ABSTRACT

FLUORESCENT DETERMINATION OF CARDIOLIPIN USING 10-N-NONYL ACRIDINE ORANGE

by Pakritsadang Kaewsuya

Cardiolipin (CL) plays an essential role as a marker for cell apoptosis. Quantitative detection of phospholipids (PLs) by UV absorbance is problematic due to the presence of only isolated double bonds in the structure. Although 10-*N*-nonyl acridine orange (NAO) has been utilized for fluorescent detection of liposomes and mitochondria through its interaction with CL, specific quantitative determination of CL in solution using NAO is uncommon. In this work, we have developed a fluorescence quenching method for CL using NAO. The optimum excitations are 499 and 518 nm at low and high NAO concentration, respectively. The optimum emissions are varied from 518 to 530 nm. The interaction of sodium n-dodecyl sulfate (SDS), sometimes used for lipid extraction from cells, and other PLs such as phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethnolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), sphingomyelin (SM) and cholesterol, with NAO is investigated. The fluorescence intensity of 0.5 μ M NAO signal is strongly quenched by SDS below 25% methanol in water. With a methanol content above 50%, no quenching of NAO by SDS or the PLs with the exception of PG above 8 μ M is observed.

Using 50-50 methanol-water, the fluorescence as a function of reaction time for the NAO-CL interaction is quite stable from 3 to at least 15 min. Concentrations of 5, 10, 20, and 50 μ M NAO are considered and 20 μ M NAO provides a linear fluorescence response from 0.2 – 10 μ M CL. The detection limit is 0.2 μ M and the limit of quantification is 0.6 μ M. Acridine orange (AO) and phenosafranin (PSF) dyes are less effective as fluorescent probes for CL. CL in whole cell and membrane samples is quantitatively determined by standard addition to be in the 0.2-1.5 μ M range. The increase of CL as compared to the controls is not significantly different in all samples subjected to cell death using staurosporine.

FLUORESCENT DETERMINATION OF CARDIOLIPIN USING 10-N-NONYL ACRIDINE ORANGE

A Thesis

Submitted to the Faculty of Miami University

in partial fulfillment of

the requirements for the degree of

Master of Science

Department of Chemistry and Biochemistry

by

Pakritsadang Kaewsuya

Miami University

Oxford, Ohio

2007

Advisor______ Dr. Neil D. Danielson Reader______ Dr. Thomas L. Riechel Reader______ Dr. Shouzhong Zou Reader______

Dr. Richard L. Bretz

TABLE OF CONTENTS

		Page
List of Figures	5	iii
List of Tables		vi
List of Abbrev	riations and Symbols	vii
Dedications		ix
Acknowledger	nents	Х
Chapter 1	Introduction and Background	1
Section 1.1	Fluorescence Spectroscopy	2
Section 1.2	Fluorescent Probes	6
Section 1.3	Cell Membrane and Phospholipids	10
Section 1.4	Cardiolipin	12
Section 1.5	Purpose	19
References		20
Chapter 2	Fluorescent Determination of Cardiolipin using 10-N-Nonyl	
	Acridine Orange	24
Section 2.1	Introduction	25
Section 2.2	Experimental	27
Section 2.3	Results and Discussion	33
Section 2.4	Conclusions	64
References		65
Appendix		69

LIST OF FIGURES

		Page
Figure 1.1	A simple Jablonski diagram.	3
Figure 1.2	Components of a spectrofluorometer.	5
Figure 1.3	Examples of (a) intrinsic probes and (b)-(c) extrinsic covalently	
	bound probes.	7
Figure 1.4	Examples of hydrophobic, hydrophilic or amphiphilic probes.	9
Figure 1.5	Biomembrane structure.	10
Figure 1.6	Structure of a typical phospholipid (phosphatidylcholine).	11
Figure 1.7	(a) Phospholipid bilayer and (b) micelle.	12
Figure 1.8	Structure of typical CL with fatty acyl groups $(R_1, R_2, R_3, and R_4)$.	13
Figure 1.9	Structure of 1, 1', 2, 2'-tetralinoleoyl cardiolipin (CL, 18:2).	15
Figure 1.10	Structure of 1, 1', 2, 2'-tetraoleoyl cardiolipin (CL, 18:1).	16
Figure 1.11	Structure of a typical mitochondrion.	18
Figure 2.1	Structure of 10-N-nonyl acridine orange (NAO).	27
Figure 2.2	Structures of surfactants.	30
Figure 2.3	Structures of (a) acridine orange (AO) and (b) phenosafranin (PSF).	32
Figure 2.4	Visible absorbance spectra of 0.5-100 μ M NAO in 50% methanol.	35
Figure 2.5	(a) Fluorescence excitation and emission spectra of 0.5 μ M NAO in	
	50% methanol (slit widths = 2.5 nm).	36
	(b) Fluorescence excitation and emission spectra of 1.0 μ M NAO in	
	50% methanol (slit widths = 2.5 nm).	37
	(c) Fluorescence excitation and emission spectra of 10 μ M NAO in	
	50% methanol (slit widths = 2.5 nm).	38
	(d) Fluorescence excitation and emission spectra of 20 μM NAO in	
	50% methanol (slit widths = 2.5 nm).	39
	(e) Fluorescence excitation and emission spectra of 50 μ M NAO in	
	50% methanol (slit widths = 2.5 nm).	40
	(f) Fluorescence excitation and emission spectra of 100 μM NAO	
	in 50% methanol (slit widths = 2.5 nm)	41

- Figure 2.6 The fluorescence intensity of emission at different concentrations of NAO in 50% methanol ($\lambda_{\text{excit.}}$ / $\lambda_{\text{emiss.}}$ = 450/530 nm, 499/430 nm, and 518/530 nm, slit widths = 2.5 nm).
- Figure 2.7 Effect of methanol contents (25%, 50%, 60%, and 75%) in water on the fluorescence interaction between NAO (50 µM) and various concentrations CL. ($\lambda_{\text{excit.}}$ / $\lambda_{\text{emiss.}}$ = 518/530 nm, slit widths = 2.5 nm).
- Figure 2.8 Effect of methanol content (0%, 5%, 10%, and 25%) in water on the fluorescence interaction between 0.50 µM NAO and SDS at different concentrations. ($\lambda_{excit.}$ / $\lambda_{emiss.}$ = 499/518 nm, slit widths = 5 nm).
- Figure 2.9 Effect of methanol contents (50%, 75%, and 100%) in water on interaction between 0.50 µM NAO and SDS at different concentrations. ($\lambda_{\text{excit.}}$ / $\lambda_{\text{emiss.}}$ = 499/518 nm, slit widths = 5 nm). 45
- Fluorescence decay curve of NAO in 50% MeOH at low Figure 2.10 concentrations (0.05 μ M, 0.1 μ M, 0.2 μ M, 0.3 μ M, and 0.4 μ M, $\lambda_{\text{excit.}} / \lambda_{\text{emiss.}} = 496/518 \text{ nm}, \text{ slit widths} = 5 \text{ nm}$). 46
- Figure 2.11 Fluorescence decay curve of NAO in 50% MeOH at high concentrations (1 μ M, $\lambda_{excit.}$ / $\lambda_{emiss.}$ = 499/518 nm, slit widths = 5 nm), (20 μ M, and 10 μ M $\lambda_{excit.}$ / $\lambda_{emiss.}$ = 518/530 nm, slit widths = 2.5 nm).
- Fluorescence-reaction time profiles for various ratios of CL to NAO Figure 2.12 concentrations in 50% MeOH (2/1, $\lambda_{\text{excit.}}$ / $\lambda_{\text{emiss.}}$ = 496/518 nm, slit widths = 5 nm), (2/10, 2/20, 10/20, and 20/20, $\lambda_{\text{excit.}}$ / $\lambda_{\text{emiss.}}$ = 518/530 nm, slit widths = 2.5 nm).
- Figure 2.13 Fluorescence profiles using a low concentration of NAO (0.1 μ M) as a function of concentration for various PLs ((PA, 12:0), (PA, 18:0), (PC, 18:1), (PG, 18:0), and (PS, 180.5), $\lambda_{\text{excit.}} / \lambda_{\text{emiss.}} = 499/518 \text{ nm}$, slit widths = 5 nm). 50

Page

42

43

44

47

48

- Figure 2.14 Fluorescence profiles using a high concentration of NAO (20 μ M) as a function of concentration for various PLs ((PA, 18:0), (PC, 18:1), (PG, 18:0), and (PS 18:05), (λ_{excit} / $\lambda_{emiss.} = 518/530$ nm, slit widths = 2.5 nm). 51
- Figure 2.15 Linearity of fluorescence as a function of cardiolipin concentration using NAO in 50 % methanol at 5 μ M (-x-), 10 μ M, ($\lambda_{excit.} / \lambda_{emiss.} =$ 499/530 nm, slit widths = 2.5 nm), 20 μ M, and 50 μ M, ($\lambda_{excit.} / \lambda_{emiss.}$ = 518/530 nm, slit widths = 2.5 nm). 52
- Figure 2.16 Fluorescence calibration curve of CL with 20 μ M NAO in 50% methanol in water, ($\lambda_{excit.} / \lambda_{emiss.} = 518/530$ nm, slit widths = 2.5 nm). 53
- Figure 2.17 Visible absorbance curves of various CL concentrations at different concentrations of NAO (10 μ M and 20 μ M) ($\lambda_{abs.}$ = 497 nm)
- Figure 2.18Comparison of fluorescence profiles using NAO 20 μ M ($\lambda_{excit}/\lambda_{emiss}$ = 518/530 nm, slit width = 2.5/2.5 nm), AO 20 μ M ($\lambda_{excit}/\lambda_{emiss}$ =499/524 nm, slit width = 2.5/2.5 nm), and PSF 20 μ M ($\lambda_{excit}/\lambda_{emiss}$ =522/570 nm, slit width = 5/5 nm) as the various concentrations ofCL.Figure 2.19Predicted interaction of AO and CL.57
- Figure 2.20Predicted interaction of NAO and CL.58
- Figure 2.21Predicted interaction of AO and CL.59

55

LIST OF TABLES

Page

Table 1.1	Main molecular species of mammalian cardiolipin.	14
Table 1.2	Phospholipid compositions of organelle membranes from rat liver.	17
Table 2.1	The fluorescence intensity (FI) values of samples spiked with 1 μM	
	CL and recovery ratios.	60
Table 2.2	CL concentration in various cell membrane samples.	63

LIST OF ABBREVIATIONS AND SYMBOLS

AO	Acridine Orange
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CL	Cardiolipin
Cer	ceramides
DPH	1,6-Diphenyl-1,3-5-hexatriene
FI	Fluorescence intensity
FRET	Fluorescence Resonance Energy Transfer
FW	Formula weight
Gb	glycosphingolipids
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
k	Rate constant
<i>k</i> _r	Rate constant for fluorescence
<i>k</i> _{nr}	Rate constant for the competing non-radiative route
μΜ	Micromoles per liter
MW	Molecular Weight
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NAO	10-N-Nonyl Acridine Orange
ppm	Part per million
PSF	Phenosafranin
PLs	Phospholipids
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SM	Sphingomyelin

rpm	Revolution per minute
SDS	Sodium n-Dodecyl Sulfate
TG	Triglycerides
UV	Ultraviolet-Visible

DEDICATION

To my father, Chim, my mother, Pow, my sisters, Sunee, Ratiya, Kammul and Chayaporn and my brothers, Wichan and Korakort.

