environment, however, this is in conflict with the rest of other experiments that nitroxide experiencing different environment. It confirms that we should treat the two nitroxide covalently attached to the same WALP peptide as entity and look at a WALP peptide as a unit. In conclusion, by comparing independent double dissociation and
dependent double dissociation models, results reaffirmed that double spin labeled WALP peptides undergo two sequential proton dissociations and pK<sub>a</sub> difference between two spin labels is correlated to columbic energy and can be used to assess the dielectric constant.

Comparing pK<sub>a</sub> values of WALP (2cys) peptides labeled with IKMTSL or IMTSL at the same position but incorporated into liposome with various lipid composition confirmed that lipid bilayer surface charge density affect pK<sub>a</sub> by mostly contributing to \( \Delta pK_a^{el} \) term in equation for calculating local pK<sub>a</sub> values. Figure 3.11 shows the effect of lipid bilayer surface charge density on effective pK<sub>a</sub> values of WALP peptides. pK<sub>a</sub> values calculated by fitting titration curves to dependent double dissociation model are summarized in Table 3.09-3.10. Replacing DOPC with DOPG in the IKMTSL labeled WALP3, 21 peptides shifts the experimental pK<sub>a1</sub> by 2.6 pH units, replacing DOPC and DOPG mixture at 1:1 molar ratio with DOPG in the IMTSL labeled WALP3, 21 peptides shifts the experimental pK<sub>a1</sub> by 1.5 pH units.
Figure 3.11 Titration curves for IKMTSL labeled WALP3, 21 incorporated into DOPG LUVs (red), DOPC/DOPG LUVs at a 1:1 molar ratio (purple), DOPC LUVs (blue), experimental data adapted from [65] (left). Titration curves for IMTSL labeled WALP3, 21 incorporated into DOPG LUVs (red), DOPC/DOPG LUVs at a 1:1 molar ratio (purple), experimental data adapted from [65] (right). Solid lines are the best fit to dependent double dissociation model Eq.42.

Table 3.09 $pK_a$ values obtained from titration curves for IKMTSL labeled WALP3,21 incorporated into LUVs of various lipid composition using dependent double protonation model Eq.42. Experiments were carried out by Dr. M. Donohue, and data were analyzed by Erkang Ou.

<table>
<thead>
<tr>
<th>Spin label, lipid type</th>
<th>$pK_{a2}$</th>
<th>$pK_{a1}$</th>
<th>$\Delta pK_a$</th>
<th>Single $pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKMTSL, DOPC</td>
<td>5.27 ± 0.09</td>
<td>6.06 ± 0.09</td>
<td>0.79</td>
<td>5.67 ± 0.04</td>
</tr>
<tr>
<td>IKMTSL DOPC50:DOPG50</td>
<td>6.82 ± 0.06</td>
<td>7.69 ± 0.05</td>
<td>0.87</td>
<td>7.26 ± 0.03</td>
</tr>
<tr>
<td>IKMTLS, DOPG</td>
<td>7.43 ± 0.05</td>
<td>8.65 ± 0.06</td>
<td>1.22</td>
<td>7.99 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3.10 $pK_a$ values obtained from titration curves for IMTSL labeled WALP3,21 incorporated into LUVs of various lipid composition using dependent double protonation model Eq.42. DOPG experiment was carried out by Dr. M. Donohue, and data were analyzed by Erkang Ou.

<table>
<thead>
<tr>
<th>Spin label, lipid type</th>
<th>$pK_{a2}$</th>
<th>$pK_{a1}$</th>
<th>$\Delta pK_a$</th>
<th>Single $pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMTSL DOPC50:DOPG50</td>
<td>3.28 ± 0.11</td>
<td>3.39 ± 0.11</td>
<td>0.11</td>
<td>3.36 ± 0.04</td>
</tr>
<tr>
<td>IMTLS, DOPG</td>
<td>4.58 ± 0.03</td>
<td>4.95 ± 0.03</td>
<td>0.37</td>
<td>4.77 ± 0.01</td>
</tr>
</tbody>
</table>
Since the first pK\textsubscript{a1} is determined by the intrinsic pK\textsubscript{a} of the probe, the pK\textsubscript{a} shift due to the electric field from the surface charges and the shift due to local dielectric constant. In Figure 3.12 we plot pK\textsubscript{a1} values as a function of Cys α-carbon distance from the center of the bilayers. pK\textsubscript{a} values for single labeled WALP are shown on the same graph for comparison.

