THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Analysis of phospholipids in cellular membranes with LC and imaging mass spectrometry

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Cover picture; Part of Figure 10. *A TOF SIMS image of a cluster of PC12 cells showing K^+ in red, PC in green, and PE in blue.*

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ABSTRACT

Imaging mass spectrometry enables the creation of molecule specific images from the surface of a solid sample in vacuum. To solve the issue of bringing single cells into vacuum without altering their native distribution of molecules, a freeze fracture device that fits the time of flight secondary ion mass spectrometry (TOF-SIMS) IV instrument has been developed. This makes it possible to get a snapshot of the chemical distribution across frozen hydrated single cells that are only 10-20 µm in diameter. The cells of interest in this thesis are rat pheochromocytoma (PC12) cells. PC12 cells resemble and act like neurons in the sense that upon stimulation they release dopamine, which is a substance used for communication between neurons. In previous studies using these model cells, the rate of this release has been shown to change after the cells have been incubated with different phospholipids. To investigate the amount of phospholipids that have accumulated in the plasma membrane of PC12 cells after an overnight incubation, the combination of the freeze fracture device and the TOF-SIMS IV instrument was utilized. Relative to the endogenous phospholipid the results show that 0.5% of phosphatidylcholine (PC) and 1.3% of phosphatidylethanolamine (PE) had accumulated in the plasma membrane. Together with previous results on changes in the release of dopamine in PC12 cells, this suggests that the phospholipid composition of the plasma membrane of neurons is highly regulated. This gives a hint as to the importance of phospholipids during this highly important cellular process.

The technique of liquid chromatography (LC) mass spectrometry (MS) does not provide molecular information in images but has the ability to separate similar molecules in a sample. This is of high importance when analyzing a specific molecule in a complex sample. Anaerobic ammonium oxidizing (anammox) bacteria reside in sediment on the ocean floor. These bacteria are highly important to the environment because they convert biologically available nitrogen into dinitrogen gas (N2), which is returned to the atmosphere. By denitrifying biologically available nitrogen they limit the risk of over fertilization in the ocean. They are also believed to contribute greatly to the global N2 production. By combining LCMS with an extensive sample clean up procedure a phospholipid biomarker for viable anammox bacteria has been used to detect the location of anammox bacteria in a sediment core sample.

Keywords: Mass spectrometry, TOF-SIMS, phospholipid, cells, anammox

Populärvetenskaplig sammanfattning på svenska

Enskilda mänskliga celler är små, endast en eller två hundradels millimeter breda. Det gör att det krävs speciell mätutrustning för att kunna urskilja olika delar inuti cellen. Vanligtvis används ett mikroskop som förstorar cellen och visar dess konturer, men ett mikroskop ger ingen information om de kemiska molekyler som finns i cellen och på dess yta. I denna avhandling presenteras en provhållare som tillsammans med ett speciellt mätinstrument kan avbilda de kemiska molekyler som finns på ytan av en enskild cell. Provhållaren laddas med celler i lösning och fryses sedan snabbt ner till -196°C. Den snabba nedfrysningen av cellerna och deras innehåll gör att man får en ögonblicksbild av var de olika kemiska molekylerna befann sig i cellen vid tidpunkten för nedfrysning. Med den här tekniken kan man skapa bilder där man inte bara ser cellens konturer utan även vilka molekyler som finns och var de befinner sig i cellen.

Varför vill man då veta vilka kemiska molekyler som finns var i en cell? Jo, för att cellen är den minsta levande komponent som finns och de kemiska processer som sker där har stor betydelse för hur cellen fungerar i vår kropp. Som exempel behöver vår hjärna särskilda celler som kan kommunicera med varandra genom kemiska processer. Denna livsnödvändiga kommunikation har visat sig vara beroende av de kemiska molekyler som finns i cellens membran. Genom att avbilda av de kemiska molekylerna i en enskild cells membran kan man mäta förändringar. Resultat i denna avhandling, tillsammans med tidigare resultat, visar att kommunikationshastigheten hos de studerade cellerna påverkas av att en viss molekyl i membranet förändras med knappt en procent. Det antyder att kommunikationen mellan celler i hjärnan är starkt beroende av den kemiska sammansättningen i den enskilda cellens membran. Detta resultat kan vara en del i pusslet för vidare forskning om att förstå mekanismerna bakom hur vi lär oss och hur vi minns.