ACKNOWLEDGEMENT

I would like to express my sincerest gratitude to Dr. Neil D. Danielson, Chairperson and thesis advisor, for his professional guidance on my research and for his kindness, hospitality, and encouragement.

I also appreciate my thesis committee, Dr. Thomas L. Riechel, Dr. Shouzhong Zou, Dr. Richard L. Bretz and Dr. Gary A. Lorigan for their valuable suggestions. I have been extremely fortunate having them as my committee. Also thanks to Daryoush Ekhterae at the Department of Surgery, School of Medicine, University of Pennsylvania, Philadelphia who provided the membrane samples.

Appreciation is extended to my coworkers in the lab, Martin M. Waichigo, Erin Myers and all of my friends for their help and friendship, particularly, Nathan P. Grove and Joshua D. Miller and Christopher H. Landis who are my lab assistants. Specially, I express my thanks to my mother and father for encouraging me to get through everything, and my special sister, Sunee Kaewsuya, who passed away in January, 2000; she is the one who always supported and encouraged me whenever I felt unconfident and sad, and also thanks to my brothers and sisters for their love and encouragement.

CHAPTER 1

INTRODUCTION AND BACKGROUND

SECTION 1.1: FLUORESCENCE SPECTROSCOPY

For microscopy techniques, fluorescence is currently probably the most widely used analytical detection technique other than the eye. It enables the molecular composition of the structures being observed to be identified through the use of fluorescently-labeled probes of high chemical specificity. The reason for the high sensitivity of fluorescence techniques is that the emission signal is measured above a low background level due to elimination of light source interference. This is inherently more sensitive than comparing two relatively large signals as in absorption spectroscopy. The sensitivity of fluorescence techniques is as much as 1000 times more sensitive than absorption spectroscopy (1-2).

Fluorescence is the result of a three stage process that occurs in molecules of certain structure (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes (3-4). A fluorescent probe is often a fluorescent dye designed to localize within a specific area of a biological specimen. The process responsible for fluorescence is illustrated by the simple electronic state diagram (Jablonski diagram) shown in Fig. 1.1.

Stage 1: Excitation

In the ground state, the molecule will be in the lowest vibrational energy levels of S_0 . At room temperature the higher vibrational energy level is usually not populated. A photon of energy (hv_A) supplied by an external UV or visible light source is absorbed by the fluorophore, creating an excited electronic singlet state (S_1 , S_2). The magnitude of the absorbed energy determines which vibrational level, S_1 or S_2 , becomes populated. This process is very fast and happens within 10^{-15} s.

Stage 2: Excited-State Lifetime

The excited state exists for a finite time which occurs typically for 1–10 nanoseconds. During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1 is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission initiates. The second is not all of the molecules excited by light absorption return to the ground state (S_0) by fluorescence emission. A non-radiative route is quite likely. Other processes such as collisional quenching and fluorescence resonance energy transfer (FRET) can be affected.

FRET is non-radiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore. FRET results in the excitation of the nearby acceptor fluorophore that emits a photon when it returns to the ground state. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted to the number of photons absorbed, is a measure of the relative extent to which these processes occur. The response of a fluorophore will depend on the molar absorptivity and the quantum yield.

Stage 3: Fluorescence Emission

A photon of energy (hv_F) is emitted, returning the fluorophore to its ground state S₀ and the fluorescence spectrum is generally independent of the excitation wavelength. The energy of this photon is lower because of energy dissipation during the excited-state lifetime. Therefore, a longer wavelength is observed in relation to the excitation photon (hv_A). The difference in energy or wavelength represented by ($hv_A - hv_F$) is called the Stokes shift (see Fig. 1.1). The Stokes shift is observed because of the energy lost from a higher excited state to the lowest excited state due to vibrational relaxation or internal conversion. The Stokes shift is a fundamental of the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background which is isolated from excitation photons.



Figure 1.1 A simple Jablonski diagram. Electronic singlet states, S₀, S₁, and S₂, and three vibrational energy levels are shown (hv_A and hv_F represent absorption and fluorescence, k_r is the rate constant for fluorescence, and k_{nr} is the rate for the competing non-radiative route).

Fluorescence Instrumentation

The sensitivity of fluorescence is dependent on both the fluorophore and the instrument. Fluorescence instruments are primarily of four types, each of them providing particularly different information (2-3).

1) Spectrofluorometers and microplate readers measure the average properties of bulk samples.

2) Fluorescence microscopes resolve fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects.

3) Fluorescence scanners, including microarray readers, determine fluorescence as a function of spatial coordinates in two dimensions for macroscopic objects such as electrophoresis gels, blots, and chromatograms.

4) Flow cytometers measure fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantitated.

A typical component of a fluorometer is composed of four essential elements of fluorescence detection systems:

1) An excitation source

2) A clear four sided quartz cuvette

3) Wavelength filters to isolate emission photons from excitation photons and

4) A detector

Molecules in solution are usually excited by UV light. The excitation source is usually a deuterium or xenon lamp. Broad-band excitation light from a lamp passes through an excitation monochromator, which passes only a selected wavelength. If a laser is used as the source, the excitation monochromator is not needed. The fluorescence is dispersed by emission monochromator and detected by a photomultiplier tube. Scanning the excitation monochromator gives the excitation spectrum, while scanning the emission monochromator gives the fluorescence spectrum. A block instrument diagram is shown in Fig. 1. 2.



Figure 1.2 Components of a spectrofluorometer.

Fluorescence Signals

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance which is defined by the Beer–Lambert law as the product of the molar extinction coefficient, optical path length, and solute concentration. In dilute solutions, fluorescence intensity is linearly proportional to these parameters. When sample absorbance exceeds about 0.05 in a 1 cm pathlength, the relationship becomes nonlinear and measurements may be distorted by artifacts such as self-absorption and the inner-filter effect (2-3). Because fluorescence quantitation is dependent on instrument settings such as slit widths, fluorescent reference standards are essential for calibrating measurements made at different times or using different instrument configurations.

SECTION 1.2: FLUORESCENT PROBES

To detect an analyte with a fluorescent detector, the analyte must naturally fluoresce or be tagged with a fluorescent dye. For a molecule giving fluorescence naturally, it must meet some structural requirements. The compounds should contain fused ring aromatic functional groups with low energy $\pi \to \pi^*$ transition levels such as indole nitrogen heterocycles.

Fluorophores currently used as fluorescent probes offer sufficient permutations of wavelength range, Stokes shift and spectral bandwidth to meet requirements imposed by instrumentation. The fluorescence output of a given dye depends on the efficiency of which it absorbs and emits photons, and its ability to undergo repeated excitation/emission cycles. Absorption and emission efficiencies are most handily quantified in terms of the molar extinction coefficient (ϵ) for absorption and the quantum yield for fluorescence. Both are constants under specific environmental conditions. The value of ϵ is specified at a single wavelength which is generally the absorption maximum, whereas the quantum yield is a measure of the total photon emission over the entire fluorescence spectral profile. Fluorescence intensity per dye molecule is proportional to the product of ϵ and the quantum yield. The range of these parameters among fluorophores of current practical importance is roughly 5000 to 200,000 cm⁻¹M⁻¹ for ϵ and 0.05 to 1.0 for the quantum yield (8).

Because of a consequence of the strong influence of the surrounding medium on fluorescence emission, fluorescence molecules are currently used as probes for the investigation of physicochemical, biochemical, and biological systems. Fluorescent probes can offer important information in various fields including biological membranes, proteins, nucleic acids, surfactant solutions, polymers, solid surfaces, living cell, and fluoroimmunochemistry (6-7). The fluorescent probes can be classified into three categories (8);

 Intrinsic probes. They are ideal probes such as tryptophan (Fig. 1.3a) used as a fluorescence probe to obtain information about protein tertiary structure in solid state on beta-lactoglobulin (βLg) and interferon alpha-2a (IFN) (9). An exciting, new development for biological studies has been the expansion of the use of naturally fluorescent proteins as fluorescent probes.

2) Extrinsic covalently bound probes. Examples of this type which are attached to phospholipids and polystyrene are shown in Fig. 1.3b-c. Liposomes containing 5 mol % acyl chain labeled 3-palmitoyl-2-(1-pyrenedecanoyl)-L-alpha-phosphatidylcholine has been used to bind annexin V (proteins that bind to membranes). A decrease in the excimer-to-monomer fluorescence ratio was observed (10). Polystyrene were studied as a function of molecular weight using the fluorescence response of anthracene chemically bonded to the polymer chain ends. The fluorescence spectra of the anthracene were collected at 380 and 600 nm for an excitation and emission, respectively (11).



(b) 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine





(c) polystyrene labeled with anthracene

Figure 1.3 Examples of (a) intrinsic probes and (b)-(c) extrinsic covalently bound probes. (cont.)

3) Extrinsic noncovalently associating probes. The synthesis of molecules with covalently bound specific probes can be difficult so noncovalently associating probes have become more important. The specific interactions can be governed by their chemical nature. The hydrophobic, hydrophilic, or amphiphilic property of this probe is practically significant. Some examples are shown in Fig 1.4. Especially, 1,6-diphenyl-1,3-5-hexatriene (DPH) has been widely used as a fluorescent probe for post-column detection for phosphatidylchlolines (PC), triglycerides (TG), glycosphingolipides (Gb) and ceramides (Cer) in microboreliquid chromatography (12-14). DPH exhibits weak fluorescence in water but enhanced fluorescence in a nonpolar (lipid) environment. Asymmetric chromatographic peaks for a PC mixture were observed when the percentage of water was optimized at 12.5% in methanol. Solubility of DPH is limited so a higher percent water in methanol which would result in a lower fluorescence background could not be used. This technique provides a wide range of linear response of PC concentrations from 5 ppm to 100 ppm. This detection approach generated a linear response with a variety of PLs which is contrast to evaporative light scattering detection (ELSD). 1-Pyrenedodecanoic acid (anionic membrane probe) has been used to study the uptake of fluorescent fatty acids into cultured cells (15).

