Interaction of Antimicrobial Peptides With Model Cell Membranes.

Lucille Dionne smith Wright

Louisiana State University and Agricultural & Mechanical College

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INTERACTION OF ANTIMICROBIAL PEPTIDES WITH MODEL CELL MEMBRANES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Chemistry

by

Lucille Smith Wright
B.S., Jackson State University, 1991
May, 2000
A DEDICATION

(For my husband Raymond Wright)

Thanks for hanging in there with me. I love you........and you, too Ayanna.
ACKNOWLEDGMENTS

I give many thanks for the indwelling spirit of the Creator that has sustained me through this journey. I am grateful for the people, who along the way, continually encouraged me and sacrificed their time, energies, and resources to see me to the completion of my studies. Through this humbling experience, I have gained a sense of peace and maturity that will carry me into the next phase of life which I have learned to cherish everyday.

It would take volumes of space to credit all the positive influences that I have encountered over the years so I ask forgiveness of those I fail to mention by name. Just know you are genuinely appreciated and thanked.

I thank first my major professor Paul Russo. On any of “more than a few” occasions he could have dismissed me because of my personal challenges but he didn’t. He never judged my academic worth based on my limitations and I am ever indebted to him for that. My fellow group members, past and present, served as a constant source of support and helped me unselfishly whenever I needed them. Tahir, Debbie, Keonuk, Daewon, Stephen, Brian, Mike, Sybil, and Garrett, you guys are true gems. I especially acknowledge Daewon and Keonuk for the wisdom of their culture that they shared with me. I also acknowledge the assistance of Dr. Stephen Bishop. He unselfishly placed my well being higher than his personal goals and never complained about the delays he experienced because of me.

On the homefront I have to thank my husband Raymond and my daughter Ayanna for their patience and unconditional love. I acknowledge also the
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Chris. I thank my in-laws, Dr. and Mrs. Raymond Wright, for all the phone
calls, cards, and babysitting over the years. A special thank you goes to my
brother-in-law Rev. Derrick Wright for the prayers and words of upliftment.

I give more “special thank you’s” to those who aided me spiritually locally
as I was challenged every day to find meaning in the frustrations I experi­
enced. To the members of the Ausar Auset Society, Tua! Mut Aqhati, I owe
you dearly for your insight and help. To my special friends in the Africentric
Focus/Maat Study Group, thank you for helping me to realize “Sankofa”.

Finally I pay tribute to the two who nurtured me from the womb; my
parents. My father’s spirit, which I constantly feel around me, I hope will be
lightened by my success. No words can adequately describe the appreciation
I have for my mother whose life exemplifies faith in action. She always
encouraged me to never give up and to use my faith in God as a source of
inner strength. Thank you for being there for me unconditionally. I love you.

Tua Neter
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<tr>
<td>AOM</td>
<td>Acoustooptic modulator</td>
</tr>
<tr>
<td>AC(t)</td>
<td>Magnitude of ac signal</td>
</tr>
<tr>
<td>CONTIN</td>
<td>LaPlace inversion algorithm</td>
</tr>
<tr>
<td>DAD</td>
<td>Data acquisition design</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMPC</td>
<td>Dimyristoylphosphatidylcholine</td>
</tr>
<tr>
<td>DOPC</td>
<td>Dioleoylphosphatidylcholine</td>
</tr>
<tr>
<td>FPR</td>
<td>Fluorescence photobleaching recovery</td>
</tr>
<tr>
<td>FWHH</td>
<td>Full width at half height</td>
</tr>
<tr>
<td>(KLAKKLA)₃</td>
<td>Lys,Leu,Ala-containing model peptide</td>
</tr>
<tr>
<td>(KLGKKLG)₃</td>
<td>Lys,Leu,Gly-containing model peptide</td>
</tr>
<tr>
<td>KLS</td>
<td>Kinetic light scattering</td>
</tr>
<tr>
<td>L:P</td>
<td>Lipid to peptide</td>
</tr>
<tr>
<td>LSU</td>
<td>Louisiana State University</td>
</tr>
<tr>
<td>LUV's</td>
<td>Large Unilamellar Vesicles</td>
</tr>
<tr>
<td>MAPS</td>
<td>Model amphipathic α-helical peptides</td>
</tr>
<tr>
<td>MLV's</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>OMA</td>
<td>Optical multichannel analyzer</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene</td>
</tr>
<tr>
<td>REV's</td>
<td>Reverse-phase evaporation vesicles</td>
</tr>
<tr>
<td>SLS</td>
<td>Static light scattering</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>B</td>
<td>baseline of the autocorrelation function</td>
</tr>
<tr>
<td>B</td>
<td>background scattered light for the sample</td>
</tr>
<tr>
<td>C</td>
<td>polydispersity parameter</td>
</tr>
<tr>
<td>D</td>
<td>dark signal</td>
</tr>
<tr>
<td>$D_{app}$</td>
<td>apparent diffusion coefficient</td>
</tr>
<tr>
<td>$D_m$</td>
<td>mutual diffusion coefficient</td>
</tr>
<tr>
<td>$D_s$</td>
<td>tracer self diffusion coefficient</td>
</tr>
<tr>
<td>f</td>
<td>coherence parameter</td>
</tr>
<tr>
<td>g</td>
<td>geometry term</td>
</tr>
<tr>
<td>$G^{(2)}(\tau)$</td>
<td>second order intensity autocorrelation function</td>
</tr>
<tr>
<td>$g^{(1)}(\tau)$</td>
<td>normalized electric field autocorrelation function</td>
</tr>
<tr>
<td>I</td>
<td>intensity of scattered light</td>
</tr>
<tr>
<td>K</td>
<td>spatial frequency</td>
</tr>
<tr>
<td>L</td>
<td>Striped pattern spacing</td>
</tr>
<tr>
<td>n</td>
<td>refractive index</td>
</tr>
<tr>
<td>q</td>
<td>scattering vector magnitude</td>
</tr>
<tr>
<td>R</td>
<td>measured signal</td>
</tr>
<tr>
<td>$R_h$</td>
<td>hydrodynamic radius</td>
</tr>
<tr>
<td>$R_g$</td>
<td>radius of gyration</td>
</tr>
<tr>
<td>S</td>
<td>stray light</td>
</tr>
<tr>
<td>$\Gamma$</td>
<td>decay time</td>
</tr>
<tr>
<td>$\lambda_0$</td>
<td>laser wavelength in vacuo</td>
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</table>
\( \theta \)  
scattering angle

\( \phi \)  
offset angle

\( \tau \)  
lag time in dynamic light scattering
ABSTRACT

This research was part of a multidisciplinary effort aimed at the elucidation of the mechanism of membrane lysis by model peptides. The focus of the dissertation was to characterize the average size dimensions of model cell membranes before and after being treated by model peptides. Experiments with neutral dioleoylphosphatidylcholine (DOPC) vesicles and negatively charged dioleoylphosphatidylcholine/dioleoylphosphatidylglycerol (DOPC/DOPG) vesicles were conducted using the model peptides (KLAKKLA)$_3$ and (KLGKKLG)$_3$. A melittin study was conducted alongside for comparison. Changes in membrane integrity were studied as a function of lipid-to-peptide (L:P) ratio using three techniques: kinetic light scattering (KLS), dynamic light scattering (DLS), and fluorescence photobleaching and recovery (FPR).

The document is divided into four chapters. The first chapter gives a general introduction. The second chapter introduces the KLS concept along with latex calibrations and preliminary measurements of surfactant/vesicle interactions. In chapter 3 the interaction between neutral and negatively charged vesicles composed of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol with the model peptides (KLAKKLA)$_3$ and (KLGKKLG)$_3$ is examined as a function of lipid-to-peptide ratio. Chapter 4 outlines a complement FPR study to chapter 3. Also presented in this chapter are results from experiments of the same lipid systems with melittin.
CHAPTER 1
INTRODUCTION
1.1 LYTIC PEPTIDES

1.1.1 Definition and Amphipathic Character

The interaction of lytic peptides with model cell membranes has posed a long-standing challenge to the area of biophysical research. The term "lytic" refers to the peptide's ability to disrupt/restructure cell membranes. In nature lytic peptides play a key role in the defense systems of many animal species such as insects, amphibians, and mammals. Isolation, purification, and characterization from their biological sources have led to the discovery of several distinct classes of lytic peptides. The more studied types include cecropins, defensins, and magainins. In part, the potent antimicrobial properties of lytic peptides have been attributed to their amphipathic α-helical character. The amphipathic α-helix is defined as an α-helical region of a protein in which the amino acid residues are distributed in the secondary structure to form opposing polar and nonpolar faces. The amphipathic α-helix was first recognized in myoglobin in 1965. In 1969, it was postulated that an amphipathic α-helical structure might account for lipid-protein interactions in the apolipoproteins of plasma lipoproteins. The amphipathic α-helix has been proposed as an important structural feature of several peptide hormones and cytotoxic peptides. To conclude their studies Kaiser & Kezdy reported that "membrane affinity is determined by the ability of the peptide to assume an amphiphilic structure" and "the amphiphilicity of the peptide is defined by its secondary structure". Other properties that are reported to help account for the antimicrobial activity of
lytic peptides include net cationic charges and relatively short lengths. The importance of charge is demonstrated by the preferential binding of antimicrobial peptides to bacteria which are negatively charged over mammalian cells which are neutral in charge.⁹ Peptide lengths of 23-29 amino acid residues are typical. Lear proposed in 1988 that a length of 20 residues was necessary for effective lytic activity through ion channel formation.¹⁰ This number was modified in 1991 when peptides of 8-12 amino acid residues were also shown to display lytic activity via ion channels.¹¹

Of all the naturally occurring lytic peptides, melittin is probably the most widely studied.¹²⁻¹⁶ It is the principle toxin in bee venom and possesses both powerful antimicrobial and cytolytic properties.¹⁷ Several mechanisms propose to account for its activity but none has been successfully proven. Among the more widely reported mechanisms are membrane micellization, ion channel formation, and wedge-shaped insertion into the membrane to induce cytolysis.¹⁸⁻²⁰

1.1.2 Model Amphipathic α-Helical Peptides

Antibiotics continue to be used as primary agents against infectious diseases. The subsequent production of antibiotic-resistant "super bugs" has created the need for newer, more potent disease-fighting alternatives. Lytic peptides have become drug prototypes for the design and synthesis of new peptide analog varieties that promise comparable and better limits of activity.²¹ The rational design of model amphipathic α-helical peptides
(MAPS) not only addresses this need but additionally aids fundamental investigations of the structure/function relationships in peptide/membrane interactions. Systematically varying the residues of the parent species to probe the effects of charge distribution, modifying the size/shape of the hydrophobic region of the peptide, and adjusting peptide length usually dominate the approach of design strategies. Pioneering work in this area has been performed by many authors. It is the main thrust of this manuscript to focus on the characterization of the membrane interactions of two model peptide analogs with high antimicrobial activity and low mammalian cell toxicity, (KLAKKLA)$_3$ and (KLGKKG)$_3$. A collaborating group carried out their design and synthesis. Details of their design, synthesis, and characterization can be read elsewhere.