I avhandlingen beskrivs också en ny metod för hur särskilda kemiska molekyler i kombination med speciell mätutrustning används för att hitta bakterier som lever på havsbotten. De eftersökta bakterierna är viktiga för naturen eftersom de motverkar både döda havsbottnar och övergödning i havet. Den framtagna metoden gör det möjligt att följa på vilket djup och var på havsbotten dessa bakterier lever.

Part A

Part B

Research papers

I - Time of flight mass spectrometry imaging of samples fractured in situ with a spring-loaded trap system. **Lanekoff I**, Kurczy ME, Hill R, Fletcher JS, Vickerman JC, Winograd N, Sjövall P, Ewing AG. Anal Chem 2010 (82) 6652-6659

II - An in situ fracture device to image lipids in single cells using TOF SIMS. **Lanekoff I**, Kurczy ME, Adams KL, Malm J, Karlsson R, Sjövall P, Ewing AG. Surface and Int. Sci. 2011 (43) 257-260

III – Relative quantification of phospholipid accumulation in the PC12 cell plasma membrane following phospholipid incubation using TOF-SIMS Imaging. **Lanekoff I**, Sjövall P, Ewing AG. Anal Chem In press

IV – Analysis of intact ladderane phospholipids, originating from viable anammox bacteria, using RP-LC-ESI-MS. **Lanekoff I**, Karlsson R. Anal and Bioanal Chem 2010 (397) 3543-3551

Contribution report

I – Planned and performed experiments in collaboration with M. Kurczy and P. Sjövall. Responsible for data analysis and for writing the paper.

II - Planned and performed experiments in collaboration with M. Kurczy and P. Sjövall. Responsible for data analysis and for writing the paper.

III - Planned and performed experiments in collaboration with P. Sjövall. Responsible for data analysis and for writing the paper.

IV – Planned and performed experiments. Responsible for data analysis and for writing the paper.

PUBLISHED PAPER NOT INCLUDED IN THIS THESIS

HAMLET interacts with lipid membranes and perturbs their structure and integrity. Mossberg AK[1], Puchades M[2], Halskau Ø[3], Baumann A[3], **Lanekoff I**[2], Chao Y[4], Martinez A[3], Svanborg C[1,4], Karlsson R[2]. PLOS ONE 2010 (5)

LIST OF ABBREVIATIONS

ANAMMOX – ANaerobic AMMonium OXidizing
Ar – Argon
Bi – Bismuth
CE – Capillary Electrophoresis
CID – Collision Induced Dissociation
Da – Dalton
DCM – Dichloromethane
ESI – Electro Spray Ionization
FID – Flame Ionization Detector
GC – Gas Chromatography
IPA – Isopropanol
LC – Liquid Chromatography
LLE – Liquid Liquid Extraction
LMIG – Liquid Metal Ion Gun
LN2 – Liquid Nitrogen
ms – millisecond
m/z – mass to charge ratio
MeOH – Methanol
MS – Mass Spectrometry
NH4Ac – Ammonium acetate
N2 – Dinitrogen gas
nm – nanometer
PC – Phosphatidylcholine
PC12 – Rat pheochromocytoma cells
PE – Phosphatidylethanolamine
PG - Phosphatidylglycerol
PS – Phosphatidylserine
Q – Quadrupole
QqQ – Triple quadrupole
QTOF – Quadrupole – Time Of Flight
Rf – Radiofrequency
RP – Reversed Phase
SM – Sphingomyeline
SPE – Solid Phase Extraction
Srm – Single reaction monitoring
TOF-SIMS – Time Of Flight Secondary Ion Mass Spectrometry

1 - Introduction

In this thesis mass spectrometry is used to analyze phospholipids originating from cellular membranes in eukaryotic and prokaryotic cells. The first step in mass spectrometry analysis is to convert molecules in the sample to ions. This can be done in several ways and in Papers I-III the impact of primary ions is used while electrospray ionization is used in Paper IV. The ions created from the sample are then brought into a mass analyzer where they are separated based on their mass to charge ratio, *m/z*. Mass spectrometry enables the analysis of ionized sample molecules with almost identical masses. This ability to detect similar ions is a key feature when complex biological samples are analyzed. The work in this thesis has had two main aims that are briefly described below;

A) The chemical processes within a single cell are many and highly important for the function of the cell. The ability to perform single cell analysis is therefore desirable to further understand the organization of individual molecules and parts within the cell. Cellular interactions also create functions in the body, organs and tissue making them vital in health and disease. Despite the complexity of the chemical processes within and between single cells, every cell is also more or less affected by the molecules surrounding it. An aim of this thesis was to enable a snapshot analysis of the chemical distribution within a single cell using imaging mass spectrometry and to further use this to quantify the accumulation of exogenous phospholipids in the plasma membrane of single cells.