For some molecules are not fluorescent; indirect fluorometric detection can be applied. Fluorescence quenching, one of these indirect methods, is a process which decreases the intensity of the fluorescence emission. Fluorescence quenching is the result of molecular interactions including possibility of molecular rearrangements, excited-state reactions, collisional quenching, energy transfer, and ground state complex formation. A novel oligonucleotide probe consists of a fluorophore and an intercalator. Its fluorescence was quenched by the intercalator in the absence of a target sequence. The probe emitted marked fluorescence due to the interference in quenching by intercalation when having hybridization with a target sequence (16).



(a) 1,6-diphenyl-1,3-5-hexatriene (DPH)



(b) 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3-5-hexatriene (TMA-DPH)



(c) 1-pyrenedodecanoic acid

Figure 1.4 Examples of hydrophobic, hydrophilic or amphiphilic probes.

SECTION 1.3: CELL MEMBRANE AND PHOSPHOLIPIDS

Cells are surrounded by membranes composed of proteins and lipids such as phospholipids (PLs). The purpose of the membrane is to control what goes in and out of the cell and to permit communication with other cells, for example through receptors on the surface. Biomembranes are not rigid but exhibit considerable movability. The fluid mosaic model of the membrane is shown in Figure 1.5. A membrane or phospholipid bilayer is a two-dimensional, spherical shape separating an inside section from an outside section. Membranes are barriers for hydrophilic molecules and ions because of the hydrophobic core of the phospholipid bilayer.



Figure 1.5 Biomembrane structure. (http://www.people.virginia.edu/~rjh9u/cellmemb .html)

In addition to lipids, a membrane also contains proteins that control the transport of hydrophilic and charged, and varied size molecules in and out of the cell and intracellular organelles. The importance of the lipid bilayer membrane is its ability to function as an electrical capacitor. This enables charge separation and thus the storage of electro-chemical energy in the form of ion gradients.

The main structural components of biological membranes are the phospholipids. A phospholipid molecule consists of a hydrophilic polar head group (which points towards the outside environment and the cytoplasm) and a hydrophobic tail. The polar head group contains one or more phosphate groups. Most phospholipid head groups belong to phosphoglycerides, which contain glycerol joining the head and the tail (17-18). Examples of phosphoglycerides include phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). The hydrophobic tails, made up of fatty acyl chains, repel the water and point in. The fatty acyl chain in biomembranes usually contains an even number of carbon atoms and may be saturated or unsaturated. The typical phospholipid is shown in Figure 1.6.

The phospholipids are the hydrophilic circles with hydrophobic tails. Since most of the cell and area surrounding the cell are made up of water, these fatty acid tails always push away from the water. This causes a bilayer as a micelle, which is a single layer circle of phospholipids with the tails pointing in shown in Figure 1.7.

Since the fatty chains can vary in length and degree of unsaturation, each PLs class has numerous molecular species with different chemical and biological properties. Thus identification and quantitation of PLs in biological samples has been of great interest. It was found that the level of PLs was decreased in blood plasma and increased in erythrocytes of sickle cell patients when compared to normal subjects. The tumorous tissue of liver cancer contains less PLs than normal tissue (17-18).



Figure 1.6 Structure of a typical phospholipid (phosphatidylcholine).





(http://courses.cm.utexas.edu/jrobertus/ch339k/overheads-2/ch12_lipid-bilayer.jpg)

There are 10 different main types of lipids in cell membranes from rat liver. Each type of cell will have a differing percentage of each lipid, protein, and carbohydrate. The main types of lipids are;

-cholesterol
-diphosphatidylglycerol (cardiolipin (CL))
-glycolipids
-phosphatidylethnolamine
-phosphatidylinositol
-phosphatidylcholine
-phosphatidylserine
-phosphatidylglycerol
-phosphatidic acid and
-sphingomyelin

SECTION 1.4: CARDIOLIPIN

Among PLs, cardiolipin is a unique PL with a dimeric structure, four fatty acid chains, and two negative charges linked by a glycerol backbone. The structure is shown in Fig. 1.8. Each phosphate group of cardiolipin contains one acidic proton, but these protons have varying acidic properties ($pK_1 = 2.8$ and $pK_2 > 7.5$). The weak acidity of the second phosphate is probably due to formation of a stable intramolecular hydrogen bond with the central 2'-hydroxy group (19-20). CLs have been noted for the unique, usually C18 fatty acid chains. The data in Table 1.1 shows the different CL forms for the bovine heart and rat liver. The diacylglycerol groups in this case compose most of 18:2-18:2, 18:2-18:1, and 18:3 (21-22).



Figure 1.8 Structure of typical CL with fatty acyl groups (R₁, R₂, R₃, and R₄).

CL is the most abundant in mammalian hearts from which most commercial cardiolipin is obtained. The majority of CL found in bovine heart is the linoleoyl chain (CL-18:2) as shown in Fig. 1.9, comprising 87% of all aliphatic long chains (23-24). The oleoyl group (Fig. 1.10) is present in about 8% of all fatty acyl chains.

CL is not present in all eukaryotic cell membranes and is synthesized in prokaryotes as well (20, 25). CL is a considerable component (18%) of the mitochondrial inner membrane as shown in Table 1.2 (26). CL is crucial for the functionality of several mitochondrial proteins. Its distribution between the inner and outer leaflet of the mitochondrial internal membrane is crucial for ATP synthesis (Fig. 1.11).

Source of		Unsaturated fa	ttty acyl groups		Abundance
cardiolipin	Residue R ₁	Residue R_2	Residue R ₃	Residue R4	(mole %)
Bovine heart	18:2	18:2	18:2	18:2	48
	18:3	18:2	18:2	18:2	21
	18:2	18:3	18:2	18:2	I
	18:2	18:2	18:3	18:2	ı
	18:2	18:2	18:2	18:3	ı
	18:2	18:1	18:2	18:2	15
	18:2	18:2	18:1	18:2	I
Rat liver	18:2	18:2	18:2	18:2	57
	18:2	18:2	18:2	18:2	35
	18:2	18:2	18:1	18:2	ı

Table 1.1 Main molecular species of mammalian cardiolipin (20).



Figure 1.9 Structure of 1, 1', 2, 2'-tetralinoleoyl cardiolipin.

(* 18 and 2 = no. of carbon and double bond in each fatty acyl chain, respectively)



Figure 1.10 Structure of 1, 1', 2, 2'-tetraoleoyl cardiolipin.

	Percent	age of total pho	spholipids in m	nembranes from	ı different orga	nelles
	Mitochondria	Microsomes	Lysosomes	Plasma membrane	Nuclear membrane	Golgi membrane
Cardiolipin	18	1	1	-	4	1
Phosphatidylethanolamine	35	22	14	23	13	20
Phosphatidylcholine	40	58	40	39	55	50
Phosphatidylinositol	5	10	5	8	10	12
Phosphatidylserine	1	7	7	6	3	9
Phosphatidic acid	ı	1	1	1	2	\sim
Sphingomyelin	1	1	20	16	3	8
Phospholipids (mg/mg protein)	0.175	0.374	0.156	0.672	0.500	0.825
Cholesterol (mg/mg protein)	0.00	0.014	0.038	0.128	0.038	0.078

Table 1.2 Phospholipid compositions of organelle membranes from rat liver (18).



Figure 1.11 Structure of a typical mitochondrion. (http://en.wikipedia.org/wiki/Mitochondrial membrane# note-Alberts)

Mitochondria serve as "cellular power plants" because their primary function is to convert organic materials into energy in the form of ATP via the process of oxidative phosphorylation. CL as an important constituent of the inner mitochondrial membrane serves as an insulator and stabilizes the activity of protein complexes important to the electron transport chain. Moreover, the CL level in the mitochondrial membrane is used as a marker of many diseases. It was discovered that cytochrome c was released from mitochondria and mitochondrial CL level decreased in apoptosis disease (22-23, 28-31). CL levels also decreased in other diseases such as thyroid dysfunction (32-33), aging, and oxidative stress (34-37).

SECTION 1.5: PURPOSE

In this study, we have developed a fluorescence quenching method for detection of CL using 10-N-nonyl acridine orange (NAO) dye as a fluorescent probe. The phospholipid probe DPH discussed previously is not expected to offer specificity for CL. Many previous studies have used NAO as a fluorescent probe for CL detection, in *vivo*, on different models of artificial membranes, liposomes, and also extended to mitochondria in different respiratory states (38-43). Our overall goal is to investigate fluorescence methods in solution, in *vitro*, for CL and other PLs. Application of method for automation using flow injection and possibly liquid chromatography is envisioned.

REFERENCES

- Albani, J. R., Structure and Dynamics of Macromolecules: Absorption and Fluorescence Studies, Elsevier, 2004.
- Brand, L. and Johnson, M. L., Eds., "Fluorescence Spectroscopy" (1997) Methods in Enzymology. 278, Academic Press
- 3) Guilbault, G. G., Ed., Practical Fluorescence, Second Edition, Marcel Dekker, 1990.
- Powe, A. M., Fletcher, K. A., St. Luce, N. N., Lowry, M., Neal, S., McCarroll, M.E., Oldham, P. B., McGown, L. B., Warner, I. M., "Molecular fluorescence, phosphorescence and chemiluminescence spectrometry" (2004) *Anal. Chem.* 76, 4614–4634.
- Mathies, R. A., Peck, K., Stryer, L. "Optimization of high-sensitivity fluorescence detection" (1990) *Anal. Chem.* 62, 1786–1791.
- Giuliano, K. A., Post, P. L., Hahn, K. M., Taylor, D. L. "Fluorescent protein biosensors: measurement of molecular dynamics in living cells" (1995) *Annu. Rev. Biophys. Biomol. Struct.* 24, 405-434.
- Johnson, I. D. "Fluorescent probes for living cells" (1998) *Histochem. J.* 30, 123–140.
- 8) Valcur, B. Molecular fluorescence: Principle and applications, Wiley-vch, New York, 2002.
- Sharma, V. K., Kalonia, D. S. "Steady-state tryptophan fluorescence spectroscopy study to probe tertiary structure of proteins in solid powders" (2003) *J. Pharm. Sci.* 92, 890-899
- Meers, P., Daleke, D., Hong, K., Papahadjopoulos, D. "Interactions of annexins with membrane phospholipids" (1991) *Biochemistry* 30, 2903-2908.
- Turrión, S.G., Olmos, D., Ekizoglou, N., González-Benito, J. "Fluorescence response from anthracene labeled polystyrene to study its thermal transitions" (2005) *Polymer* 46, 4023-4031.
- Caudron, E., Zhou, J, Y., Chaminade, P., Baillet, A., Prognon, P. "Fluorescence probe assisted post-column detection for lipid analysis in microbore-LC" (2005) *J. Chromatogr. A.* 1072, 149-157.