1.2 MODEL CELL MEMBRANES

1.2.1 Defining Characteristics

Liposomes have evolved tremendously as models for the study of biological membrane structure and function. Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules. Phosphatidylcholine molecules are the most common lipids and are used extensively as the principal phospholipid in liposomes because of their low cost, neutral charge, and chemical inertness. They were first observed in 1947 by Bernard in his studies of myelin figures formed by ammonium oleate. It was later in 1962, when electron microscope images of phospholipid dispersions in water were observed by
Bangham and Horne, that their existence was verified and the new structures were first given the name “liposomes”. The value of liposomes as model membrane systems derives from the fact that liposomes can be constructed from natural constituents such that they resemble biological cells. As a result they can be used to study many cellular processes, particularly transport/delivery phenomena. The application of liposomes as delivery systems includes the delivery of enzymes, anticancer drugs, fungicides, antiparasite drugs, anti-bacterials, and antiviral drugs. Liposomes have also been useful in the diagnostic imaging of tumors and as cosmetic vehicles for the delivery of moisturizers and anti-inflammatory agents to the skin.

1.2.2 Preparation and Classification

The ever-increasing market for the study and application of liposomes has produced a strong driving force for the development of well-characterized and reproducible methods for their manufacture. In general, all methods of manufacture follow a common scheme: 1) drying down of lipids from an organic solvent stock; 2) hydration of resulting film in a buffer solution; 3) purification/filtration of vesicles formed; and 4) analysis of the final product. No matter the application, it seems that the most important aspect of the liposome’s involvement with other molecular species is the nature of its surface properties. The lipid surface can be manipulated by varying the phospholipid constitution and by the addition of molecules that modify either chemically or physically membrane integrity.
formation of phospholipid membranes is a spontaneous process in aqueous solution, most manufacturing techniques focus on customization of the final product. The major classifications of this final product proceed according to vesicle size and lamellar characteristics.\textsuperscript{43} Multilamellar vesicles (MLV's) vary in size from 100-10000 nm and consist of at least five lamellae. Small unilamellar vesicles (SUV's) range in size from 20 –50 nm and are defined as liposomes at or close to their lower size limits. Large unilamellar vesicles (LUV's) include liposomes of diameter 1000 nm or more. Figure 1 gives a depiction of these general classes. Other types of liposomes are defined according to the technique of manufacture. Unilamellar vesicles of different sizes and encapsulation volumes can be generated by a variety of techniques either followed exclusively or in combination. Common choices include sonication, reverse-phase evaporation (REV's), ether injection, detergent analysis, hand-shaking/vortexing, and extrusion.\textsuperscript{31,44} The popular methods are especially useful for the manufacture of unilamellar vesicles of uniform size. This is important as monodisperse populations are more readily adaptable to theoretical principles. Liposomes used in the following chapters were prepared using a combination of the handshaken/mechanical dispersion method and extrusion. A complete overview of the process is provided elsewhere.\textsuperscript{31}

1.2.3 Characterization by Light Scattering

Dynamic and static light scattering have emerged as standard techniques for characterizing liposome suspensions. The mean geometric
Figure 1.1 Classification of vesicles (liposomes).
size, shape, size distribution, and molecular weight can be obtained from light scattering data. The techniques are nonperturbing, fast, and reliable. Kolchens et al characterized the size distribution of extruded vesicles using DLS and correlated the observed data with filter pore size, extrusion pressure, and lipid concentration. For vesicles prepared by the freeze-thaw extrusion method it was found that the larger the pore size of the filter, the more dependent the vesicle size. Filter sizes used for the experiment were 100nm, 200nm and 400nm. Lipid concentration in the range 0.1-10 mg/ml had no effect on the resulting size. Zanten et al demonstrated that the vesicle wall thickness could be estimated using static light scattering measurements for scatterers 40-115nm in diameter. Information about vesicle size distributions was recently extracted by Strawbridge et al using an integrated approach to SLS with which light scattering intensity measurements at several angles could be recorded simultaneously.

1.3 LIGHT SCATTERING STUDIES OF PEPTIDE/MEMBRANE INTERACTIONS

Cell membrane perturbation has been studied extensively by light scattering techniques. In order to gain insight into the molecular mechanisms of lysis, peptide-induced changes in the morphology of cell membranes have been investigated by several authors. The most widely studied model for peptide/membrane interactions is melittin with mixed and/or neutral phospholipid membranes. It appears the action of melittin restructures, by fusion or fragmentation, model membranes to form new entities whose size and shape depend, in part, on the make-up of the
lipid surface, physical state of the lipids, and the lipid-to-peptide ratio. Light scattering studies of melittin interacting with lipid membranes revealed that the average size of small unilamellar phosphatidylcholine vesicles increased upon addition of melittin up to a critical ratio of peptide to lipid (~ 1:10) after which a sharp decrease in size is observed. It was hypothesized that initially melittin induced fusion of the vesicles and at some critical lipid to peptide value micellization occurred. Neutral phosphatidylcholine membranes were reported to be more susceptible to micellization by melittin than phosphatidylserine membranes, which are negatively charged. This result, in part, is due to melittin's positive charge. Electrostatic interactions with negatively charged membranes may keep it on the surface of the membrane. On the other hand, interaction with a neutral membrane allows for insertion of the peptide into the lipid bilayer with greater ease thereby allowing leakage, reorganization, or some other morphological change. It has also been shown that dynamic light scattering can be used to monitor the effect of melittin on lipid bilayers of variable acyl chain length. Faucon et al showed that the rate of disappearance of small lipid-melittin complexes increased as the acyl chain length increased. In other fundamental membrane studies, the mediation of cell fusion by polyethylene glycol (PEG) was followed by dynamic light scattering. It was determined for vesicle sizes less than 77 nm that the diameters increased dramatically when treated with high concentrations of PEG. The adsorption of Casein, a mixture of four proteins from milk, onto polystyrene...
latices has been studied by monitoring the increase in the hydrodynamic radius of the latex particles as protein is added.\textsuperscript{54} Integrated light scattering spectroscopy was used to measure the swelling of POPC/POPG large unilamellar vesicles (LUV 60:40; mol:mol) upon titration with the E1 colicin channel peptide.\textsuperscript{55}

1.4 REFERENCES

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CHAPTER 2
A VERSATILE INSTRUMENT FOR RAPID AND TIME-RESOLVED STATIC LIGHT SCATTERING FROM POLYMER SOLUTIONS

1 This chapter is derived from the paper, "Static light scattering instrument for rapid and time-resolved particle sizing in polymer and colloid solutions", Review of Scientific Instruments 67 (10), October 1996.

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2.1 INTRODUCTION

Static light scattering, SLS, is an analytical technique by which the angular distribution of scattered intensity is collected from a polymer solution. Because of its reliable and non-perturbing nature, the SLS experiment has been employed in the study of a variety of biopolymer problems.1-3 The key hardware component of conventional SLS spectrometers is a photomultiplier tube attached to a revolving arm which allows manual rotation about the horizontal plane of scattering. The task of manually changing angles takes too much time and prevents the observation of dynamical processes inside the sample. As a result, a number of SLS instruments that feature automated data acquisition devices have been built and some reviewed in the literature.4,5

Our instrument exploits a three lens focusing scheme coupled to an intensified silicon diode array. The main feature is that we have tailored the system to monitor dynamic processes in polymer solutions. The present report offers a complete description of the apparatus and results of calibrations performed with uniform polystyrene latex suspensions. We also summarize data from one of the first applications of our machine: following the interaction of Triton-X 100 with vesicle suspensions composed of dimyristoylphosphatidylcholine.

2.2 DESIGN OF SLS INSTRUMENT

2.2.1 Description

Figure 2.1 shows a schematic of the basic components of the modified SLS apparatus. The light source is a 5 mW He-Ne laser. The sample is housed in a cylindrical copper chamber which is mounted onto a centering device. Light scattered by the sample is directed through an optical system...
of three lenses and a diaphragm. The configuration chosen is that which optimized the number of angles resolved by the detector.

Figure 2.1 Schematic of key features of SLS instruments.

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Figure 2.2 Close-up of scattered beam path.
We are using a model 1454 EG&G intensified detector with a 512-element, silicon photodiode array. The whole assembly is mounted atop a Troyke goniometer which permits rotation of the detection system about the horizontal axis of the sample cell. This enables the selection of angular windows forty consecutive degrees in range from ~20 degrees to ~120 degrees. The key is that parallel laser rays get focused to a point on the detector. Figure 2.2 shows parallel nonaxial light emanating from within the scattering volume at an angle $\theta$ to the incident beam. After reducing the incident light levels with neutral density filters, the laser may be directly aimed at the array detector. By rotating the laser, it was found that each pixel of the detector corresponds to 0.17702° of an angle (see Figure 2.3). Such an arrangement also permitted determination of angular resolution. As shown in Figure 2.4 the full angular width at half intensity of the laser at 0° scattering angle was found to be 0.7208° (4 pixels). Insertion of a sample (water) broadened the value to 1.2614° (7 pixels).