For IKMTSL double labeled WALP (2cys) peptides incorporated into DOPC LUVs, the experimental pK\textsubscript{a1} values drop as the label is moved close to the bilayer center and the plot of pK\textsubscript{a} vs. the depth of the label yields a linear relationship. The corresponding IKMTSL spin labeled WALP (1cys) peptides exhibit similar trend pK\textsubscript{a} values 0.2 pH lower than the first pK\textsubscript{a} of double labeled WALP (2cys). This small difference might be attributed to somewhat different location of the single label within the membrane as compared with the position on labels of double labeled peptide. Single label can slightly shift the peptide with respect to bilayer, while in double labeled WALP potential displacement of WALP due to first label is counteracted by the second label. For IKMTSL double labeled WALP (2cys) peptides incorporated into either DOPC/DOPG mixture or DOPG LUVs, the experimental pK\textsubscript{a1} values don’t follow the gradual lowering trend when labeling position approaching lipid bilayer center. Because of electrostatic interactions (charge-charge and charge-dipole) between the positive protonated nitroxide and lipid bilayer surface, a positional displacement of the nitroxide towards the bilayer surface or even peptide relocation within the lipid bilayer might happen, which we called it snorkeling effect. The snorkeling of ionizable spin labels was confirmed by distance measurements between spin label pairs carried out using Q-band double electron-electron resonance (DEER) experiment. DEER experiments confirmed that the
average spin-spin distance increased upon protonation of labels compared to the neutral states [65].

For IMTSL double labeled WALP (2cys) peptides incorporated into DOPG liposome, the experimental pK$_{a1}$ values drop as the label is moved close to the bilayer center, yielding a linear relationship. The corresponding IMTSL single labeled WALP (1cys) peptides exhibit similar trend, however the single pK$_a$ values are much lower than WALP (2cys) and resulting in a much steeper gradient. We suspect that in the later WALP (1cys) DOPG lipids were not fully charged. This needs to be validated with further experiments.

Figure 3.12 Calculated pK$_{a1}$ values corresponding to WALP (2cys) dependent double dissociation (filled circle) and calculated pK$_a$ values corresponding to WALP (1cys) single dissociation (open circle) as a function of spin label attachment site’s respective alpha carbon distance from the bilayer center. IKMTSL labeled WALPs in DOPG (red) DOPC/DOPG (purple) and DOPC (blue) bilayers (left). IMTSL labeled WALPs in DOPG (red) and DOPC/DOPG (purple) bilayers (right).
Figure 3.13 Probability profiles of different molecular components of DOPC, adapted with modification from [152]. The WALP peptide is drawn as a ribbon within the pink box.

The hydrophobic L-A repeats of WALP peptides are assumed to locate in the hydrophobic interior of the lipid bilayer and the capping tryptophan sidechains partitioned into the polar head group region serve as the anchors ensuring the peptide consistent registration in the lipid bilayer [148]. It was demonstrated that the WALP peptides is theoretically 34.5 Å in length (1.5 Å alpha helical rise for 23 residues) and matches well with the hydrophobic thickness of the DOPC/DOPG bilayers (27 Å) and total thickness of the DOPC/DOPG bilayer (50 Å) [149]. WALP1,23 labeling position is around head group and carbonyls region and is attached to α-carbon distance from center of 17Å. WALP3,21 labeling position is between hydrocarbon core and carbonyls region and label is attached to α-carbon located 14Å from center of the bilayer. In WALP5, 19 labeling position is close to hydrocarbon core edge and the label is attached to α-carbon located at 11Å from center of the bilayer. WALP6, 18 label is attached to α-carbon
located at 9.5Å from the bilayer center, WALP8, 16 is attached to α-carbon located at 6.5Å from the bilayer center.

In Chapter 1 we have discussed the electrostatic potential across lipid bilayer and its decomposition into three major components, surface potential, transmembrane potential and dipole potential. Surface potential of WALP peptides should be constant within lipid bilayer. Since we utilized gA to ensure inner leaflet and outer leaflet pH are always fully equilibrated, we expect the absence of the transmembrane potential. Dipole potential for WALP3,21 to WALP8,16 is assumed to be constant, thus we expect constant total electrostatic potential for WALP3,21 to WALP8,16. The relative gradient in local dielectric constants could be determined from the first protonation pKₐs using the calibration plot of pKₐ of the probe as a function of dielectric constant of solvent reported for MTSL [94] and IKMTSL [99] and introduced in chapter 2. For example, the difference in the first pKₐ for IMTSL labeled WALP3,21 and WALP8,16 in DOPG lipids is 0.51 pH units, resulting in estimation of the gradient of dielectric constant of 13.3 between these locations. Results of calculations using this approach are shown in Table 3.11 as Δε.