- 13) Zhou, J. Y., Chaminade, P., Gaudin, K., Prognon, P., Baillet, A. "Postcolumn fluorescence as an alternative to evaporative light scattering detection for ceramide analysis with gradient elution in non-aqueous reversed-phase liquid chromatography" (1999) J. Chromatogr. A. 859, 99-105.
- 14) Kitsos, M., Gandini, C., Massolini, G., Lorenzi, E. "High-performance liquid chromatography post-column derivatization with fluorescence detection to study the influence of ambroxol on dipalmitoylphosphatidylcholine levels in rabbit eustachain tube washing" (1991) J. Chromatogr. 553, 1-6.
- 15) Nahas, N., Fibach, E., Giloh, H., Gatt, S. "Use of the fluorescence activated cell sorter for studying uptake of fluorescentfatty acids into cultured cells" (1987) *Biochim. Biophys. Acta.* 917, 86-91.
- 16) Yamane, A. "MagiProbe: a novel fluorescence quenching-based oligonucleotide probe carrying a fluorophore and an intercalator" (2002) *Nucleic Acids Res.* 30, 97-101.
- Hanahan, D. J. A Guide to Phospholipid Chemistry, Oxford University Press, New York, 1997.
- 18) Ceve, G. Phospholipids handbook, Marcel Dekker, Inc., New York, 1993.
- 19) Kates, M., Syz, J. Y., Gosser, D., Haines, T. H. "pH-dissociation characteristics of cardiolipin and its 2'-deoxy analogue" (1993) *Lipids* 28, 877-882.
- Schlame, M., Rua D., Greenberg, M. L. "The biosynthesis and functional role of cardiolipin" (2000) *Prog. Lipid Res.* 39, 257-288.
- Wang, Y., Krull, I. S., Liu, C., Orr, J. D. "Derivatization of phospholipids" (2003) J. Chromatogr. B. 793, 3-14.
- 22) McMillin, J. B., Dowhan, W. "Cardiolipin and apoptosis" (2002) *Biochim. Biophys. Acta*. **1585**, 97-107.
- 23) Hanahan, D. J. A Guide to Phospholipid Chemistry, Oxford University Press, New York, (1997) Chapter 1.
- 24) Schlame, M., Rua D., Greenberg, M. L. "The biosynthesis and functional role of cardiolipin" (2000) Prog. Lipid Res. 39, 257-288.

- 25) Wright, M. M., Howe, A. G., Zaremberg, V. "Cell membranes and apoptosis: role of cardiolipin, phosphatidylcholine, and anticancer lipid analogues" (2004) *Biochem. Cell Biol.* 82, 18-26.
- 26) Gorbenko, G. P., Molotkovsky, J. G., Kinnunen, P. K. "Cytochrome c interaction with cardiolipin/phosphatidylcholine model membranes: Effect of cardiolipin protonation" (2006) *Biophy. J.* **90**, 4093-4103.
- 27) Hatch, G. M. "Regulation of cardiolipin synthesis in the heart" (1996) Mol. Cell Biochem. 159, 139-148.
- 28) Zhao, K., Zhao, G. M., Wu, D., Soong, Y., Birk, A. V., Schiller, P. W., Szeto, H.H. "Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury" (2004) *J. Biol. Chem.* 279, 34682-34690.
- 29) Iverson, S. L., Orrenius, S. "The cardiolipin-cytochrome *c* interaction and the mitochondrial regulation of apoptosis" (2004) *Arch. Biochem. Biophys.* **423**, 37-46.
- 30) Fernandez, M. G., Troiano, L., Moretti, L., Pedrazzi, J., Salvioli, S., Cortazar, I. C., Cossarizza, A. "Changes in intramitochondrial cardiolipin distribution in apoptosisresistant HCW-2 cells, derived from the human promyelocytic leukemia HL-60" (2000) *FEBS Lett.* **478**, 290-294.
- 31) Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., Greenberg, M. L. "Absence of cardiolipin in the *crd1* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function" (2000) J. Biol. Chem. 275, 22387-22394.
- 32) Cao, S. G., Cheng, P., Angel, A., Hatch, G. M. "Thyroxine stimulates phosphatidylglycerolphosphate synthase activity in rat heart mitochondria" (1995) *Biochim. Biophys. Acta.* 1256, 241-244.
- 33) Paradies, G., Ruggiero, F. M. "Stimulation of phosphate transport in rat-liver mitochondria by thyroid hormones" (1990) *Biochim. Biophys. Acta.* **1019**, 133-136.
- 34) Paradies, G., Ruggiero, F. M. "Age-related changes in the activity of the pyruvate carrier and in the lipid composition in rat-heart mitochondria" (1990) *Biochim. Biophys. Acta.* 1016, 207-212.

- 35) Paradies, G., Ruggiero, F. M. "Effect of aging on the activity of the phosphate carrier and on the lipid composition in rat liver mitochondria" (1991) *Arch. Biochem. Biophys.* 284, 332-337.
- 36) Paradies, G., Ruggiero, F. M., Petrosillo, G., Quagliariello, E. "Age-dependent decline in the cytochrome *c* oxidase activity in rat heart mitochondria: role of cardiolipin" (1997) *FEBS Lett.* **406**, 136-138.
- 37) Paradies, G., Ruggiero, F. M., Gadaleta, M. N., Quagliariello, E. "The effect of aging and acetyl-L-carnitine on the activity of the phosphate carrier and on the phospholipid composition in rat heart mitochondria" (1992) *Biochim. Biophys. Acta.* 1103, 324-326.
- 38) Petit, J. M., Maftah, A., Rantinaud, M. H., Julien, R. "Direct cardiolipin assay in yeast using the red fluorescence emisstion of 10-*N*-nonyl acridine orange" (1995) *Eur. J. Biochem.* 228, 113-119.
- 39) Gohil, V. M., Jeremic, J. G., Schlame, M., Greenberg, M. L. "Binding of 10-N-nonyl acridine orange to cardiolipin-deficient yeast cells: implications for assay of cardiolipin" (2005) *Anal. Biochem.* 343, 350-352.
- 40) Fernandez, M. G., Ceccarelli, D., Muscatello, U. "Use of the fluorescence dye 10-Nnonyl acridine orange in quantitative and location assays of cardiolipin: a study on different experimental models" (2004) *Anal. Biochem.* **328**, 174-180.
- 41) Petit, J. M., Huet, O., Gallet, P. F., Maftah, A., Rantinaud, M. H., Julien, R. "Direct analysis and significance of cardiolipin transverse didstribution in mitochondrial inner membranes" (1994) *Eur. J. Biochem.* **220**, 871-879.
- 42) Petit, J. M., Maftah, A., Rantinaud, M. H., Julien, R. "10-N-Nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria" (1992) *Eur. J. Biochem.* 209, 267-273.
- 43) Maftah, A., Petit, J. M., Julien, R. "Specific interaction of the new fluorescent dye 10-N-nonyl acridine orange with inner mitochondrial membrane" (1990) *FEBS Lett.* 260, 236-240.

CHAPTER 2

FLUORESCENT DETERMINATION OF CARDIOLIPIN USING 10-N-NONYL ACRIDINE ORANGE

SECTION 2.1: INTRODUCTION

Cardiolipin (CL) is found almost exclusively in the inner membranes of mitochondria. The role of CL in biological membranes is as a proton trap within membranes that accomplish oxidative phosphorylation leading to ATP synthesis (1, 2). The reducing potential of mitochondrial NADH is used to supply the energy for ATP synthesis via oxidative phosphorylation. Oxidation of NADH with phosphorylation of ADP to form ATP is supported by the mitochondrial electron transport assembly and ATP synthase. Two principal conditions are required to precede an oxidative phosphorylation. First, the inner mitochondrial membrane must be physically intact so that protons can only re-enter the mitochondrion by a process coupled to ATP synthesis. Secondly, a high concentration of protons must be present on the outside of the inner membrane.

Mitochondria are involved in many types of apoptosis or programmed cell death. Apoptosis has been identified recently as a component of much cardiac pathology (3-4). Detachment of cytochrome c from the mitochondrial inner membrane is a necessary first step for cytochrome c release into the cytosol and initiation of apoptosis (5-9). A decrease of CL in the mitochondrial inner membrane is utilized as a marker for cell apoptosis (10-14). Also loss of CL content in mitochondria has been found from rat heart subjected to isomia and ischemia-reperfusion (15-16) and in patients with rheumatoid arthritis which is a chronic inflammatory disease (17). The CL level is also decreased during the course of human-aging (18).

Quantitative detection of phospholipids (PLs) by UV absorbance is problematic due to the lack of chromophores in the structure and few fluorescent methods have been developed. Aminophospholipids can be derivatized with 1-dimethylaminonaphthalene-5sulfonyl chloride and succinimidyl 2-naphthoxyacetate with fluorescence detection (19). Some phospholipids through interaction with fluorescent compounds can change the fluorescence emission. For example, 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) has been used in conjunction with microbore liquid chromatography as a fluorescent probe for phosphatidylcholine (PC) and other phospholipids classes (20-21). The interaction phenosafranin (PSF) - (3, 7-diamino-5-phenylphenazinium chloride) with PC was followed with fluorescence at 572 nm (22). Both DPH and PSF are not expected to be effective
probes for CL detection because DPH is able to bind to many kinds of phospholipids and PSF has a phenyl not a long chain alkyl substitutes.

Recently 10-*N*-nonyl acridine orange (NAO), structure shown in Fig. 2.1, was mostly used as a specific fluorescence probe for CL detection in *vivo*. The incorporation of NAO in inner mitochondrial membranes has been detected as the red fluorescence emitted by formation of NAO dimers (23). Increasing amounts of CL in thin-walled vesicles added to NAO changed the green fluorescence (525 nm) to red fluorescence (640 nm). Also NAO has been utilized for fluorescent detection of liposomes and mitochondria through its interaction with CL (24-28). However, specific quantitative determination of CL in solution using NAO is still unrevealed.

NAO has been added to mitochondrial samples isolated from NS1 cell grown for determination of cardiolipin. They used capillary electrophoresis detection with laser-induced fluorescence detection at 495 nm and 525 nm for excitation and emission, respectively (29). NAO also can be added to the running electrolyte for visible spectrophotometric detection of CL by capillary electrophoresis (CE) (30). A 0.1 mM solution of NAO was used for CL absorbance detection at 497 nm in on-line CE but low sensitivity and nonlinear calibration curves as sub μ M CL levels were limitations (30). The 0.1 mM NAO concentration was too high for use with conventional flow injection or liquid chromatography spectrophotometric detection.