2.2.2 Principles of Operation

A brief explanation of how intensity measurements are made is provided here. It is assumed that no light is lost due to absorption in the sample. Also ignored are the variations in laser intensity and the use of filters to reduce incident beam intensity. Light detected by the photodiode array consists of the scattered intensity from the sample ($I$) which is of interest to us, stray light ($S$), background scattered light ($B$), and a dark signal ($D$). In addition, the scattered intensity observed contains a geometry term, ($g$), which must be determined for each detector element, or angle. This term accounts for the volume that the detector "sees" at each angle along with variations due to reflections, lens aberrations, and other instrumental flaws. The equation below describes the measured signal ($R$):
eq. 1  \[ R = g(l + B) + S + D \]

where all terms except \( g \) are measured per unit time. This is the master equation and it applies to any scattering angle. By incorporating the subscripts "s" (sample) and "bs" (blank) into the equation we obtain the following expressions:

eq. 2  Sample: \[ R_s = g(l_s + B_s) + S_s + D_s \]

eq. 3  Blank: \[ R_{bs} = g(0 + B_{bs}) + S_{bs} + D_{bs} \]

Subtraction of the measured blank signal from the measured sample signal yields:

eq. 4  \[ R_s - R_{bs} = g(l_s + (S_s - S_{bs}) + (D_s - D_{bs}) \]

The geometry term is found by measuring the signal from a Rayleigh standard. The same equations apply and by inserting the subscripts "r" (Rayleigh standard) and "br" (blank) we obtain:

eq. 5  \[ R_r - R_{br} = g(l_r + (S_r - S_{br}) + (D_r - D_{br}) \]

Great care is taken to ensure that the stray light and dark signal are negligible. Then, since \( l_r \) is a constant of angle for a valid Rayleigh standard, the geometry term can be computed. Absolute intensities are not required for this type of experiment so a normalized "g" is computed by
dividing the function $gl$ through by its maximum value. To reference the intensities to $l$, we divide equation 4 by equation 5:

![Graph](image)

**Figure 2.3** Plot of instrument response to pixel vs angle adjustment. Measurements were taken at $0^\circ$ offset angle.
Figure 2.4 Intensity peak of laser beam at $0^\circ$ offset angle observed before and after insertion of a sample (water). The effect was a slight broadening of the linewidth.
The validity of this approximate form depends upon the suppression of the stray light and dark count. If \( I_r \) is set to unity the desired measurement of \( I_s \) can be obtained. The angular dependence of \( I_s \) is well approximated by the Guinier-Debye expression:

\[
\text{eq.7} \quad \ln I_s = \ln I_0 - q^2 R_g^2 / 3 + 
\]

where \( I_s \) is the intensity of scattered radiation, \( q \) is the magnitude of the scattering vector, \( n \) is the refractive index, \( \lambda \) is the laser wavelength in vacuo, \( \theta \) is the scattering angle, and \( R_g \) is the radius of gyration. The Guinier-Debye expression is used to obtain measurements of the radius of gyration from plots of \( \ln I_s \) versus \( q^2 \).

2.3 EXPERIMENTAL METHODS

Uniform polystyrene latex microspheres (10% solids) of diameters 497 nm, 87 nm, and 173 nm were purchased from Duke Scientific Corporation. A latex of diameter 297 nm was obtained from IDC Spheres. Sizes were confirmed by dynamic light scattering. Latex/water suspensions were
prepared by adding 3 ml of dust-free water from a Barnstead Nanopure purifier to one drop of the appropriate latex suspension.

The lipid dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma Chemicals. Chloroform and methanol were purchased from Aldrich Chemicals. Triton-X 100 was purchased from Sigma. Vesicle suspensions were prepared by the “Handshaken” method.\textsuperscript{6} In a 100-150 ml round-bottomed flask, 10 mg of the lipid was first dissolved in 2 ml of a chloroform/methanol (2:1) solution. Removal of the solvent by rotary evaporation resulted in the formation of a thin lipid film. The film was allowed to dry in a vacuum oven overnight. A suspension of multilamellar vesicles (MLV’s) was prepared by hydrating the dried lipid film with 2.5 ml of a 10 mM NaCl and 2.5 mM sodium phosphate buffer (pH = 7.4). Glass beads were added to the flask and the container was vigorously shaken. The MLV’s were extruded through a 100 nm polycarbonate filter on a Liposofast extrusion apparatus to obtain monodisperse suspensions of vesicles with a diameter of 65-80 nm. The size was determined by both our instrument and dynamic light scattering. Then 0.2 mM sample aliquots were prepared by diluting the appropriate amount of stock solution with 3ml of buffer. All suspensions were filtered through 0.2 μM Whatman (PVDF) membranes before analysis. Triton was added at a 40:1 surfactant:lipid ratio. A microemulsion composed of 9.1 ml H\textsubscript{2}O, 116 μl hexadecane, 302 μl n-butanol, and 0.532 g Brij-96 was used as the Rayleigh standard.

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2.4 TESTING AND RESULTS

We calibrated the instrument with monodisperse latex suspensions of known diameter. The sizes were first checked by dynamic light scattering to ensure validity. Measurements performed at two different offset angles are summarized in Table 2.1. At an offset angle of 27° only the 497 nm and 297 nm latexes could be measured. At the 90° offset angle latexes of diameters 297 nm, 173 nm, and 87 nm could be measured. The criterion used for these studies was that the modified SLS machine had to measure the average radius of gyration accurately to within an error margin of five percent.

The ability of the instrument to follow changes in the average radius of gyration in real-time was demonstrated with a lipid/surfactant study. The detector was programmed to begin data sampling immediately after addition of Triton-X 100 to a DMPC suspension (see experimental methods for sample details). The experiment was carried out at 25° Celsius and at an offset angle of 90 degrees. Intensity data were collected at ten second intervals. Figure 2.5 shows the plot obtained for \( R_g \) versus time. The average radius of gyration increased from \( \sim 65 \text{ nm} \) to \( \sim 250 \text{ nm} \) for the DMPC vesicles. This result was confirmed by dynamic light scattering.

2.5 CONCLUSIONS

A static light scattering instrument has successfully been adapted to study dynamical behavior. Our study of a DMPC suspension treated with Triton-X 100 showed that \( R_g \) increased 5 fold within a ten minute period.
The dips in the plot are likely due to inadequate mixing. Future work includes developing better stirring/mixing methodology and mechanics.

Table 2.1 Test measurements of polystyrene latex.

<table>
<thead>
<tr>
<th>Offset angle, $\phi$ (deg)</th>
<th>True $R_g$ (^a) (nm)</th>
<th>Measured $R_g$ (^b) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>208</td>
<td>201</td>
</tr>
<tr>
<td>27</td>
<td>115</td>
<td>120</td>
</tr>
<tr>
<td>90</td>
<td>112</td>
<td>115</td>
</tr>
<tr>
<td>90</td>
<td>63.5</td>
<td>65</td>
</tr>
<tr>
<td>90</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>

\(^a\) From dynamic light scattering, applying the factor $(3/5)^{1/2}$ appropriate for uniform spheres, to the hydrodynamic radius.

\(^b\) Error estimated to be ± 5% for all measurements except for the 11 nm $R_g$ whose error was slightly higher.
Figure 2.5 Plot of the radius of gyration, $R_g$, vs time for 0.2 mM DMPC vesicles treated with 8 mM Triton-X 100.
2.6 REFERENCES


CHAPTER 3
THE EFFECT OF TWO MODEL AMPHIPATHIC $\alpha$-HELICAL PEPTIDES ON DIOLEOYLPHOSPHATIDYLCHOLINE VESICLES: A LIGHT SCATTERING STUDY
3.1 INTRODUCTION

The previous chapter presented the design and fundamental principles for a unique instrument capable of following polymer solution behavior in real-time. Test experiments demonstrated its utility in monitoring the changes in the radius of gyration, $R_g$, for a vesicle suspension treated with Triton-X 100. In this chapter the technique, kinetic light scattering (KLS) will be used to capture the dynamical trends in vesicle size that occur during peptide/membrane interactions. A brief overview of the technique is provided below but a more detailed explanation of the unique instrument design is given in chapter 2 and elsewhere.1

Dynamic and static light scattering have already emerged as standard techniques for characterizing the membrane disruption of phospholipid vesicles.2-5 The usual application is to measure the average particle size of the vesicles before and after addition of some membrane-active agent. While this information is very valuable, the rate at which the interaction occurs is also important, and often ignored. Non-specific turbidity experiments are often used for such kinetic measurements.6-7 This chapter will examine the particle size evolution of dioleoylphosphatidylcholine (DOPC) vesicles treated with two model amphipathic $\alpha$-helical peptides. DOPC is a neutral phospholipid whose fluid phase extends to a very low temperature of -18°C.

The study of vesicle/peptide interactions is important for elucidating the mechanisms of lysis. Because of their amphipathic structure, it has been
suggested that lytic peptides interact directly with and permeate the membrane by the formation of concentration-dependent ion channels via a 'barrel-stave' mechanism (see Figure 3.1b). This mechanism involves three steps: 1) binding of monomers to the membrane; 2) insertion into the membrane to form a pore; and 3) progressive recruitment of monomer peptide to increase the pore size. This is in marked contrast to data from recent studies that suggest a 'carpet-like' mechanism. In this mechanism the peptide: 1) binds parallel to the surface of and begins to cover the membrane without much penetration; 2) self-associates once a critical concentration has been reached; and 3) either through aggregation and/or pore-like behavior creates defects in the lipid packing of the bilayer (see Figure 3.1a).