If location of the labels are symmetric and both nitroxides are located in area of the same dielectric environment with the same local electric field potential from the surface charges, using experimentally determined ΔpKₐ = |pKₐ1 - pKₐ2| and measured spin-spin distance r (experiments were conducted by Dr. M. Donohue) the dielectric constant ε for the medium between the two interacting labels can be determined from Eq. 43.
\[
\varepsilon_r = \frac{e_0^2}{4\pi\varepsilon_0 kT \ln(10) \Delta pK_a} \quad \text{Eq. 43}
\]

Table 3.11 Gradient in dielectric constant \(\Delta\varepsilon\) is calculated from \(pK_a\) calibration introduced in Table 2.02. For double labeled WALP-IMTSL in DOPG LUVs the absolute value of dielectric constant \(\varepsilon\) is calculated using Eq. 43.

<table>
<thead>
<tr>
<th>WALP</th>
<th>IMTSL DOPG (\Delta\varepsilon)</th>
<th>IMTSL DOPG (\varepsilon)</th>
<th>IKMTSL DOPC (\Delta\varepsilon)</th>
<th>Peptide</th>
<th>IKMTSL DOPC (\Delta\varepsilon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP1,23</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td>WALP1</td>
<td>-</td>
</tr>
<tr>
<td>WALP3,21</td>
<td>Reference</td>
<td>22.7</td>
<td>Reference</td>
<td>WALP3</td>
<td>Reference</td>
</tr>
<tr>
<td>WALP5,19</td>
<td>Ref – 9.9</td>
<td>8.1=22.7–14.6</td>
<td>Ref – 1.3</td>
<td>WALP5</td>
<td>Ref – 6.3</td>
</tr>
<tr>
<td>WALP6,18</td>
<td>Ref – 13.3</td>
<td>6.9=22.7–15.8</td>
<td>Ref – 7.4</td>
<td>WALP6</td>
<td>Ref – 9.1</td>
</tr>
<tr>
<td>WALP8,16</td>
<td>NA</td>
<td>NA</td>
<td>Ref – 13.1</td>
<td>WALP8</td>
<td>Ref – 17.8</td>
</tr>
</tbody>
</table>

Ideally if nitroxides covalently attached to WALP (2cys) peptides are perfectly symmetric within the bilayer, the determined dielectric constant \(\varepsilon\) absolute values for liposome composed with different lipids and surface charge density should result in similar values, and the values should agree with gradients in dielectric constant \(\Delta\varepsilon\) between different labeling positions calculated from \(pK_a\) calibration introduced in Table 2.02. However because of snorkeling effect of protonated nitroxides and rotational flexibility of spin label with certain linker length [65], upon titration, protonation may cause a positional displacement of the nitroxide towards the bilayer surface or even drag the peptide to relocate and rest at the region with higher dielectric permittivity and lower Gibbs energy to stabilize the positively charged label within the lipid bilayer. Thus the calculated \(\Delta pK_a = |pK_{a1} - pK_{a2}|\) is not determined solely by the additional columbic interaction defined. Another potential source of error is in utilization of the calibration curve. The calibration curve reported in [94, 99] was measured for solvents with the range of dielectric constant from 40 to 80. In our calculations we assumed the same slope
could be used for solvents with much lower dielectric constant, assumption that was not verified experimentally. Theoretical model states that hydrocarbon interior with a distance of 0-10Å from lipid bilayer has a dielectric constant of 2, carbonyls area with a distance of 10-15Å from lipid bilayer has a dielectric constant of 7, interfacial between phosphate, head groups and ester area with a distance of 15-20Å from lipid bilayer has a dielectric constant of 180 [144]. For IMTSL labeled WALP (2cys) incorporated into DOPG liposome, the dielectric constant ε determined for WALP6,18 WALP5,19 and WALP3,21 was higher than the values predict for the bulk membrane. Although isobutyl and methyl side-chains of WALP peptide are similar in hydrophobicity to the hydrocarbon chains of lipids, a contribution of the peptide backbone dipole moment can result in a higher local dielectric constant ε at the peptide-lipid interface. In addition, transmembrane WALP peptides may perturb and deform the local bilayer structure thus allowing water and ions penetrate deeper and increase the local dielectric permittivity.