B) A second aim in the thesis involved the marine environment. Many molecules such as decomposition products from dead biological material and manmade molecules from waste water end up in the ocean sediment. These sediments are also the home of many living organisms and bacteria, making the number of available molecules for analysis extraordinarily large. Thus, the specific aim of this part of the thesis was to develop a method using the specificity of a combined liquid chromatography (LC) and mass spectrometry (MS) method to identify a specific bacterium in ocean sediment with a phospholipid biomarker.

2 - Biological cells

2.1 The cell

The cell is the basic unit of life and the body of a full grown human consists of about 100 000 billion eukaryotic cells. For the human body to work properly there is also 1-2.2 kg of prokaryotic cells, bacteria, mostly working in the large intestine. Prokaryotic cells are small, only between 1 and 2 µm in diameter, and they usually have all their molecular content free floating in the cytoplasm. The cell body of a eukaryotic cell is larger, about 10-30 µm in diameter. Inside the cytoplasm of the eukaryotic cell there are many different kinds of organelles as shown in the schematic in Figure 1A. These organelles perform specific tasks making it possible for the cell to survive, procreate and communicate. Some organelles shown in Figure 1A are a) the mitochondria which is responsible for the energy production of the cell; c) the endoplasmatic reticulum which is a site for protein and lipid synthesis and; d) the nucleus which contains the cell's genetic information, its DNA; e) the golgi apparatus which packages and transports synthesized proteins to the appropriate site within the cell. This schematic figure might give the impression that a large part of the cell is empty, consisting only of water. However, this is not the case. The cytosol surrounding the organelles in the cell body is filled with macromolecules, small molecules and metabolites. It has a highly complex structure and processes such as transport, synthesis and metabolomics of molecules readily take place.

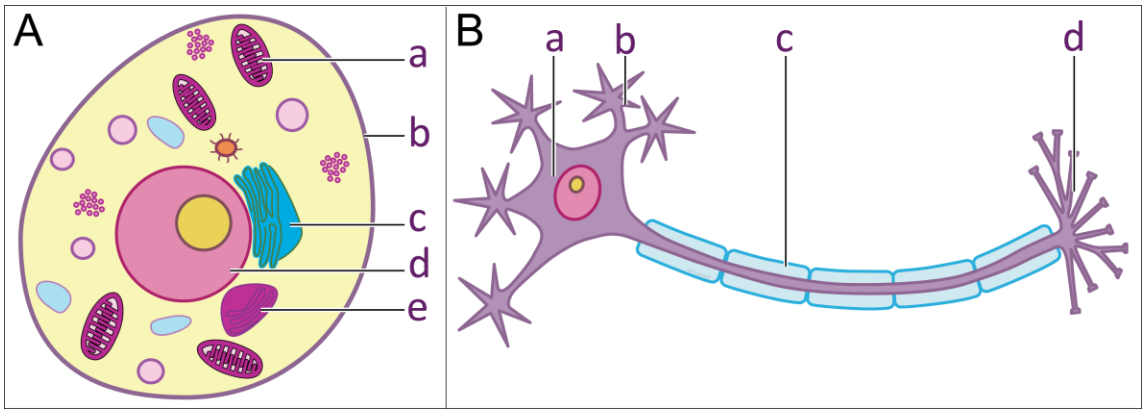

***Figure 1**. **A**) Schematic of a cross section of a cell: a) mitochondria, b) plasma membrane, c) endoplasmatic reticulum, d) nucleus, e) golgi apparatus. **B**) Schematic of a neuron: a) cell body, b) dendrites, c) myelinated axon, d) axon terminal with synapses.*