Assessing the physical changes membranes undergo after disruption is one of the major focal points of mechanistic studies. From a light scattering perspective it is considered that the action of a lytic peptide may have one of the following effects on the vesicles (see Figure 3.2): 1) the peptide could have no effect at all and the light scattering results would return the same values before and after addition; 2) The peptide may cause the fusion or aggregation of the membranes (3.2a and 3.2d). In this case the light scattering data would return a size increase with the added possibility of shape changes; 3) The peptide could completely obliterate the vesicle. In this case the individual lipid molecules form small micellar structures (3.2b). The light scattering data would show this as a drastic decrease in size if the
signal is strong enough; and 4) The peptide could cause a slight swelling of
the vesicle due to partial insertion and/or adsorption onto the membrane
(3.2c). It should also be mentioned that the peptides may not interact with
all vesicles in the sample. This would lead to a polydisperse population of
affected and non-affected vesicles. Polydispersity can be detected with an
angle-dependent dynamic light scattering study. The problem is that shape
changes are also detected in this manner too.

Much research has gone into the design and subsequent synthesis of
model peptides that exhibit membrane activity. The peptides for these
experiments, (KLAKKLA)_3 and (KLGKKLG)_3 have been provided by
collaborators. Details of their design and synthesis appear elsewhere.

Reported below are results of DLS and KLS experiments that examine the
effect of lipid-to-peptide ratio on the rate of interaction of the peptide/vesicle
suspensions. The data are analyzed for changes in the hydrodynamic radii
of the liposomes, rate of interaction, the diffusive character, and
polydispersity.

3.2 BACKGROUND

3.2.1 Dynamic Light Scattering

There are two types of laser light scattering: time averaged or static light
scattering and time dependent or dynamic light scattering. Quasi-elastic or
dynamic light scattering has become the standard method for the
measurement of diffusion coefficients for macromolecules. A typical
Figure 3.1 Cartoon depictions of possible mechanisms of action of lytic peptides: (a) peptide adsorbs onto surface of bilayer (carpet mechanism); (b) peptide inserts into bilayer (barrel-stave mechanism).
Figure 3.2 Cartoon depictions of possible effects of peptides on vesicles: (a) fusion; (b) micellization; (c) swelling; and (d) aggregation.
apparatus is shown in Figure 3.3. The key hardware components are a laser source, optical system for harnessing scattered light (usually some sort of lens train), detector, and signal analyzer. Under the usual conditions of homodyne mixing and Gaussian statistics for the scattered field, the experiment tracks the intensity autocorrelation function, the decay of which is characterized by the particle diffusion coefficient. The correlation function is given by:

\[ G^{(2)}(\tau) = B(1 + f g^{(1)}(\tau)^2) \]

Where \( G^{(2)}(\tau) \) is the second order intensity autocorrelation function, \( B \) is the baseline, \( f \) is the coherence parameter \( (0 < f < 1) \), and \( g^{(1)}(\tau) \) is the first order electric field autocorrelation function. For translational diffusion in a monodisperse solution, \( g^{(1)}(\tau) \) can be expressed as a single exponential:

\[ g^{(1)}(\tau) = e^{-\Gamma \tau} \]

where \( \Gamma \), the average decay rate, is related to the diffusion coefficient, \( D \), through the following relationship:

\[ \Gamma = q^2 D \]

3.2.2 Static Light Scattering

In a static light scattering experiment the angular distribution of scattered intensity, or the particle form factor \( P(\theta) \), is measured. The initial form of \( P(\theta) \) provides a means for determining the mean square z average radius of gyration. It is well approximated by the Guinier-Debye relationship:
Figure 3.3 Dynamic light scattering apparatus.
\[
\frac{l(q)}{l(0)} = 1 - \frac{q^2 R_g^2}{3} + \ldots
\]

where \(l(q)\) is the intensity of scattered radiation at scattering vector \(q = (4\pi n \sin(\theta/2))/\lambda_o\); \(n = \) solution refractive index, \(\theta = \) scattering angle, \(\lambda_o = \) laser wavelength in vacuo), and \(R_g\) is the radius of gyration. From a plot of \(\ln(l)\) vs \(q^2\) an absolute measurement of \(R_g\) can be obtained.

3.2.3 Kinetic Light Scattering

The "kinetic" light scattering technique was inspired by the need for a more thorough and accurate description of vesicle solutions undergoing disruption by the action of a solubilizing agent. In addition, greater efficiency of data acquisition was sought. The technique is similar to static light scattering in that the scattered intensity of a sample is measured as a function of angle to yield the root of the z-average of the squared radius of gyration. The difference is that instead of measuring the scattered intensity one angle at a time, an optical multichannel analyzer capable of detecting a 40 degree angle range instantaneously is implemented. The element of kinetic analysis is incorporated through the use of data acquisition design (DAD) software.

Figure 3.4 shows the general setup. See chapter 2 for a more complete description of the hardware and sampling protocol. In gist, an optical multichannel analyzer (OMA) of 512 pixels records the scattered intensity profile over a 40 degree angle range (Figure 3.5). This 40 degree range is
defined by an offset angle or the angle that the rotating arm of the laser makes with the 256th pixel (center) of the OMA. The intensity profile obtained is then corrected for background scattering and geometry effects (Figure 3.6). The background scattering is the underlying scattering from the solvent matrix. The geometry factor describes the volume the detector sees at a given scattering angle and also the light gathering efficiency of the lens train and detector at different angles. It accounts for other effects too, such as reflections. After the geometry correction and background subtraction, Ln(I) vs q^2 is plotted where the slope = - R_g^2/3 (Figure 3.7).

Figure 3.4 General schematic for kinetic light scattering apparatus.
Figure 3.5 Typical raw intensity profile for a vesicle suspension, uncorrected for geometry and background.
Figure 3.6 Intensity profile corrected for geometry and background effects.
Figure 3.7 Guinier plot for DMPC vesicles.
3.3 EXPERIMENT

3.3.1 Materials

L-α-dioleoylphosphatidylcholine (DOPC) was purchased from Sigma Chemicals in a rubber-capped chloroform solution, at concentration 20mg/ml and stored in the freezer at < 0°C. Additional chloroform was purchased from Aldrich Chemicals. Sodium chloride (NaCl), and sodium phosphate hepta hydrate (Na₂HPO₄·7H₂O) were purchased from EM Sciences. The peptides, (KLAKKL)₃ and (KLGKLG)₃ were obtained from the peptide facility at Louisiana State University under the direction of Dr. Mark McLaughlin.

3.3.2 Liposome Preparation

Vesicle suspensions were prepared by the “handshaken” method.¹⁵ Into a 100-150 round-bottomed flask, 10 mg of the lipid was introduced using disposable BD latex-free syringes and 23G1 needles. Additional chloroform (1-2ml) was added via pipette to ensure a large enough volume for optimal film formation. The flask was immediately covered with aluminum foil and the chloroform was removed under a Buchler Instruments rotary evaporator for 15-20 minutes. The resulting film was allowed to dry further overnight in a Napco Model 5831 vacuum oven. The completely dry lipid film was hydrated with 2.5 ml of a 2.5mM sodium phosphate, 10mM NaCl (pH = 7.4-7.5) to form a suspension of multilamellar vesicles (MLV’s). The MLV’s were extruded 10 complete cycles through a 100 nm pore-size Avestin polycarbonate filter on a Liposofast-Basic manual extrusion apparatus to

41
obtain vesicles of uniform diameter 60 – 80 nm. Monodispersity was validated by a multi-angle dynamic light scattering experiment. The data are plotted in the form of $\Gamma/q^2$ vs $q^2$ to produce a curve, the flatness of which is an indicator of polydispersity. All sample and stock solutions were maintained by refrigeration at 5°C.

3.3.3 Peptide Solutions

The peptide resins, (KLAKKLA)$_3$ and (KLGKKLG)$_3$, were obtained as powders. Resins were typically 45-60% peptide by mass. Each was dissolved in 1 ml of a 2.5mM sodium phosphate, 10mM NaCl buffer (pH = 7.4-7.5). The resulting solutions were passed through a 0.02 μm Anatop "low-protein binding" filter and kept under refrigeration at 5° C.

In a control experiment, it was discovered that the manner in which the peptide solutions are cleared of unwanted dust and debris affects the resulting activity with vesicles. The peptide samples used in this control study were approximately 4-5 yrs old and had been stored as solids in either plastic (KLAKKLA)$_3$ or glass (KLGKKLG)$_3$ vials. The solid (KLGKKLG)$_3$ sample was “clumpy” and its solution was visibly cloudy. Given these observations the experiments were performed with the intent of only ascertaining whether filtering vs centrifuging makes a difference. No inferences will be made as to why the specific effects in this study were observed because the samples were obviously not ideal for serious scrutiny. Both peptide solutions were made with a 2.5 mM phosphate, 10 mM NaCl buffer, pH = 7.4-7.5. DOPC suspensions were prepared as above and
treated with either centrifuged or filtered preparations of (KLAKKLA)₃ and (KLGKKLG)₃ at a L:P molar ratio of 500:1. Measurements were taken using DLS at a 45° scattering angle. Table 3.1 shows the “before and after” results. (KLAKKLA)₃ seemed to be the more sensitive. Centrifuging it before addition to a DOPC suspension produced a 13% increase in size while filtering it first produced a 20% increase in the average particle size. It seemed here that filtration actually enhanced the peptide’s activity. The results with (KLGKKLG)₃ contrasted greatly. A centrifuged (KLGKKLG)₃ essentially cleared the DOPC suspension while the filtered one had no effect at all. Because the starting peptide solution in this case was cloudy it is probable that it had aggregated and the process of filtering actually removed it from the solution. The lesson from this experiment is that these aged peptides behaved “differently” according to the method of choice for cleaning the solutions. It is also demonstrated that the peptides can pass filters if not aggregated. The extent to which previous experiments are affected can not be gleaned, as the samples used in this study may not have been in optimal condition. In those original experiments, however, the solutions were clear. In future studies, the better method may be centrifugation because at least with this one no question of sample loss or activity due to passing through a membrane can be raised. It should be pointed out that the protocol for all studies performed in this research used the filtration method with the exception of all melittin experiments. In those studies centrifugation was used.
An alternative technique for conducting this control experiment would have been UV absorption. In conversation with others in the research cluster, it was learned that this particular experiment would not be ideal. Because of very small extinction coefficients and unexplained sensitivities of the peptides to the solution environment, early experiments performed showed that the results obtained are essentially inconclusive.