In this work, we have developed a fluorescence quenching method for CL using NAO. The excitation and emission wavelengths were optimized. To ensure specific interaction of NAO with CL in the presence of a surfactant or other PLs, the percentage of methanol in the solvent was optimized. The optimization of NAO concentration was studied. The linearity, detection limit (DL), and limit of quantification (LOQ) were also estimated for this method. The sensitivity was compared between fluorescent and UV-visible methods. The selectivity of the NAO dye was also compared to other analogous dyes, acridine orange (AO) and PSF. CL in spiked cell extract samples was determined with good recovery.



Figure 2.1 Structure of 10-N-nonyl acridine orange (NAO).

SECTION 2.2: EXPERIMENTAL

Chemicals and Materials

Cardiolipin, 1, 1', 2, 2'-tetralinoleoyl cardiolipin, (heart, bovine, CL, 18:2, FW = 1493.92) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Other phospholipids such as 1,2-dilauroyl-*sn*-glycero-3-phosphate (PA, 12:0, FW = 557.65), 1,2-distearoyl-*sn*-glycero-3-phosphate (PA, 18:0, FW =725.98), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC, 18:1, FW= 786.15), 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG, 18:0, FW = 801.07), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (PE 18:0, FW = 748.08), L- α -phosphatidylserine (PS 18:05, FW= 812.05), sphingomyelin (SM) were also purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol was purchased from Aldrich. All structures of biological surfactants and SDS are shown in Fig. 2.2. N-nonyl acridine orange, (3, 6-bis (dimethylamino)-10-nonylbromide), (NAO, FW = 472.51) was obtained from Molecular Probes, Portland, OR. Acridine orange, 3, 6-bisdimethylaminoacridine, (AO, FW = 265.36) was purchased from Aldrich and the structure is shown in Fig. 2.3. Phenosafranin, 3, 7-diamino-5-phenylphenazinium chloride, (PSF, FW = 322.79) was purchased from Sigma-Aldrich; the structure is also shown in Fig. 2.3. Methanol (HPLC grade, 99.9% v/v)

and absolute ethanol (HPLC grade) were obtained from Pharmaco Products Inc. Sodium dodecyl sulfate (SDS, FW = 288.38) was from Aldrich. Distilled, deionized, filtered water was generated using an E-Pure water treatment system (Barnstead / Thermolyne, Dubuque, IA). The whole cell and membrane samples were prepared under the direction of Dr. Daryoush Ekhterae at the Department of Surgery, School of Medicine, University of Pennsylvania, Philadelphia.

Instrumentation

Fluorescence spectra were recorded on a Perkin-Elmer Model LS50B luminescence spectrometer with a 1x1x4 cm quartz fluorescence cell. UV-VIS absorbance spectra were measured using Agilent 8453 UV-visible spectrophotometer with 1x1x4 cm quartz cell.

Procedure

The stock solutions of all PLs and surfactants were prepared in ethanol and stored at 4 °C. Dilutions were done in different percentages of methanol in water; a 50-50 mixture was optimum. A 3 mL volume of each sample was measured in a 1x1x4 cm quartz cuvette. Stock solutions of 100 μ M NAO were made in different methanol - water solutions. The incubation time of NAO with CL or the PLs was about 15 min. in the darkness. Several PLs solutions were diluted to the range 0-100 μ M. Biological membrane samples were diluted with 3 mL of 50% methanol-50% water. A spiked sample of 1 μ M CL was added and 300 μ L of each sample were used.

Preparation of the whole cell and membrane sample extracts (D. Ekhterae, University of Pennsylvania)

For the whole cell (Neo) samples, a volume of 2 mL of 0.3N NaOH/0.1% SDS was placed on a confluent monolayer T-75 culture dish and allowed to sit in the incubator overnight. For the membrane samples, cells were suspended in a buffer containing 340 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH=7.5), 1 mM diothiothreitol, and 1 x proteases inhibitors from Roche Biochemicals, Indianapolis, IN at 4 °C. The cells were disrupted by

passage through a 23 gauge needle 12 times at 4 °C. The cells were then centrifuged for 5 minutes at 1000 x g at 4 °C to remove the unbroken cells. The supernatant were then centrifuged for 20 minutes at 12000 rpm at 4 °C to obtain the pellet (the membrane fraction).

The total lipids were extracted from the membrane fraction as described below. For lipid extraction, a volume of 800 μ L of the prior NaOH/SDS solution was placed it in a conical tube for lipid extraction. After spiking with 5-alpha-cholestane (our internal standard used for cholesterol analysis), a 2 mL volume of methanol was added and vortexed carefully. To the same tube, a 1 mL volume of chloroform was added and again carefully vortexed. These processes were repeated one more time for a total volume of chloroform = 2 mL. Then 1 ml of deionized water was added and again the mixture vortexed. After centrifugation at 2000 rpm for 15 minutes, the chloroform (the bottom layer) was carefully removed and a 1 mL portion was placed into one tube for cholesterol analysis. The protein wafer at the interface of the aqueous (top) layer and the chloroform (bottom) layer was left undisturbed by tilting the tube slightly. The aqueous layer which may have entered the tip of the Pasteur pipette was released by passing a few bubbles through prior to the chloroform uptake.

Both tubes of chloroform were placed into a nitrogen evaporator, maintained at 40 °C, until brought to dryness. The tubes were carefully labeled, covered with parafilm and placed in the freezer until analysis.



1,2-dilauroyl-*sn*-glycero-3-phosphate (PA, 12:0, FW = 557.65)



1,2-distearoyl-*sn*-glycero-3-phosphate (PA, 18:0, FW =725.98)



1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC, 18:1, FW= 786.15)





1,2-distearoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (PG, 18:0, FW= 801.07)



sphingomyelin





Figure 2.2 Structures of surfactants (cont.).





Figure 2.3 Structures of (a) acridine orange (AO) and (b) phenosafranin (PSF).

SECTION 2.3: RESULTS AND DISCUSSION

Visible absorbance spectra of NAO at a concentration in a range of 0.5-100 μ M were also studied (Fig. 2.4) to anticipate fluorescence excitation wavelengths. It showed that the optimum absorbance was relatively constant at 497 nm for all concentrations of NAO in 50% methanol and also exhibited a shoulder at 460 nm.

Using a Perkin-Elmer LS-50 spectrofluorometer with slit widths set at 2.5 nm and using a 1x1x4 cm quartz cell, the optimum excitation and emission wavelengths were determined as a function of concentration of NAO in 50% methanol. It showed that the excitation peak was separated into three peaks, according to Fig. 2.5 (a-f), at 450, 499 and 518 nm. So these excitation wavelengths were investigated. The excitation and emission wavelengths were examined as a function of concentration of NAO (Fig. 2.6). It showed that high fluorescence intensity was obtained using 499/530 nm as excitation and emission wavelengths at low concentrations of NAO less than 10 μ M and 518/530 nm at a concentration and emission wavelengths at 499/530 nm at low concentrations of NAO and at 518/530 nm at high concentrations of NAO are selected. Considering the fluorescence intensity, the optimum concentration of NAO was selected in the range 20-50 μ M because this concentration range provided stable and high fluorescence intensity.

According to Fig. 2.7, fluorescence quenching of NAO by CL occurred when the methanol content was 25% or 50% in water. At 25 and 50 % methanol in water, the fluorescence intensity decreased with increasing of CL concentration. At the first point without CL, NAO exists only in its monomeric form. The unbound or free NAO in the solution was measured by the green fluorescence emission at 530 nm. Upon addition of CL, aggregation between the aromatic rings in NAO structure was likely formed with CL so fluorescence intensity decreased according to the loss of the monomeric form. According to a previous study using NAO binding mitochondria and liposome (23), when NAO interacted with CL, the emission wavelength shifted from green fluorescence emission (525 nm) to red fluorescence emission (640 nm) because of its aggregation with increased π - π interactions between the aromatic rings in NAO structure (1-2). The red fluorescence in our experimental condition was not observed because of the difference of a solvent system so

the green fluorescence emission was chosen to investigate interaction between NAO and PLs. The advantage of the green fluorescence is that it was 14 fold stronger compared to the red fluorescence (26).

Fluorescence intensity was quenched to the minimum point when the concentration of CL reached a value of 25 μ M. This implies that the mole ratio of CL to NAO is about 2:1; a result in agreement with previous observations for the NAO dye which was used as a fluorescent probe with emission at 640 nm for CL determination (1, 25) in which maximum formation of a nonfluorescent dimer was observed at the lowest value of fluorescence signal. It indicates that two molecules of NAO react with one molecule of CL. In a related previous study, when the concentration of SDS was added after the lowest point, the fluorescence signal was enhanced because the micelle formation of SDS induced the breaking of AO dimer (31).

It was concluded that NAO can not completely approach a dimer form at 60% or 75% methanol content since no significant change in fluorescence intensity with various concentrations of CL was observed. The intensity of free NAO at 70% methanol-30% water was the highest compared to 60%, 50% and 25% methanol. This implies that NAO is more readily soluble in methanol than in water. Moreover, fluorescence intensity was quenched to the lowest value of 25 μ M CL at 25% methanol because a hydrophobic effect through the C9 aliphatic chain in NAO is dominant.

To ensure a specific interaction of NAO with CL in the presence of a surfactant, the percentage of methanol in the solvent was optimized using sodium n-dodecyl sulfate (SDS) which may be present in biological samples. It was found that fluorescence intensities of NAO were quenched by the presence of the SDS surfactant in 0%, 5%, 10% or 25% methanol. The fluorescence intensity of NAO signal was strongly quenched at 5 μ M SDS below 25% methanol and at 20 μ M SDS with 25% methanol- 75% water as shown in Fig. 2.8. With a solvent methanol content above 50%, no quenching of NAO even at low concentrations of SDS was observed as presented in Fig. 2.9. Similar to CL, the hydrophobic interaction between NAO and SDS is apparently weak at a methanol percentage of 50% or higher and therefore the 50-50 methanol-H₂O solvent ratio was used in all further studies.

Because the NAO dye is light sensitive, the fluorescence decay of NAO itself at low and high concentration in 50% methanol solvent was investigated. It was observed that fluorescence intensity of NAO is stable to at least 15 min as shown in Fig. 2.10 and Fig. 2.11 for low and high NAO concentrations, respectively. Fluorescence as a function of reaction time between the interaction of CL and NAO, as shown in Fig. 2.12, is stable after 3 min to at least 15 min. To make sure that the reaction is complete, a reaction time of 15 min was used.



Figure 2.4 Visible absorbance spectra of 0.5-100 µM NAO in 50% methanol.



Figure 2.5 (a) Fluorescence excitation and emission spectra of 0.5 μ M NAO in 50% methanol (slit widths = 2.5 nm).