3.3.4 Effect of silica support on dielectric environment and protonation of pH sensitive labels at protein-membrane interface.

IKMTSL spin labeled WALP (1cys) and WALP (2cys) peptides were incorporated into DOPC vesicles and then SUVs were coated on silica beads. The titration results were compared with the corresponding LUVs titrations. Representative EPR spectra from IKMTSL labeled WALP peptides incorporated in silica supported lipid bilayers are shown in Figure 3.14.
The appearance of broad component in the low-field part of the spectra upon titration is attributed to a protonated fraction of the nitroxide. Note that spectra from WALP peptides incorporated in silica supported lipid bilayer exhibit additional splitting in the central component of the spectra. Such spectra are indicative of slower and more anisotropic motion, indicating that dynamics of WALP peptide is affected by the silica support. In chapter 2 we have demonstrated that the rotational diffusion of lipids in silica supported membranes is slower than in LUVs and silica supported lipid bilayers are characterized by higher order parameter. These silica-induced changes in lipid organization affect motion of the WALP peptides.

WALP1-IKMTSL incorporated into DOPC bilayer was exceptionally affected by silica beads support. Instead of relatively sharp protonation transition we observed continuous changes in the EPR spectra as function of pH. However this abnormal behavior was not observed for silica supported WALP1,23-IKMTSL incorporated into DOPC bilayer.
Figure 3.14 Representative intensity normalized CW X-band EPR spectra from WALP1-IKMTSL incorporated into DOPC LUVs (upper left), WALP1-IKMTSL incorporated into silica supported DOPC bilayer (upper right), WALP1,23-IKMTSL incorporated into DOPC LUVs (lower left), WALP1,23-IKMTSL incorporated into silica supported DOPC bilayer (lower right).
Figure 3.15 Titration curves for silica supported WALP1-IKMTSL incorporated into DOPC bilayer (dark red), WALP1-IKMTSL incorporated into DOPC LUVs (red), red solid line is the best fit to single dissociation model Eq.32, WALP1,23-IKMTSL incorporated into DOPC LUVs (blue), blue solid line is the best fit to dependent double dissociation model Eq. 42, silica supported WALP1,23-IKMTSL incorporated into DOPC bilayer (dark blue), dark blue solid line is the best fit to dependent double dissociation model Eq.42.

For WALP1-IKMTSL incorporated into silica supported DOPC bilayer, titration data points cannot be fitted to generate titration curve as shown in Figure 3.15. At first we suspected that this abnormal pK$_a$ shift of WALP1-IKMTSL incorporated into supported DOPC bilayer is attributed to significant displacement of the peptide moving to lipid bilayer surface where it is susceptible to silica beads surface charge. However, WALP3, WALP5, and WALP6 all exhibit the same unusual pK$_a$ shift, even WALP8, for which spin labeling position is closer to lipid bilayer center, shows the same titration behavior.
Figure 3.16 Nitroxide intensity decay for 0.074mM WALP1-IKMTSL incorporated into DOPC LUVs (red open circle), 0.078mM WALP1-IKMTSL incorporated into silica supported DOPC bilayer (dark red filled circle), 0.12mM WALP8-IKMTSL incorporated into silica supported DOPC bilayer (dark pink filled circle) induced by five-fold molar excess of ascorbic acid at pH=3.0 and under continuous N\textsubscript{2} flow at room temperature. The kinetic decay curves are the best fit to a single exponential equation.