Table 3.1 Results of DLS Control Study of Peptide Filtration Protocol

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rₙ of DOPC vesicles</th>
<th>Altered in buffer</th>
<th>Centrifuged peptide (500:1)</th>
<th>Filtered peptide (500:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(KLAKKLA)₃</td>
<td>73.1 ± 1.8</td>
<td>83.1 ± 2.5</td>
<td>90.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>(KLGKKG)₃</td>
<td>68.1 ± 1.3</td>
<td>-ᵇ</td>
<td>69.8 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ average of 5 runs using method of cumulants
ᵇ decay signal too fast to measure

3.3.4 Preparation of Light Scattering Suspensions

Samples were prepared in dust-free environmental sample vials, 8 dram. The vials were prepared using a cleansing protocol consisting of first washing the vial with a small amount of Alconox cleanser and then rinsing the vial repeatedly with ultra-pure water, final filter size 0.2 µm, until no dust could be detected using the viewing lens on the light scattering apparatus. The ultra-pure water source was a Barnstead Nanopure system fed by a Millipore Milli-R/Q water purifier.
Appropriate dilutions were prepared by adding filtered buffer solution to aliquots of the lipid stock solution. The lipid-to-peptide ratios examined were 1000:1, 500:1, 100:1, 10:1, and 1:1. All vesicle suspensions were filtered through a 0.2 μm Whatman polyvinylidene (PVDF) filter. An appropriate amount of peptide, corresponding to the desired lipid-to-peptide ratio, was then added to the vesicle suspension.

3.3.5 Methods

Kinetic light scattering measurements were performed using a 5 mW He-Ne laser operating at $\lambda_o = 632.8$ nm and at an offset angle of 90°. Data sampling was controlled by a computer-driven optical multichannel analyzer and intensity profiles were collected in 3 s intervals for 180 s each run. Temperature control of the samples was achieved using a locally-made water-jacketed cell holder connected to a Neslab circulating bath.

Dynamic light scattering measurements were made on a multiangle machine at scattering angles of 20°, 45°, 60°, 90°, and 120°. The light source was a Melles Griot He-Ne laser operating at 30 mW with maximum output at 632.8nm. Scattered light was collected on a photomultiplier tube and monitored by a Pacific Instruments laboratory photometer. Sample runs were measured using an ALV-5000 multiple tau digital correlator. Analyses were performed using the method of cumulants. The temperature was maintained at 25 ± 0.1 °C for the duration of each experiment using a Neslab water bath.
3.4 RESULTS

3.4.1 Preliminary Findings

In preliminary light scattering studies, it was sought to determine the gross response, if any, of DOPC vesicles to (KLAKKLA)_3 and (KLGKKLG)_3. Figures 3.8 and 3.9 show the superposition of 10 KLS intensity traces, collected at 30 s intervals, for 0.2mM DOPC vesicles treated with 0.04mM (KLGKKLG)_3 and (KLAKKLA)_3 respectively. While a significant increase in intensity is observed for vesicles treated with (KLAKKLA)_3, no increase at all

![Graph showing intensity profile](image)

Figure 3.8 Superposition of intensity profiles from a 0.04mM (KLGKKLG)_3 and 0.2mM DOPC vesicle suspension.
Figure 3.9 Superposition of intensity profiles taken from a 0.04mM (KLAKKLA)$_3$ and 0.2mM DOPC vesicle suspension.
Figure 3.10 Plot of $R_g$ vs time for 0.2mM DOPC vesicles treated with 0.04mM (KLAKKLA)$_3$. 
was seen for those treated with (KLGKKLG)$_3$. The average particle size of the vesicles treated with (KLAKKL)$_3$ increased from 65nm to ~80nm. Those treated with (KLGKKLG)$_3$ exhibited no change at all in the average particle size. Because of the minimal response of (KLGKKLG) toward the vesicle system using KLS, all further experiments in this chapter were performed using (KLAKKL)$_3$ only.

3.4.2 Interaction of (KLAKKL)$_3$ with DOPC using KLS

The plot obtained of $R_g$ vs time, Figure 3.10, for the DOPC/(KLAKKL)$_3$ experiment revealed that the bulk of the interaction occurred in the first few minutes as the average particle size stabilized within that period. Subsequent measurements revealed that this average particle size was still unchanged one week later. Hence the peptide concentration dependence studies tracked only the first 180s of vesicle changes after addition of (KLAKKL)$_3$ in order to extract the most meaningful information.

Experiments were performed with a lipid concentration of 0.3mM with the exception of the 1:1 study where a concentration of 0.03mM lipid was used. This was done in order to avoid using a concentration of (KLAKKL)$_3$ that would encourage aggregation of the peptide and also to avoid lipid concentrations that would produce number fluctuations or other artifacts in the light scattering.

The interaction was monitored immediately after addition of peptide to the vesicle suspension. Sixty traces for each lipid-to-peptide ratio were collected. All were individually analyzed for the value of $R_g$ and plotted as a
function of time. Figures 3.11-3.15 show the evolution of particle size for all
ratios. Each ratio was run twice. Visual inspection of the plots revealed a
possible mixing problem for the 100:1 and 1:1 ratios analyzed. A similar
occurrence of "dips" happened in the case of the DMPC/Triton experiment
in chapter 2. An improved systematic way of delivering the peptide to the
solution could probably solve this. Even so, the trend was still evident. For
all lipid-to-peptide ratios, a marked increase in $R_g$ was observed. By
inspection it appeared that this fairly quick and steady increase exhibited
scaled linearly with time. At smaller concentrations of peptide, 1000:1 L:P
and 500:1 L:P, the increase in $R_g$ was minimal, ~ 1.5-2nm, which is
analogous to results reported by Strawbridge et al who attributed the slight
change in size to vesicle swelling due to peptide binding. In the
experiment a light scattering instrument of different design but similar
capability was used to monitor the effect of the channel-forming colicin E1
peptide on 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC)
and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG)
vesicles. Studies of magainins and alamethicin have revealed a
concentration dependent orientation and hence activity of the peptides. At
low concentrations it is hypothesized that the peptide is surface bound. At
high concentrations a more "inserted" type of orientation leads to
subsequent disruption. This could also account for a slight increase in $R_g$ in
these experiments if the lipid packing is affected enough to detect. At
higher concentrations of peptide a more significant increase in $R_g$ was
observed, the more dramatic one occurring at the 100:1 L:P ratio. Two possible explanations for this increase exist. The increase, from ~70nm to >100nm, could possibly be explained by vesicle fusion. The other interpretation of the increase could be aggregation. Finally, as can been seen in Figure 3.16, a comparison of rates (obtained using a linear regression routine in Microcal Origin) from the L:P experiments at various L:P ratios showed a general upward trend with increasing concentration of peptide.

3.4.3 Interaction of (KLAKKLAL)₃ with DOPC using DLS

To obtain more insight into the interaction of the peptide with DOPC vesicles, the effect of (KLAKKLAL)₃ on the hydrodynamic size, $R_h$, the mutual diffusion coefficient, $D_m$, and the size distribution of the vesicle suspensions was examined by dynamic light scattering. The same concentrations and sample volumes as in the KLS study were used. All experiments were performed from the same lipid and peptide stock solutions. For all DLS experiments vesicle suspensions were measured at 5 different angles before and after peptide addition. The "after" measurement was taken at a 5 minute "incubation" limit.

Table 3.2 shows the general changes in $R_h$ for each L:P ratio before and after peptide addition. Values were taken from 60° data. In all cases, a third-order polynomial fit to the correlation functions, using ALV-5000 software, was applied to extract $R_h$ using the cumulants method. Once again, there was a trend toward greater changes in the average particle size.
Figure 3.11 Plot of $R_g$ vs time for 1000:1 L:P ratio.
Figure 3.12 Plot of $R_g$ vs time for 500:1 L:P ratio.
Figure 3.13 Plot of $R_g$ vs time for 100:1 L:P ratio.
Figure 3.14 Plot of $R_g$ vs time for 10:1 L:P ratio.
Figure 3.15 Plot of $R_g$ vs time for 1:1 L:P ratio.
Figure 3.16 Rate comparison for all L:P ratios.
at higher concentrations of peptide. The DLS data did not show as big of an increase in size for the 100:1 L:P ratio which was 38% less than the KLS result. The most significant percent increases occurred at the 1:1 (~17%) and 10:1 (~26.1%) L:P ratios. Similar changes were seen by KLS at the same ratios, ~24% and ~19% respectively. The gross effects observed are consistent with earlier findings: mild increases in the average size at low concentrations of peptide and relatively larger increases in the average size at higher concentrations of peptide.

Additional information is available from the angular dependence of the DLS measurements. For all L:P ratios a master plot of the apparent diffusion coefficient ($D_{\text{app}}$) vs the squared wave vector ($q^2$) was obtained to illustrate how diffusion in these systems was affected. Figure 3.17 shows a decreasing trend in $D_{\text{app}}$ with increasing concentration of peptide. This occurred with a significant positive change in slope for larger concentrations of peptide. This observation usually signals the presence of polydispersity which was also evidenced by the broadening in the particle size distributions discussed ahead. At the lower concentrations this result could be rationalized as distributing of the peptide among the population of vesicles and/or with the occasional formation of aggregated or fused vesicles. At the higher concentrations of peptide it is possible that the peptide induces a more distinct heterogenous population that includes fused/aggregated vesicles coexisting alongside the original structures. Another possible explanation for the positive change in slope would be a change in shape as
DLS is sensitive to the geometry of the particle. Figure 3.18 elaborates further on the polydispersity issue. The parameter "C", whose value is obtained from the following equation,

\[ D_{\text{app}} = D_0(1 + Cq^2) \]

is associated with polydispersity and/or a shape change. It is plotted as a function of L:P ratio. A marker to the far right of the plot shows C for a uniform vesicle suspension. There is a marked increase in C toward higher concentrations of peptide indicated by the data.