Figure 2.5 (b) Fluorescence excitation and emission spectra of 1.0 μ M NAO in 50% methanol (slit widths = 2.5 nm).



Figure 2.5 (c) Fluorescence excitation and emission spectra of 10 μ M NAO in 50% methanol (slit widths = 2.5 nm).



Figure 2.5 (d) Fluorescence excitation and emission spectra of 20 μ M NAO in 50% methanol (slit widths = 2.5 nm).



Figure 2.5 (e) Fluorescence excitation and emission spectra of 50 μ M NAO in 50% methanol (slit widths = 2.5 nm).



Figure 2.5 (f) Fluorescence excitation and emission spectra of 100 μ M NAO in 50% methanol (slit widths = 2.5 nm)



Figure 2.6 The fluorescence intensity of emission at different concentrations of NAO in 50% methanol ($\lambda_{\text{excit.}} / \lambda_{\text{emiss.}} = 450/530 \text{ nm} (-\diamondsuit-)$, 499/530 nm (- \blacksquare -), and 518/530 nm (- \blacktriangle -), slit widths = 2.5 nm).



Figure 2.7 Effect of methanol contents (25% (- \blacktriangle -), 50% (- \blacksquare -), 60% (-x-) and 75% (- \blacklozenge -)) in water on the fluorescence interaction between NAO (50 μ M) and various concentrations CL. ($\lambda_{excit}/\lambda_{emiss.} = 518/530$ nm, slit widths = 2.5 nm).



Figure 2.8 Effect of methanol contents (0% (- \bullet -), 5% (- \blacksquare -), 10% (- \blacktriangle -), and 25% (- \blacklozenge -)) in water on the fluorescence interaction between 0.50 μ M NAO and SDS at different concentrations. ($\lambda_{excit.} / \lambda_{emiss.} = 499/518$ nm, slit widths = 5 nm).



Figure 2.9 Effect of methanol contents (50% (- \blacklozenge -), 75% (- \blacksquare -), and 100% (- \blacktriangle -)) in water on interaction between 0.50 µM NAO and SDS at different concentrations. ($\lambda_{excit.}$ / $\lambda_{emiss.}$ = 499/518 nm, slit widths = 5 nm).



Figure 2.10 Fluorescence decay curve of NAO in 50% MeOH at low concentrations (0.05 μ M (- \blacklozenge -), 0.1 μ M (- \bullet -), 0.2 μ M (- \blacktriangle -), 0.3 μ M (- \blacksquare -), and 0.4 μ M (- \square -), $\lambda_{excit.} / \lambda_{emiss.} = 496/518$ nm, slit widths = 5 nm).



Figure 2.11 Fluorescence decay curve of NAO in 50% MeOH at high concentrations (1 μ M (- \blacklozenge -), $\lambda_{excit.} / \lambda_{emiss.} = 499/518$ nm, slit widths = 5 nm), (20 μ M (- \blacktriangle -), and 10 μ M (- \blacksquare -), $\lambda_{excit.} / \lambda_{emiss.} = 518/530$ nm, slit widths = 2.5 nm).



Figure 2.12 Fluorescence-reaction time profiles for various ratios of CL to NAO concentrations in 50% MeOH (2/1(- \diamond -), $\lambda_{excit.} / \lambda_{emiss.} = 496/518$ nm, slit widths = 5 nm), (2/10 (- \Box -), 2/20 (- \blacktriangle -), 10/20 (- \blacksquare -), and 20/20 (- \bullet -), $\lambda_{excit.} / \lambda_{emiss.} = 518/530$ nm, slit widths = 2.5 nm).

To consider the interference from other PLs at both low and high concentrations to NAO-CL interaction, other PLs and lipid were selected with a variety of functional groups including 1,2-dilauroyl-*sn*-glycero-3-phosphate, 1,2-distearoyl-*sn*-glycero-3-phosphate, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine,1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], and L- α -phosphatidylserine. No significant fluorescence quenching of NAO with a 50% methanol solvent by these PLs was noted at low concentration of NAO (0.1 μ M), Fig. 2.13. At a high concentration of NAO (20 μ M), Fig. 2.14., 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] did appear to quench the fluorescence signal at concentrations above 8 μ M. In general, PL concentrations in biological samples would be expected to be less than this. In addition, no quenching of NAO (20 μ M) by three other cell membrane compounds, 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sphingomyelin, and cholesterol, was observed in this same CL range. This suggests that PLs are not able to bind to NAO according to the previous study (26-27) which mentioned that NAO has a low affinity for other anionic PLs and no ability to bind to zwitterionic PLs.

The fluorescence response CL was optimized at different NAO concentrations, 5 μ M, 10 μ M, 20 μ M and 50 μ M in 50 % methanol, as presented in Fig. 2.15. It revealed that nonlinearity from 0 - 2 μ M CL was obtained by using 5 and 10 μ M concentrations of NAO. Linearity is improved to 10 or 20 μ M CLby using either 20 or 50 μ M NAO. The sensitivity was enhanced slightly at 20 μ M NAO. The upturn in fluorescence above 15 μ M CL and 30 μ M CL using 20 and 50 μ M NAO respectively was similar to that reported previously (31). It was pointed out that the fluorescence intensity would be decreased due to formation of aggregation of NAO and then the fluorescence was relatively stable because of the interaction of NAO by excess CL.

Fig 2.16 shows a calibration curve for CL from 0-10 μ M using 20 μ M NAO. The RSD data (n=3) for 10 points ranged from 1% to 5%. The linear regression equation was F = -26.68[CL] + 381.28. The correlation coefficient (R²) of 0.9929 indicated a good linearity of this method. The detection limit and limit of quantitation were estimated at 0.20 and 0.60 μ M CL, respectively (see appendix).



Figure 2.13 Fluorescence profiles using a low concentration of NAO (0.1 μ M) as a function of concentration for various PLs ((PA, 12:0) (- \blacklozenge -), (PA, 18:0) (- \blacksquare -), (PC, 18:1) (- \blacktriangle -), (PG, 18:0) (-x-), and (PS, 180.5) (- \circ -), $\lambda_{\text{excit.}} / \lambda_{\text{emiss.}} = 499/518$ nm, slit widths = 5 nm).



Figure 2.14 Fluorescence profiles using a high concentration of NAO (20 μ M) as a function of concentration for various PLs ((PA, 18:0) (- \blacklozenge -), (PC, 18:1), (- \blacksquare -), (PG, 18:0) (- \blacktriangle -), and (PS 18:05) (- \bullet -)), ($\lambda_{\text{excit.}} / \lambda_{\text{emiss.}} = 518/530$ nm, slit widths = 2.5 nm).



Figure 2.15 Linearity of fluorescence as a function of cardiolipin concentration using NAO in 50 % methanol at 5 μ M (-x-), 10 μ M (- \blacktriangle -), ($\lambda_{excit.}$ / $\lambda_{emiss.}$ = 499/530 nm, slit widths = 2.5 nm), 20 μ M (- \blacksquare -), and 50 μ M (- \blacklozenge -), ($\lambda_{excit.}$ / $\lambda_{emiss.}$ = 518/530 nm, slit widths = 2.5 nm).



Figure 2.16 Fluorescence calibration curve of CL with 20 μ M NAO in 50% methanol in water, ($\lambda_{excit.} / \lambda_{emiss.} = 518/530$ nm, slit widths = 2.5 nm).

Spectrophotometric absorbance at 497 nm using NAO at 10 μ M and 20 μ M was investigated for the determination of CL (Fig. 2.17.). It revealed that CL linearity from 0-8 μ M is poor when using 10 μ M concentrations of NAO. Linearity is improved from 0 - 10 μ M by using 20 μ M NAO. The RSD data (n=3) for 11 points ranged from 1% to 9%. The linear regression equation was A = -0.08[CL] + 1.06 with only a fair correlation coefficient (R²) of 0.9898.

The AO and PSF dyes at 20 μ M as fluorescence indicators for CL were studied for comparison to NAO dye at 20 μ M (Fig. 2.18). AO provided a non linearity from 0 - 10 μ M CL. No electrostatic interaction between the AO and the phosphate groups of CL is possible as shown in Fig. 2.19 and an interaction between the apolar chromophore of AO and the alkyl chain of CL is insufficient to provide linearity.

The spectral change due to the NAO and CL interaction was likely because of a hydrophobic interaction between the nonyl chain of NAO and linoleoyl chains of CL and also an electrostatic interaction between the positively charged NAO and the negatively charged CL (Fig. 2.20). Because of the solvent composition, the results suggest that the hydrophobic interaction plays a dominant role in binding the NAO to CL. The nonyl chain of NAO should be bound to the linoleoyl chains of CL. The decrease in fluorescence emission corresponded to the disappearance of monomeric form of NAO. Linearity was observed for CL concentrations lower than 10 μ M and for higher concentrations, the green fluorescence intensity was almost stable. This suggests that the NAO-CL interaction was enhanced by the C9 aliphatic tail of the NAO structure.

Positive linearity is obtained from $0 - 20 \ \mu\text{M}$ by using 20 μM PSF (Fig. 2.18). The RSD data (n=3) for 11 points ranged from 0.5% to 2%. The correlation coefficient (R²) was 0.9913 but this dye also provided a low sensitivity compared to NAO-CL. The linear regression equation was F = 4.25[CL] + 267. As shown in Fig. 2.21, increasing amounts of CL may have induced the rigidity of the benzyl substitute group at the quaternary ammonium group of PSF leading to increasing of fluorescence intensity. The hydrophobic effect involving a phenyl ring is weaker than that of a C9 alkyl chain. Any interaction between PSF and CL was weaker than that of AO or NAO with CL because a slit width at 5/5 nm was needed to better detect fluorescence.



Figure 2.17 Visible absorbance curves of various CL concentrations at different concentrations of NAO (10 μ M (- \blacksquare -), and 20 μ M (- \blacklozenge -)) ($\lambda_{abs.} = 497$ nm).



Figure 2.18 Comparison of fluorescence profiles using NAO 20 μ M ($\lambda_{excit}/\lambda_{emiss} = 518/530$ nm, slit width = 2.5/2.5 nm, (- \blacktriangle -)), AO 20 μ M ($\lambda_{excit}/\lambda_{emiss} = 499/524$ nm, slit width = 2.5/2.5 nm, (- \blacksquare -)) and PSF 20 μ M ($\lambda_{excit}/\lambda_{emiss} = 522/570$ nm, slit width = 5/5 nm, (- \blacklozenge -)) as the various concentrations of CL.



Figure 2.19 Predicted interaction of AO and CL.



Figure 2.20 Predicted interaction of NAO and CL.



Figure 2.21 Predicted interaction of PSF and CL.