To check if the conformation of WALP1-IKMTSL changes from transmembrane orientation to surface associated when membrane is coated on silica beads, we employed accessibility experiments. Ascorbic acid reduction experiment was carried out to compare the rate of nitroxide intensity decay for both liposome and silica supported samples as shown in Figure 3.16. At pH = 3.0, ascorbic acid in its neutral form effectively penetrates into membrane. Silica supported WALP1-IKMTSL incorporated into DOPC exhibits EPR signal decay that could be approximated by exponential decay with effective rate constant $k = 0.0228 \pm 0.0005$ min\textsuperscript{-1} while
the signal intensity decay for the corresponding liposome samples is characterized by $k = 0.0159 \pm 0.0004\text{min}^{-1}$. The difference in the rate is attributed to a difference in the position of the label in silica supported bilayer compared with LUVs. However, WALP8-IKMTSL incorporated into silica supported DOPC was found to exhibit much slower nitroxide intensity decay and less solvent accessibility compared to WALP1-IKMTSL incorporated into DOPC (because of limited experiment time of 3h, the decay curve cannot be fitted to a single exponential equation), indicating WALP8-IKMTSL indeed was inserted into lipid bilayer. We concluded that WALP remains in transmembrane orientation in silica supported bilayers. Further experiments needed to examine the abnormal pK$_a$ behavior of silica supported samples.

Protonation of the double labeled WALP (2cys)-IKMTSL incorporated into DOPC bilayer was also affected by silica beads support, showing shift in both, pK$_{a1}$ and pK$_{a2}$ and larger $\Delta$pK$_a$. For WALP1,23-IKMTSL incorporated into silica-supported DOPC bilayer the first protonation shifted to somewhat higher value, from 6.28 to 6.56 and the shift could be explained by the increase in the bilayer surface potential due to silica, as we have demonstrated in chapter 2. The second pK$_{a2}$, however, shifts to much lower value than observed in LUVs. Similarly, the first pK$_{a1}$ of WALP3,21 slightly increases for silica supported system, and the second pK$_a$ drops below the corresponding value in LUVs. The difference between the first pK$_{a1}$ and second pK$_{a2}$ for the silica supported systems is almost 1 unit of pH larger than in LUVs. Effect of silica on the first pK$_{a1}$ of WALP5,19 diminishes, however the second pK$_{a2}$ of WALP5,19 is shifted to the lower values by 0.7 units compared to the second pK$_{a2}$ in LUVs. More experiments needed to elucidate the mechanism of reduction in second pK$_{a2}$. 
pKₐ values reflect local environment of a side chain and may have significant consequences for structure and function of a membrane protein. Silica beads support may alter the polarity, water and proton profiles within the lipid bilayer and at the peptide-lipid interface, potentially disrupting the optimal conditions for lipid-peptide interaction.

**Figure 3.17** Titration curves for WALP3,21-IKMTSL (left) and WALP5,19-IKMTSL (right). spin labeled WALP incorporated into DOPC LUVs (blue open circle), silica supported spin labeled WALP incorporated into DOPC bilayer (blue filled circle). Solid lines are the best fit to dependent double dissociation model Eq.42.

**Table 3.12** pKₐ values obtained from titration curves for WALP (2cys)-IKMTSL incorporated into DOPC bilayers and silica supported lipid bilayer using dependent double dissociation model Eq.42.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>LUVs</th>
<th>Silica supported lipid bilayer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKₐ2</td>
<td>pKₐ1</td>
</tr>
<tr>
<td>WALP1,23</td>
<td>5.10 ± 0.04</td>
<td>6.28 ± 0.04</td>
</tr>
<tr>
<td>WALP3,21</td>
<td>5.27 ± 0.09</td>
<td>6.06 ± 0.09</td>
</tr>
<tr>
<td>WALP5,19</td>
<td>4.99 ± 0.05</td>
<td>6.00 ± 0.07</td>
</tr>
</tbody>
</table>
3.4 Conclusions

Here we employed a series of α-helical transmembrane peptides labeled symmetrically with two pH sensitive nitroxides and corresponding single labeled peptides to assess the pK\textsubscript{a} of label protonation at membrane-peptide interface and transmembrane dielectric constant profile. IMTSL with shorter linker length reports on a dielectric constant at the peptide-lipid interface that is somewhat higher than expected for corresponding depth in phospholipid bilayer. Silica bead support give rise to lipid bilayer distortion and shifts the effective pK\textsubscript{a1} of first protonation for double labeled WALP to a somewhat higher values, while the second pK\textsubscript{a2} drops to unexpectedly low values. Such strong effect of silica support on protonation state of protein sidechains may have significant consequences for structure and function of transmembrane and membrane-associated proteins, incorporated in silica-supported systems. Nanoconfinement of lipid membrane may alter the polarity, water and proton profile within the lipid bilayer at the protein-lipid interface, potentially disrupting the optimal conditions for lipid-protein interaction.
REFERENCES