For the same 60° data, the particle size distributions were obtained using CONTIN. Figures 3.19-3.23 show the changes in the distribution of \( R_h \) before and after addition of \((KLAKKLA)_3\). The results revealed a definite trend toward broadening of the distribution with increasing concentration of peptide. The 1:1 data, however, were perplexing as, although the average size increased, the change in \( R_h \) was very sharp with little broadening. At other ratios, e.g. 100:1 and 10:1, evidence of broadening is much more striking, especially in the high \( R_h \) region. Intensity increases at the lower scattering angles also indicated size changes, hence polydispersity. For example, at a L:P ratio of 10:1 the intensity increased 75% at 20°, 57% at 45°, and 31% at 60°.

The case presented for polydispersity, although reasonable, may not be sufficient evidence alone. The changes in the average size observed can also be explained by alterations in shape. The magnitude of scattering from
any one component in a DLS sample depends in part upon the particle structure factor, \( P(\theta) \), which describes the angular dependence of scattering for larger particles. It could be speculated that the vesicles, whose structures are not rigidly spherical, may bend or contort in solution and/or may do so in the presence of peptide. This is probably not a significant factor in this case because given the experimental range of sizes and the chosen scattering angle of observation, measurements probably remain in the realm of pure translational diffusion. This point is discussed further below.

3.5 CONCLUSIONS

The physical characteristics of DOPC vesicle suspensions treated with the model peptide \((\text{KLAKKLA})_3\) were investigated at 5 lipid-to-peptide ratios and monitored with respect to average changes in \(R_g\), \(R_h\), and the particle size distribution. In addition, these changes were strictly monitored with time-evolution experiments using a novel twist to the conventional light scattering treatment.

Overall the trend was clear: DOPC vesicles are clearly vulnerable to the activity of \((\text{KLAKKLA})_3\). The average changes seen in both KLS and DLS indicate that the general effect is an increase in the average particle size of the vesicle. This general increase becomes more pronounced at higher concentrations of peptide. The more profound interactions occurred at 100:1 and 10:1 L:P ratios. Smaller increases/perturbations could be explained by swelling due to adsorption onto the surface. Fusion or
aggregation of the vesicles may be probable causes of the larger changes. A viewpoint, borrowed from a concentration-dependent mechanism, describes the formation of surface-bound peptide/vesicle complexes at low concentrations of peptide and the formation of highly altered vesicles at high concentrations of peptide due to induced defects in membrane packing. This is the most likely mechanism. Angle-dependent DLS data further suggest that the resulting suspension is heterogenous, being comprised of aggregated/fused vesicles and the original structures. A less likely factor would be possible shape changes in the vesicles as this could also affect the resulting particle size and diffusion data. In DLS there is also the limitation that the experiment is only valid so long as $qR_h << 1$. To stay within the boundaries of theory, the value of $qR_h$, a measure of the spatial frequency, must satisfy the requirement $qR_g << 1$ where $R_g$ and $R_h$ are related according to the geometry of the particle. In this regime, only the overall translational motion of the particle is seen. At $qR_h >> 1$ the internal motions within the particle contribute to the signal. These would include changes in shape. The $qR_h$ values for these experiments ranged from 0.8-1.4.

The model peptide (KLGKKLG)$_3$ was not a good candidate for these studies using the chosen techniques and experimental conditions. Its minimal behavior with neutral membranes has been explained in terms of both helicity and charge by Javadpour et al.$^{13}$ (KLGKKLG)$_3$ has a relatively low helical content in sodium dodecylsulfate (SDS) micelles and

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dilaurylphosphatidylcholine (DLPC) vesicles (L:P = 30:1). α-Helicity is a known prerequisite for lytic activity. Also antimicrobial peptides display selectivity by inhibiting or killing bacteria without showing toxicity toward mammalian cells. Bacterial cell membranes are negatively charged while mammalian cell membranes are neutral. DOPC is a neutral lipid and consequently does not encourage the action of the positively charged (KLGKKLG)$_3$. Both peptides show higher α-helicity in negatively charged vesicles than in neutral ones.

Table 3.2 Average $R_h$ values for DOPC vesicles treated with varying amounts of (KLAKKLA)$_3$ before and after addition of peptide.

<table>
<thead>
<tr>
<th>L:P Ratio</th>
<th>$R_h$ before (KLAKKLA)$_3$</th>
<th>$R_h$ after (KLAKKLA)$_3$</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000:1</td>
<td>62.0 ± 0.8 nm</td>
<td>62.0 ± 0.6 nm</td>
<td>-</td>
</tr>
<tr>
<td>500:1</td>
<td>56.0 ± 0.5 nm</td>
<td>62.3 ± 0.5 nm</td>
<td>10.1</td>
</tr>
<tr>
<td>100:1</td>
<td>61.3 ± 0.6 nm</td>
<td>69.4 ± 0.3 nm</td>
<td>11.7</td>
</tr>
<tr>
<td>10:1</td>
<td>54.8 ± 0.7 nm</td>
<td>74.2 ± 0.7 nm</td>
<td>26.1</td>
</tr>
<tr>
<td>1:1</td>
<td>58.7 ± 0.5 nm</td>
<td>71.0 ± 0.5 nm</td>
<td>17.3</td>
</tr>
</tbody>
</table>

*a averages calculated on 5 runs using ALV software*
Figure 3.17 Plot of the apparent diffusion coefficient, $D_{app}$, for lipid and lipid/peptide suspensions. Symbols indicate: O DOPC vesicles; • 1000:1 L:P; △ 500:1 L:P; ▲ 100:1 L:P; □ 10:1 L:P; ■ 1:1 L:P.
Figure 3.18 Change in polydispersity parameter as a function of L:P ratio. Points obtained from linear regression analysis in Microcal Origin.
Figure 3.19 Particle size distribution of 1000:1 DOPC/(KLAKKLA)$_3$ suspension before and after peptide addition.
Figure 3.20 Particle size distribution of 500:1 DOPC/(KLAKKLA)$_3$ suspension before and after peptide addition.
Figure 3.21 Particle size distribution of 100:1 DOPC/(KLAKKLA)₃ suspension.
Figure 3.22 Particle size distribution of 10:1 DOPC/(KLAKKLA)$_3$ suspension.
Figure 3.23 Particle size distribution of 1:1 DOPC/(KLAKKLA)$_3$ suspension.
3.6 REFERENCES


7) Pownall, H.; Massey, J.B.; Kusserow, S.K.; Gotto, A. Biochemistry 1979, 18, 574.


CHAPTER 4
MONITORING PEPTIDE/MEMBRANE INTERACTIONS WITH FLUORESCENCE PHOTobleaching RECOVERY
4.1 INTRODUCTION

Peptide/membrane interactions have been explored by a variety of experimental techniques in order to gauge one or more of the essential aspects of cell disruption.\textsuperscript{1-6} Of particular interest to present studies is the effect that the peptide has on the resulting membrane morphology of the lipid system. Dynamic light scattering (DLS) has been widely used to monitor the physical properties of vesicle systems.\textsuperscript{6} In the previous chapter dynamic light scattering and static light scattering studies assessed how DOPC lipid suspensions were affected by the model peptide (KLAKKLA)\textsubscript{3} at different concentrations. In principle, DLS measures the rate of diffusion of suspended particles and is sensitive to almost any particle whose size lies between 1 and 1500 nm. In solutions of particles of various sizes this sensitivity is dominated by the larger particles present as they scatter more strongly, especially at smaller angles. Also, at large qR, DLS becomes sensitive to internal motions, such as undulating surface distortions of vesicles.

Fluorescence photobleaching recovery (FPR) has already become a popular tool for probing the lateral diffusion of lipids and proteins in artificial and biological membranes.\textsuperscript{7-11} The potential to measure the translational diffusion of liposomes themselves has not yet been fully exploited by the technique. Like DLS, FPR measures the rates of diffusion of suspended particles. The measurement is different as it can be used to measure the diffusivities of fluorescently labeled particles of practically any size typically
found in suspensions. In DLS the scattering intensity depends in part on the particle form factor $P(\theta)$ which varies according to the size and shape of the particle. This dependency is not present in FPR because it operates over a longer distance scale ($2\pi/K$ as compared to $2\pi/q$ in DLS) and is therefore not sensitive to intraparticle changes. One note of caution is that the temperature sensitivity of the vesicles must be taken into account when choosing the photobleaching conditions. This is addressed in the chapter with control experiments.

The objective in this study was to first establish the efficacy of FPR as a viable technique to follow the diffusion of labeled dioleoylphosphatidylcholine (DOPC) liposomes in aqueous solution. DOPC was chosen because of its availability, cost-effectiveness, and low phase transition temperature. It also serves as a good model for eukaryotic cells which is of importance for biophysical studies. Addition of Triton-X 100 to DOPC lipid suspensions was observed to show how FPR could monitor particle size changes. The interaction of Triton-X 100 with phosphatidylcholine vesicles has already been widely investigated by many techniques. It is generally accepted that Triton-X 100 disrupts the vesicle bilayer leading to the formation of lipid–detergent mixed micelles.

The second objective of this study was to continue the characterization of the interaction of the two model amphipathic $\alpha$-helical peptides, $(KLAKKL\Lambda)_3$ and $(KLGKKLG)_3$ with model cell membranes. For these experiments, neutral and negatively charged vesicles were used as models.
for eukaryotic and prokaryotic cells, respectively. The interaction was studied using fluorescence photobleaching recovery (FPR) and dynamic light scattering (DLS). Vesicle diffusion before and after addition of peptide was measured as a function of lipid-to-peptide (L:P) ratio and composition of the lipid bilayer. A melittin study for the same vesicle systems was also undertaken for comparison.