Determination of CL in Whole Cell and Membrane Samples (standard addition)

Biological samples are complex and sample matrix interference is likely for an analytical method without a separation step. To eliminate this problem, standard addition was introduced for detection. Spiking involved adding a known CL (1 μ M) to the unknown sample before the fluorescence measurement. Results are shown in Table 2.1.

Sample	FI before spike	FI after spike	$\Delta FI_{[NAO-CL(1\mu M)]}$ or	$\Delta FI_{[NAO-CL (1 \mu M)]}/$
	with CL	with CL	$\Delta \mathrm{FI}_{[ext{before spike-after spike}]}$	$\Delta \mathrm{FI}_{[ext{before spike-after spike}]}$
NAO	374.83 (1.58)	-	-	-
CL (1 µM)	-	361.14 (0.79)	13.69 (1.77)	-
A-1	368.61 (2.92)	356.79 (3.81)	11.82 (4.80)	1.16 (0.49)
B-1	367.01 (3.16)	356.79 (0.45)	10.22 (3.19)	1.34 (0.45)
C-1	371.29 (1.35)	354.11 (4.15)	17.18 (4.36)	0.80 (0.23)
D-1	362.77 (3.47)	350.95 (2.09)	11.82 (4.05)	1.16 (0.42)
NAO	440.58 (4.62)	-	-	-
CL (1 µM)	-	404.97 (2.79)	35.61 (5.40)	-
B'-1	409.71 (3.62)	376.66 (4.12)	33.05 (5.48)	1.08 (0.24)
C'-1	411.24 (9.31)	378.03 (6.15)	33.21 (11.16)	1.07 (0.39)
D′-1	397.57 (3.38)	365.50 (4.38)	32.07 (5.53)	1.11 (0.25)
E′-1	396.86 (11.79)	364.82 (6.42)	32.04 (13.43)	1.11 (0.49)
F′-1	406.71 (7.60)	373.87 (4.37)	32.84 (8.77)	1.11 (0.34)

Table 2.1 The fluorescence intensity (FI) values of samples spiked with 1 μM CL and recovery ratio. (*standard deviation in parenthesis - see appendix)

It was found that the difference between ΔFI [NAO-CL(1µM)] and ΔFI [before spikeafter spike] of each sample is not significantly different by using the *t*-test at the 95% confidence level (see appendix), because of the ratios of these ΔF values being quite close to the expected value of 1 (Table 2.1).

Calculation of CL in samples in standard addition

1. Based on fluorescence equation (1)

$$F = 2.3k' sbcP_o \qquad \dots \dots \dots (1)$$

So before spiked sample, the equation is;

$$F_x = k[X_i] \tag{2}$$

where k is constant and X_i is initial concentration of CL in sample And after spiked sample, the equation is;

where k is constant and S_f and X_f are final concentration of known CL and final concentration of CL in sample respectively.

Since fluorescence emission is directly proportional to analyte concentration so we can derive standard addition equation as;

$$\frac{\begin{bmatrix} X_i \end{bmatrix}}{\begin{bmatrix} S_f \end{bmatrix} + \begin{bmatrix} X_f \end{bmatrix}} = \frac{F_x}{F_{s+x}}$$
(4)

We can not use this equation directly because

- 1) CL itself does not fluoresce but NAO does
- 2) Standard curve is negative line
- 3) Intensity of each sample of signal is proportional to;

[NAO] - [CL-sample] - [CL-standard]

So we have to adjust the equation (4) fitted to this situation as equation (5)-(7)

For NAO itself	$F_1 = k [NAO]$	(5)
Before spiked sample	$F_2 = k([NAO] + [X_i])$	
After spiked sample with 1.0 µM CL

Where [NAO] is concentration of NAO (μM)

[X_i] is initial concentration of CL in sample

[X_f] is final concentration of CL in sample

 $[S_f]$ is concentration of known CL (1.0 μ M)

For example, sample B'-1

$$440.58 = k$$
 [NAO](8)

$$409.71 = k ([NAO] + [X_i])$$
(9)

Subtract (8) from (9)

 $-30.87 = k [X_i]$ (11)

Subtract (10) with (8)

$$-63.92 = k([X_f] + [S_f])$$
(12)

Divide (12) by (11)

So

2.071[X_i] = 1 + [X_f], but in this case $[X_i] = [X_f]$

Therefore

1.071[X_i] = 1
[X_i] = 0.934
$$\mu$$
M
Or 0.934 x 1493.92 x 10⁻³ = 1.40 μ g/ml

Table 2.2 shows the concentration of CL in μ M and μ g/ml units for whole cell and membrane extract sample prepared by D. Ekhterae and co-workers. The RSD for CL in the whole cell and membrane samples was about 6% and 18%. Determinations of CL in two samples of control mitochondrial membrane, L5 and L24, and the whole cell were based on standard addition. When the whole cell and the membrane samples were subjected to cell death using staurosporine, the CL concentrations increased but are not significantly different from the control samples except the L24c samples and L24c membrane using the *t*-test (see appendix).

Sample #	Description	[CL] (µM)	[CL] µg/ml
A-1	Nc	0.57 ± 0.21	0.85 ± 0.31
D-1	N+ST	1.05 ± 0.21	1.56 ± 0.31
D′-1	N Membrane + ST	1.36 ± 0.18	2.03 ± 0.27
B-1	L5c	0.77 ± 0.03	1.14 ± 0.05
B′-1	L5c Membrane	0.94 ± 0.11	1.41 ± 0.16
E'-1	L5 Membrane + ST	1.48 ± 0.53	2.21 ± 0.79
C-1	L24c	0.22 ± 0.06	0.32 ± 0.09
C′-1	L24c Membrane	0.90 ± 0.17	1.35 ± 0.25
F′-1	L24 Membrane + ST	1.04 ± 0.13	1.56 ± 0.20

Table 2.2 CL concentration in various cell membrane samples (n = 3).

N = Neo

c = Control

ST = Staurosporine-

L5 = One ARC stable clone

- ARC = apotosis repressor caspase
- L24 = Another ARC stable clone

SECTION 2.4: CONCLUSIONS

The optimum excitation and emission wavelengths are 518 and 530 nm, respectively for NAO in the 20 - 60 µM range. The NAO-CL interaction in 50-50% MeOH-H₂O solution indicated that a hydrophobic effect was likely important due to no significant change in NAO fluorescence by CL (18:2) in a solvent above 50% methanol in water. No significant effect of biological surfactants (PA, PC, PE, PS, SM and cholesterol) on NAO fluorescence in 50-50% MeOH-H₂O was noted. PG seemed to interfere above a 8 µM level. The interaction of excess NAO with CL is rapid and a stable fluorescence signal results providing a linear response to CL. The calibration curve for CL using 20 µM NAO is a linear quenching response from 0.2 to 10 µM. The dominant hydrophobic interaction between NAO and CL plays a more important role than electrostatic interactions. CL could be determined by standard addition in biological samples. In the future, we will synthesize n-alkyl acridine orange derivatives with different long chain aliphatic tails (C14 and C18) by n-alkyl bromide with acridine orange (34-38). Analogous fluorescence studies reaction of with CL will be made and compared to those found using the NAO dye (C9). The expected stronger hydrophobic interaction with the C18 or C14 -acridine orange dye with CL as well as use of a microcuvette or flow cell to minimize sample dilution should improve the detection limit.

REFERENCES

- Mileykovskaya, E., Dowhan, W., Birke, R. L., Zheng, D., Lutterodt, L. Haines, T. H. "Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces" (2001) *FEBS Lett.* 507, 187-190.
- 2. Haines, T. H., Dencher, N. A. "Cardiolipin: a proton trap for oxidative phosphorylation" (2000) *FEBS Lett.* **528**, 35-39.
- Wright, M. M., Howe, A. G., Zaremberg, V. "Cell membranes and apoptosis: role of cardiolipin, phosphatidylcholine, and anticancer lipid analogues" (2004) *Biochem. Cell Biol.* 82, 18-26.
- McMillin, J. B., Dowhan, W. "Cardiolipin and apoptosis" (2002) *Biochim. Biophys. Acta*. 1585, 97-107.
- Zhao, K., Zhao, G. M., Wu, D., Soong, Y., Birk, A. V., Schiller, P. W., Szeto, H. H. "Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury" (2004) *J. Biol. Chem.* 279, 34682-34690.
- 6. Iverson, S. L., Orrenius, S. "The cardiolipin-cytochrome *c* interaction and the mitochondrial regulation of apoptosis" (2004) *Arch. Biochem. Biophys.* **423**, 37-46.
- Petrosillo, G., Ruggiero, F. M., Paradies, G. "Role of reactive oxygen species and cardiolipin in the release of cytochrome *c* from mitochondria" (2003) *FASEB J.* 17, 2202-2208.
- Nomura, K., Imai, H., Koumura, T., Kobayashi, T., Nakagawa, Y. "Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome *c* from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-indued apoptosis" (2000) *Biochem. J.* 351, 183-193.
- Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X. S., Wang, X. "Cardiolipin provides specificity for targeting of tbid to mitochondria" (2002) *Nat. Cell Biol.* 2, 754-756.

- Fernandez, M. G., Troiano, L., Moretti, L., Nasi, M., Pinti, M., Salvioli, S., Dobrucki, J., Cossarizza, A. "Early changes in intramitochondrial cardiolipin distribution during apoptosis" (2002) *Cell Growth & Differentiation*. 13, 449-455.
- Kirkland, R. A., Adibhatla, R. M., Hatcher, J. F., Franklin, J. L. "Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy" (2002) *Neuroscience* 115, 587-602.
- Ostrander, D. B., Sparagna, G. C. Amoscato, A. A., McMillin, J. B., Dowhan, W.
 "Decreased cardiolipin synthesis corresponds with cytochrome *c* release in palmitate-induced cariomyocyte apoptosis" (2001) *J. Biol. Chem.* 276, 38061-38067.
- Fernandez, M. G., Troiano, L., Moretti, L., Pedrazzi, J., Salvioli, S., Cortazar, I. C., Cossarizza, A. "Changes in intramitochondrial cardiolipin distribution in apoptosisresistant HCW-2 cells, derived from the human promyelocytic leukemia HL-60" (2000) *FEBS Lett.* 478, 290-294.
- Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., Greenberg, M. L. "Absence of cardiolipin in the *crd1* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function" (2000) *J. Biol. Chem.* 275, 22387-22394.
- 15. Petrosillo, G., Ruggiero, F. M., Venosa, N. D., Paradies, G. "Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin in" (2003) *FASEB J.* 17, 714-716.
- Lesnefsky, E. J., Chen, Q., Moghaddas, S., Hassan, M. O., Tandler, B., Hoppel, C. L.
 "Blockade of electron transport during ischemia protects cardiac mitochondria" (2004) *J. Biol. Chem.* 270, 47961-47967.
- Cvekovic, J. T., Jonsson, S. W., Ahmed, E., Dahlqvist, R. S., Lefvert, A. K.
 "Increased levels of autoantibodies against copper-oxidized low density lipoprotein, malondialdehyde-modified low density lipoprotein and cardiolipin in patients with rheumatoid arthritis" (2002) *Rheumatology* 41, 988-995.
- Grant, G. T., Parr, T. "Decline of life's energy theory of ageing 1. revitalization of energy metabolism and ageing mitochondria" (2000) *Exp. Opin. Ther. Patents* 10, 1-11.

- 19. Wang, Y., Krull, I. S., Liu, C., Orr, J. D. "Derivatization of phospholipids" (2003) *J. Chromatogr. B.* **793**, 3-14.
- Caudron, E., Zhou, J, Y., Chaminade, P., Baillet, A., Prognon, P. "Fluorescence probe assisted post-column detection for lipid analysis in microbore-LC" (2005) *J. Chromatogr. A.* 1072, 149-157.
- 21. Zhou, J. Y., Chaminade, P., Gaudin, K., Prognon, P., Baillet, A., Ferrier, D. "Postcolumn fluorescence as an alternative to evaporative light scattering detection for ceramide analysis with gradient elution in non-aqueous reverse-phase liquid chromatography" (1999) J. Chromatogr. A. 859, 99-105.
- 22. Bhowmik, B. B., Nandy, P. "Critical liposome concentration of phospholipids from spectral studied" (1996) *J. Surface Sci. Technol.* **12**, 67-73.
- Petit, J. M., Maftah, A., Rantinaud, M. H., Julien, R. "Direct cardiolipin assay in yeast using the red fluorescence emisstion of 10-*N*-nonyl acridine orange" (1995) *Eur. J. Biochem.* 228, 113-119.
- Gohil, V. M., Jeremic, J. G., Schlame, M., Greenberg, M. L. "Binding of 10-N-nonyl acridine orange to cardiolipin-deficient yeast cells: implications for assay of cardiolipin" (2005) *Anal. Biochem.* 343, 350-352.
- 25. Fernandez, M. G., Ceccarelli, D., Muscatello, U. "Use of the fluorescence dye 10-*N*-nonyl acridine orange in quantitative and location assays of cardiolipin: a study on different experimental models" (2004) *Anal. Biochem.* **328**, 174-180.
- Petit, J. M., Huet, O., Gallet, P. F., Maftah, A., Rantinaud, M. H., Julien, R. "Direct analysis and significance of cardiolipin transverse didstribution in mitochondrial inner membranes" (1994) *Eur. J. Biochem.* 220, 871-879.
- Petit, J. M., Maftah, A., Rantinaud, M. H., Julien, R. "10-N-Nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria" (1992) *Eur. J. Biochem.* 209, 267-273.
- Maftah, A., Petit, J. M., Julien, R. "Specific interaction of the new fluorescent dye 10-N-nonyl acridine orange with inner mitochondrial membrane" (1990) *FEBS Lett.* 260, 236-240.

- Fuller, K. M., Duffy, C. F., Arriage, E. A. "Detection of the cardiolipin content of individual mitochondria by capillary electrophoresis with laser-induced fluorescence detection" (2002) *Electrophoresis* 23, 1571-1576.
- 30. Qi, L., Danielson, N. D., Dai, Q., Lee, R. M. "Capillary electrophoresis of cardiolipin with on-line dye interaction and spectrophotometric detection" (2003) *Electrophoresis* 24, 1680-1686.
- 31. Yunjing, L., Hanxi, S. "Study on acridine orange dimer as a new fluorescent probe for the determination of protein" (1999) *Anal. Commun.* **36**, 135-137.
- Miyoshi, N., Hara, K., Yokoyama, I., Tomita, G., Fukuda, M. "Fluorescence life time of acridine orange in sodium dodecyl sulfate premicellar solutions" (1988) *Photochem. Phobiol.* 47, 685-688.
- 33. Ban, K., Kasatani, K., Kawasaki, M., Sato, H. "Fluorescence decay of acridine orange-sodium dodecyl sulfate system: formation of dye-rich induced micelles in the premicellar region" (1983) *Photochem. Phobiol.* 37, 131-139.
- 34. Yamagishi, A., Masui, T., Watanabe, F. "Selection activation of reactant molecule by reversed micelles" (1981) *J. Phys. Chem.* **85**. 281-285.
- Miethke, E., Zanker, V. "Lumineszenzmessungen an verschiedenen acridinorange derivaten" (1958) Z. Phys. Chem. (Frankfurt am Main) 18, 375-390.
- James, A. D., Robinson, B. H. "Self-aggregation of N(10)-alkyl derivatives of acridine orange and their interaction with cationic and anionic surfactants" (1976) *Adv. Mol. Relax. Processes* 8, 287-304.
- Murad, M. M. "Fluorescence analysis of acridine orange adsorbate at the water/N-Heptane interface, bulk and interface" (1999) *J. Fluo.* 9, 257-263.
- 38. Robinson, B. H., White, N. C., Mateo, C. "Dynamics of small molecule-micelle interactions. A stopped-flow investigation of the kinetics of absorption of acridine (and related) dyes into anionic mecelles, and the characterization of surfactant-dye interactions" (1975) *Adv. Mol. Relax. Processes* 7, 321-338.

APPENDIX

(1) Limit of Detection (LOD) or Detection limit (DL)

$$DL = \frac{3.3 \times SD}{-S}$$
 For a negative slope

where: SD = the standard deviation of the response (blank) S = the slope of the calibration curve

So
$$DL = \frac{3.3 \times 1.584}{-(-26.894)} = 0.194 \ \mu\text{M CL} \approx 0.20 \ \mu\text{M CL}$$

(2) Limit of Quantitation (LOQ)

Г

$$LOQ = \frac{10 \times SD}{-S}$$
 For a negative slope

So
$$LOQ = \frac{10 \times 1.584}{-(-26.894)} = 0.589 \ \mu\text{M CL} \approx 0.60 \ \mu\text{M CL}$$

ΔFI [before spike-after spike]	ΔFI [NAO-CL (1μM)] /	
	ΔFI [before spike-after spike]	
If $y = a + b + c$, then	If $y = ab/c$	
$s_y = \sqrt{s_a^2 + s_b^2 + s_c^2}$	$\frac{s_y}{y} = \sqrt{\left(\frac{s_a}{a}\right)^2 + \left(\frac{s_b}{b}\right)^2 + \left(\frac{s_c}{c}\right)^2}$	
$s_y = \sqrt{(2.92)^2 + (3.81)^2} = 4.80$	y=13.69/11.82=1.158	
	$\frac{s_y}{1.158} = \sqrt{\left(\frac{1.77}{13.69}\right)^2 + \left(\frac{4.80}{11.82}\right)^2}$	
	$s_y = 1.158 \times 0.494 = 0.49$	

 $\label{eq:Example of calculation of standard deviation (s) of ΔFI [before spike-after spike]$ and ratio of ΔFI [NAO-CL (1 \mu M)] / ΔFI [before spike-after spike]$ of samples A-1$ }$

Sample A-1	Sample E'-1			
Degree of freedom = $n_1 + n_2 - 2 = 3 + 3 - 2 = 4$				
$t_{calculated} = \frac{\left \overline{x_1 - \overline{x}_2}\right }{s_{pooled}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$				
$s_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}}$				
$s_{pooled} = \sqrt{\frac{2(1.77)^2 + 2(4.80)^2}{4}} = 3.618$	$s_{pooled} = \sqrt{\frac{2(5.40)^2 + 2(13.43)^2}{4}} = 10.235$			
$t_{calculated} = \frac{ 13.69 - 11.82 }{3.618} \sqrt{\frac{9}{6}} = 0.633$	$t_{calculated} = \frac{ 35.61 - 32.04 }{10.235} \sqrt{\frac{9}{6}} = 0.427$			

Example of calculation of *t*-test between the difference of Δ FI [NAO-CL(1µM)] and Δ FI [before spike-after spike] of samples A-1 and E'-1

Sample #	Description	t-test
A-1	Nc	0.633
B-1	L5c	1.647
C-1	L24c	1.285
D-1	N+ST	0.733
B'-1	L5c Membrane	0.576
C'-1	L24c Membrane	0.335
D'-1	N Membrane + ST	0.793
E'-1	L5 Membrane + ST	0.427
F'-1	L24 Membrane + ST	0.466

t-test between the difference of Δ FI [NAO-CL(1 μ M)] and Δ FI [before spike-after spike] of each sample.

 $t_{\text{table}} = 2.776$ since all of $t_{\text{calculated}} < t_{\text{table}}$, so there are not significant difference between $\Delta \text{FI}_{[\text{NAO-CL}(1\mu\text{M})]}$ and $\Delta \text{FI}_{[\text{before spike-after spike]}}$ at 95 % confidence level.

Calculation of *t*-test between control and the membrane samples subjected to cell death using staurosporine.

Samples B'-1 and E'-1	Samples C'-1 and F'-1			
Degree of freedom = $n_1 + n_2 - 2 = 3 + 3 - 2 = 4$				
$t_{calculated} = \frac{\left \overline{x_1 - x_2}\right }{s_{pooled}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$				
$s_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}}$				
$s_{pooled} = \sqrt{\frac{2(0.11)^2 + 2(0.53)^2}{4}} = 0.383$	$s_{pooled} = \sqrt{\frac{2(0.17)^2 + 2(0.13)^2}{4}} = 0.151$			
$t_{calculated} = \frac{ 0.94 - 1.48 }{0.383} \sqrt{\frac{9}{6}} = 1.728$	$t_{calculated} = \frac{ 0.90 - 1.04 }{0.151} \sqrt{\frac{9}{6}} = 1.133$			

Sample Description		t-test
A-1 and D-1	Nc and N + ST	2.766
D-1 and D'-1	N + ST and N Membrane + ST	1.941
B-1 and B'-1	L5c + L5c Membrane	2.583
B'-1 and $E'-1$	L5c Membrane and L5 Membrane + ST	1.728
C-1 and C'-1	L24c + L24c Membrane	6.533
C'-1 and F'-1	L24c Membrane and L24 Membrane + ST	1.133

t-test between control and each samples subjected to cell death using staurosporine.

 $t_{\text{table}} = 2.776$ and since all of $t_{\text{calculated}} < t_{\text{table}}$ for membrane samples so the difference is not significant at 95 % confidence, but $t_{\text{calculated}} > t_{\text{table}}$ for L24 sample for the whole cell and membrane so the difference is significant at 95 % confidence level.