4.2 BACKGROUND

The dynamic light scattering technique and instrument are explained in Chapter 3.

Fluorescence photobleaching recovery is based on the principle of observing the rate of recovery of fluorescence due to the movement of a fluorescent particle into an area of the sample that contains the same particle but that has been rendered nonfluorescent via an intense photobleaching pulse of laser light. The rate of recovery is related to the diffusion coefficient of the particle. More specifically, a striped pattern is created by illuminating a coarse diffraction grating (Ronchi ruling) held in the rear image plane of a standard epifluorescence microscope with an intense, brief laser flash. With the laser intensity greatly attenuated, an electromechanical modulation detector system similar to that of Lanni and Ware monitors the subsequent disappearance of the pattern due to exchange of molecules that were bleached and those that were not. The ac amplitude from the modulation detector decays exponentially where:
Figure 4.1 Fluorescence photobleaching recovery apparatus.
\[ ac(t) = \exp(-K^2D_0t) \]

and

\[ K = \frac{2\pi}{L} \]

L is the distance between stripes in the sample, and \( D_0 \) is the tracer self diffusion coefficient of the fluorescently labeled particle. A diagram of key components is shown in Figure 4.1.

4.3 EXPERIMENTAL SECTION

4.3.1 Materials

The lipid dioleoylphosphatidylcholine (DOPC), dissolved in chloroform at 20 mg/ml, and Triton-X 100 were purchased from Sigma Chemicals. The lipid dioleoylphosphatidylglycerol (DOPG) was purchased from Avanti Polar Lipids in a chloroform solution with concentration 10mg/ml. The labeled phospho-lipid NBD-C_{12}-HPC (2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-sn-glycero-3-phospho-choline) was purchased from Molecular Probes. Chloroform was purchased from Aldrich Chemicals. Sodium chloride (NaCl) and sodium phosphate hepta hydrate (Na_2HPO_4·7H_2O) were purchased from EM Sciences. The peptides (KLAKKLA)_3 and (KLGKKLG)_3 were designed and synthesized in the peptide facility at Louisiana State University under the direction of Dr. Mark McLaughlin. Melittin was purchased from Sigma (70 % by HPLC) without further purification.
4.3.2 Preparation of Labeled Liposome Samples

Liposomes were prepared by the "handshaken" method. Details are given in chapter 3. The lipids were mixed at a ratio of 4:1 DOPC/DOPG and 5 wt% nbd-HPC to make fluorescent, negatively charged liposomes. For fluorescent, neutral liposomes, 5 wt% was mixed with DOPC. Aliquots of the lipid stock suspensions were passed through a 0.2 μm PVDF filter before usage. Samples with appropriate peptide concentrations and lipid composition were prepared in 1 dram environmental glass vials (Fischer Scientific) at the appropriate ratios and allowed to incubate for 5 minutes.

4.3.2.1 FPR Samples

A representative aliquot from each DLS vesicle suspension described below was extracted using a Vitrodynamics microslide, inner dimensions 0.2 mm x 0.4mm. The ends were flame-sealed. The microslide was held at 25 ± 0.1 °C during measurements.

4.3.2.2 DLS Samples

The samples for dynamic light scattering required more care in their preparation because of the sensitivity of the measurement to dust and other artifacts. Cleanliness of the sample cell was a high priority. Sample cells were prepared in a similar method to that described in chapter 3. Appropriate amounts of lipid and peptide were added at ratios of 500:1 and 1:1 L:P. An incubation period of 5 minutes was allowed after which the measurements were made. Melittin-treated samples were allowed to incubate 20 minutes. As already discussed in chapter 1, the peptide venom...
melittin is known to induce morphological changes in the bilayers of phosphatidylcholine vesicles causing distinct alterations in membrane properties.

4.3.3 Peptide Solutions

Peptide resins were obtained from the LSU peptide facility in glass vials as powders. According to usage, the needed amounts were dissolved in a 2.5 mM phosphate/10 mM NaCl buffer (pH = 7.48) and stored in 1 dram environmental glass vials. All peptide solutions were stored at < 5 °C.

Melittin (70% by HPLC) was purchased from Sigma Chemicals. As needed, specified amounts were dissolved in a 2.5 mM phosphate/10 mM NaCl buffer (pH = 7.48) and stored in 1 dram environmental glass vials. All melittin solutions were stored at < 5 °C.

4.3.4 Methods

4.3.4.1 Light Scattering Data Acquisition

Multi-angle measurements of both lipid systems alone, DOPC and DOPC/DOPG, were performed at scattering angles of 30, 45, 60, 90, and 110 degrees to ensure uniformity. All before/after peptide experiments were performed at θ = 45°. Data were acquired as five 60 second runs which were analyzed using the method of cumulants.

4.3.4.2 FPR Data Acquisition

All measurements were performed at one K value using a 10X objective and a diffraction grating of 100 lines per inch. Data acquisition time varied from 50 seconds to 600 seconds and all measurements were repeated at
least 3 times. The data were fit with one exponential using ANSCAN, an analysis package developed for FPR studies.

4.4 RESULTS

4.4.1 Sensitivity to Laser Exposure

The structure and fluidity of lipidic bilayers exhibit a well-documented temperature dependence.\textsuperscript{13} Below a characteristic temperature, $T_c$, the phase transition temperature, the liposome bilayer exists as a tightly ordered gel phase. As the temperature is raised above $T_c$, the bilayer passes into a more fluid state where freedom of motion of individual chains is higher. Because the liposomes used in these studies exhibit this temperature sensitivity, it was necessary to assess their response to laser power levels and bleaching pulses. It has already been shown that the release of encapsulated dye from dipalmitoylphosphatidyl-choline (DPPC) and dipalmitoylphosphatidyl-glycerol (DPPG) vesicles can be modulated by laser exposure.\textsuperscript{14}

Two experiments were performed, one in which two laser powers were used and another where the effect of varying the bleach time was monitored. Measurements were compared to a DLS result for the same vesicle suspension. The bleach times examined were 0.5 seconds, 3 seconds, and 6 seconds.

The lipid DOPC was used for the study (see chapter 3 for sample preparation). Measurement conditions were the same as described in section 4.3. In the first experiment, three FPR traces were collected and...
averaged at two different power levels. At the lower power level (1.1W) vesicle diffusion remained basically unchanged. Upon increasing the power to 1.8W the presence of two diffusive modes was detected.

In the second experiment, the lipid sample was exposed to three different bleach times (Figure 4.2). The shortest time, 0.5 seconds, proved to match closest to the unbleached (DLS) sample. Based upon these findings reported experiments were performed using bleach times of 1 second or less and at a laser power of ~ 1.1W.

Table 4.1 Effect of laser power exposure on DOPC vesicle

<table>
<thead>
<tr>
<th>Power</th>
<th>Diffusion (cm²/s) (FPR)</th>
<th>Diffusion (cm²/s) (DLS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.1 watts)</td>
<td>3.4x10⁻⁹</td>
<td>3.6x10⁻⁹</td>
</tr>
<tr>
<td>(1.8 watts)</td>
<td>2 modes, 4.39x10⁻⁹ &amp; 5.6x10⁻⁹</td>
<td>3.6x10⁻⁹</td>
</tr>
</tbody>
</table>

4.4.2 Preliminary FPR/DLS Measurement Comparison

These results concern the simultaneous experiments conducted with neutral DOPC vesicles with FPR and DLS to compare measurements. A plot of the apparent diffusion coefficient D_{app} vs q² is shown in Figure 4.3. The D_{app} value obtained from the FPR experiment, shown also, was 15% higher. This amounts to a difference of 20% between an Rₙ(DLS) and an Rₙ(FPR) measurement for the same lipid system. It is not clear why this occurred as other experiments not performed simultaneously produced closer agreement.
Figure 4.2 Histogram of DOPC vesicle diffusion at 3 bleach times.
Figure 4.3 \(D_{\text{app}}\) comparison plot for FPR and DLS techniques. The sample used was a 0.3 mM DOPC vesicle suspension.

\[
D_{\text{app}} \text{ from FPR} = 5.8 \times 10^{-8} \text{ cm}^2 \text{s}^{-1}
\]

\[
D_{\text{app}} \text{ from DLS} = 4.7 \times 10^{-8} \text{ cm}^2 \text{s}^{-1}
\]
4.4.3 DOPC/Triton-X 100 Experiment

It was necessary to test the FPR technique to see if gross changes in lipid vesicles could be seen. In this experiment FPR recovery traces were obtained for DOPC vesicle suspensions before and after addition of 10% Triton-X 100. Before addition of Triton-X 100 the vesicles exhibited a hydrodynamic radius of 65 nm. After treatment with the surfactant and a 5 minute incubation period the recovery time decreased sharply indicating the presence of small diffusers. This result was similar to experiments performed with DLS and is consistent with literature findings.\(^{15}\)

4.4.4 DOPC, DOPC/DOPG (KLAKKLA)\(_3\) and (KLGKKLG) Experiments

Changes in vesicle structure for DOPC and DOPC/DOPG suspensions induced by (KLAKKLA)\(_3\) and (KLGKKLG)\(_3\) were assessed at high and low peptide concentrations. All results are tabulated in Tables 4.2 and 4.3. At low concentrations of (KLAKKLA)\(_3\) (500:1) the DLS result for DOPC vesicles is similar to the one found previously in Chapter 3. A modest increase in the hydrodynamic radius, \(\sim 10\%\) is observed. The same measurement with FPR showed no change in the average particle dimensions once the error is considered. For DOPC/DOPG vesicles at a low concentration of (KLAKKLA)\(_3\), (500:1 L:P), DLS and FPR detected decreases of 3.7\% and 9.2 \% respectively. At high concentrations of (KLAKKLA)\(_3\) (1:1 L:P) the DLS result for DOPC vesicles showed an 11\% increase in \(R_h\) which was significantly lower than previously reported in chapter 3 (\(\sim 20\%\)). The FPR measurement showed a reduction (18.5\%). The peptide's interaction with
Table 4.2 Hydrodynamic radii for DOPC vesicles treated with (KLAKKLA)$_3$ and (KLGKKLG)$_3$ by DLS and FPR.

<table>
<thead>
<tr>
<th>R$_n$ (nm) for DOPC vesicles</th>
<th>By DLS$^a$ (app. % changes in parentheses)</th>
<th>R$_n$ (nm) for DOPC vesicles</th>
<th>By FPR$^b$ (app. % changes in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(KLAKKLA)$_3$</td>
<td>(KLAKKLA)$_3$</td>
<td>(KLAKKLA)$_3$</td>
<td>(KLAKKLA)$_3$</td>
</tr>
<tr>
<td>74 ± 1.2 (10.4 %)</td>
<td>73 ± 0.1 (11.4 %)</td>
<td>60.5 ± 1.9 (no change)</td>
<td>54.2 ± 1.7 (-18.5 %)</td>
</tr>
<tr>
<td>(KLGKKLG)$_3$</td>
<td>(KLGKKLG)$_3$</td>
<td>(KLGKKLG)$_3$</td>
<td>(KLGKKLG)$_3$</td>
</tr>
<tr>
<td>69.4 ± 0.9 (3.2 %)</td>
<td>68.2 ± 1.7 (16.6 %)</td>
<td>62.5 ± 5.8 (no change)</td>
<td>98.1 ± 12.7 (32 %)</td>
</tr>
</tbody>
</table>

$^a$ average of five runs analyzed by cumulants
$^b$ average of three runs analyzed by ANSCAN
Table 4.3 Hydrodynamic radii for DOPC/DOPG vesicles treated with (KLAKKLA)$_3$ and (KLGKKLG)$_3$ by DLS and FPR.

<table>
<thead>
<tr>
<th>$R_h$ (nm) DOPC/DOPG vesicles (app. % changes in parentheses)</th>
<th>$R_h$ (nm) for DOPC/DOPG vesicles (app. % changes in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(L:P 500:1)</td>
<td>(L:P 500:1)</td>
</tr>
<tr>
<td>(KLAKKLA)$_3$</td>
<td>(KLAKKLA)$_3$</td>
</tr>
<tr>
<td>56.8 ± 1.2</td>
<td>47.7 ± 2.2</td>
</tr>
<tr>
<td>(-3.7 %)</td>
<td>(-26.5 %)</td>
</tr>
<tr>
<td>(KLGKKLG)$_3$</td>
<td>(KLGKKLG)$_3$</td>
</tr>
<tr>
<td>58 ± 0.5</td>
<td>41.8 ± 1</td>
</tr>
<tr>
<td>(3.4 %)</td>
<td>(-9.1 %)</td>
</tr>
</tbody>
</table>

a average of 5 runs analyzed by cumulants
b average of 3 runs analyzed by ANSCAN
c particles too big to measure

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DOPC/DOPG vesicles at high concentrations produced a reduction in $R_h$ (~25%) by DLS but the FPR compliment revealed no change.

At low concentrations of $(KLGKKG)_3$ (500:1L:P) a slight increase (3.2%) was detected by DLS while FPR saw basically no change. At high concentrations of the peptide the DOPC vesicles experienced an increase of at least 15% by DLS. The amount is more if calculated by FPR (32%). For both techniques the measurements from both DLS and FPR for DOPC/DOPG vesicles were unattainable because of the formation of huge clusters.

4.4.5 Melittin Results

The same experiments were also performed using melittin. The results of the changes in size of DOPC and DOPC/DOPG vesicles with high/low concentrations of melittin are shown are shown in Table 4.4. Intensities at the high concentration are also listed. At the low concentration of melittin, slight changes were seen by both techniques for both lipid suspensions. At the high concentration of melittin, a drastic decrease in intensity was seen by DLS for both lipid suspensions which was accompanied by a shift toward faster decay rates. This combination of events made a complete analysis too difficult to undertake, although it is probable that a size decrease occurred because of the faster decay after addition of melittin. Likewise corresponding analyses of FPR traces were also challenging because the photobleaching constraints discussed in Section 4.4.1 coupled with the low
Figure 4.4 Typical FPR trace for DOPC vesicles.

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Figure 4.5 FPR trace for DOPC vesicles treated with melittin (1:1 L:P) ratio at the same spatial frequency as Figure 4.4.
dye content in the vesicles produced noisy data. Approximate sizes, however, were calculated. Before and after FPR traces for DOPC vesicles treated with a high (1:1 L:P) concentration of melittin are shown in Figures 4.4 and 4.5.

These results are consistent with previous reports. It has already been established that the action of melittin at high concentrations leads to the formation of micellar structures. At low concentrations slight changes in vesicle size are well documented and are attributed to the perturbations due to the initial adsorption of melittin onto the lipid membrane. These results have been confirmed for both neutral phosphatidylcholine (PC) and phosphatidylserine (PS) vesicles.

**4.5 CONCLUSIONS**

This present study used DLS and FPR to examine the membrane integrity of DOPC and DOPC/DOPG vesicle suspensions before and after addition of the peptides (KLAKKLA)_3 and (KLGKKLG)_3. In neutral DOPC suspensions (KLAKKLA)_3 induced an increase in R_n at both concentrations in experiments by DLS. The peptide showed no activity at all in the FPR complement at the low concentration (500:1 L:P) ratio and a decrease of (18.5%) at the high concentration (1:1 L:P) ratio.

At the low concentration of (KLGKKLG)_3 a small increase was detected by DLS for DOPC vesicles that was not seen by FPR. At a high concentration of (KLGKKLG)_3 the two measurements seemed to both agree that an increase of at least 10% took place in the average particle
Table 4.4 Rh values for DOPC and DOPC/DOPG vesicles treated with melittin: (a) by DLS; (b) by FPR; intensities from DLS for 1:1 experiments notated in brackets.

(a)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Rh (nm) vesicles by DLS$^a$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>w/Melittin 1:1</td>
<td>w/Melittin 500:1</td>
<td></td>
</tr>
<tr>
<td>DOPC</td>
<td>69.2 ± 0.7</td>
<td>71.2 ± 2.5</td>
<td>71.2 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[18 kHz]</td>
<td>[1 kHz]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPC/DOPG</td>
<td>59.3 ± 1</td>
<td>58.7 ± 0.9</td>
<td>58.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[22 kHz]</td>
<td>[2 kHz]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ average of five runs using method of cumulants

(b)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Rh (nm) vesicles by FPR$^a$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>w/Melittin 1:1</td>
<td>w/Melittin 500:1</td>
</tr>
<tr>
<td>DOPC</td>
<td>55.2 ± 1</td>
<td>53.0 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>DOPC/DOPG</td>
<td>37.8 ± 7.1</td>
<td>33.7 ± 2.7</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ average of three runs analyzed by ANSCAN

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dimensions of the DOPC suspension. The higher increase was seen by FPR (32%).

In the negatively charged suspensions of DOPC/DOPG at a low concentration of (KLGKKLG)$_3$ there was a small increase by DLS (3.4%) but a significant decrease by FPR (9.1%). At a higher concentration, (KLGKKLG)$_3$ induced the formation of structures too large to measure by either technique. Under a microscope the complexes formed were bright clusters. The dimensions varied in size. The effect of (KLAKKLA)$_3$ at a higher concentration was perplexing because by DLS the average size of $R_n$ dropped while the FPR measurement remained essentially unchanged.

With regard to the presence of polydispersity suggested by the dynamic light scattering experiment, the complement FPR analyses did not confirm this. All traces exhibited vesicle diffusion that remained essentially monoexponential, hence monodisperse before and after peptide addition. This observation would support the argument that shape changes may be occurring. See Figure 3.2 for a review of all possibilities. Quantitatively the two techniques did not consistently agree; the FPR data showing the greater amount of error. The FPR results would have produced better agreement given quieter data, which would especially help in those experiments where small changes occurred.

In general, the interaction of the peptides with neutral vesicles led to increases in the average particle dimensions while decreases were seen with negatively charged vesicles. This is probably due, in part, to enhanced
electrostatic interactions in the negatively charged vesicle suspensions.

Another factor in all results may lie in the role of the buffer. In conversations with Dr. Frederick Enright and Dr. Kathleen Morden, both members of the research cluster, it was learned that the use of phosphate buffers with the peptides studied sometimes produces ambiguous results. HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) was suggested as a better alternative.

4.6 FUTURE WORK

The interaction of two model peptides and with neutral and negatively charged membranes has been investigated using two hydrodynamic techniques, DLS and FPR. The resulting morphology was quantified and discussed in terms of the average particle dimensions. The morphology can also be observed directly using freeze fracture microscopy. This experiment could provide more information about the shape of peptide/lipid complexes.

4.7 REFERENCES


2) Schwarz, G.; Beschiaschvili, G. Biochimica et Biophysica Acta 1989, 979, 82.


APPENDIX
Request for Permission

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Ann Perlman
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John C. Perman
VITA

Lucille Dionne Smith was born on November 22, 1968, in Pine Bluff, Arkansas. Her parents, James Lee and Velma Robinson Smith, raised her along with three other siblings: Kharla, Craig, and Chris. She graduated in the top five percent of her class from Pine Bluff High School in May, 1987, and continued on to college at Jackson State University in Jackson, Mississippi, in the fall of 1987. She became a member of Alpha Kappa Alpha Sorority, Inc., was very active in student government, served as First Alternate Miss Jackson State University her senior year, and completed her studies with honors. She received her bachelor of science degree in chemistry in May, 1991. In the fall of 1991 she enrolled in the doctoral program at Louisiana State University as a Board of Regents Fellow. She married Raymond Ira Wright on December 26, 1992. They have one child, Terri Ayanna, age 7. Mrs. Wright will receive the degree of Doctor of Philosophy in chemistry at the May, 2000 commencement ceremony.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Lucille Smith Wright

Major Field: Chemistry

Title of Dissertation: Interaction of Antimicrobial Peptides with Model Cell Membranes

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

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Date of Examination: December 3, 1999