2.1.1 NEURONS

In the central nervous system of the human body the neurons, nerve cells, are responsible for receiving, transmitting and sending nerve impulses. A nerve impulse consists of a wave of electrical excitation, action potential, which travels along the cell by depolarization of the plasma membrane (b in Figure 1A). A typical neuron can be divided into three parts; cell body, dendrite and axon (a, b and c in Figure 1B). In the simplest models, nerve cells have receptors on their dendrites that receive a signal in the form of a neurotransmitter. This signal is then converted into an action potential which is transmitted by the axon to the axon terminal (d in Figure 1B). At the axon terminal a cascade of molecular events occurs leading the synaptic vesicles to release their neurotransmitters outside the cell. Receptors at the dendrite of the next cell in the communication chain will then recognize these neurotransmitters and the signal can be passed on further. The release of neurotransmitters, to carry out chemical signaling at the axon terminals, is generally thought to occur by a process called exocytosis and will be discussed further in section 3.1.1. An axon can be up to 1 meter long and transmit the signal at a speed of 100 m/s [1]. In order to pass the signal through the axon at this high speed most axons, at least in higher systems, are insulated with a myelin sheath. Myelination is performed by glial cells, called oligodendrocytes, which wrap themselves around the axon in several layers (c in Figure 1B).

2.2 Biological cell membranes

Every cell is surrounded by a plasma membrane that encloses and separates the cell's interior from the surrounding environment (b in Figure 1A). The plasma membrane is constructed mostly from lipids and proteins in a thin, about 5 nm, and fluid film, as shown in the schematic in Figure 2. Molecules can be transported from the outside to the inside of the cell, and vice versa, either by indirect transport through the plasma membrane or by protein mediated transport using channels (c in Figure 2) or carriers (d in Figure 2). A typical plasma membrane has an approximately 50:50 relationship in mass between lipids and proteins, although this can vary substantially. Some proteins in the plasma membrane are thought to require specific lipids to function properly and lipid rafts or domains of about 70 nm in diameter of certain lipids have been predicted to be embedded in the plasma membrane [1].

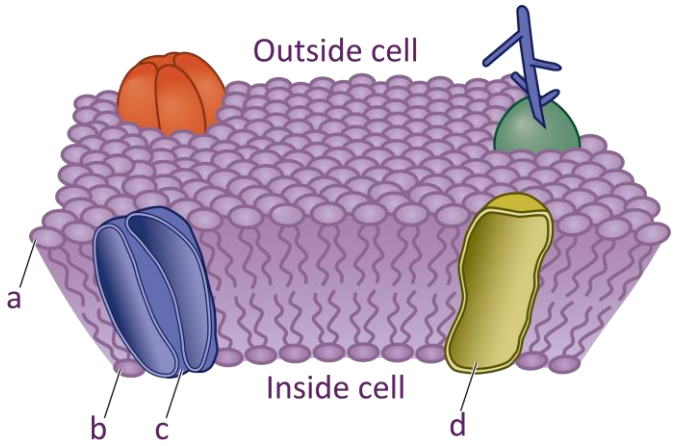

Figure 2*. Schematic of the plasma membrane a) phospholipids in the outer leaflet b) phospholipids in the inner leaflet c) protein channel d) protein carrier*

The most abundant lipids in the membrane are phospholipids (section 2.2.1). Phospholipids are organized in a bilayer in the membrane with the polar head groups facing the water phase and the hydrophobic tail groups facing each other (Figure 2). This organization minimizes the cost of free energy. The two leaflets in the phospholipid bilayer are asymmetric and the outer leaflet (a in Figure 2) has been shown to be comprised mainly of phosphatidylcholine (PC) and sphingomyelin (SM) while phosphatidylethanolamine (PE) and phosphatidylserine (PS) are almost exclusively located in the inner leaflet (b in Figure 2). This reflects the different functions of the two leaflets [2].

Membranes are also used as boundaries for organelles in the cytoplasm separating the interior of the organelle from the cytosol. Depending on the task of the organelle the membrane proteins and the composition of phospholipids are altered. In eukaryotic membranes there are also large amounts of cholesterol that can change the fluidity of the membrane and glycolipids with sugar residues that are located on the outside of the membrane. Prokaryotic membranes are often composed of one main type of phospholipid.

The processes of the membrane and the importance of specific lipids and proteins are largely unknown and are subject to many studies. To visualize the distribution of lipids in a single cell, fluorescent lipids have been developed. These can then be specifically followed using fluorescent microscopy. However, even though these fluorescent lipids mimic native lipids the cellular metabolism recognizes and treats them as if they are unique [3]. To study the abundance of certain native lipids in cells liquid chromatography (LC) combined with mass spectrometry (MS) is often used. However, in such studies the molecules in the cell are mixed together before analysis causing the localization in the cell to be unknown [4]. By instead using imaging mass spectrometry, Ostrowski et al were able to image the distribution of native phospholipids in mating *Tetrahymena* cells [5]. They discovered that as the pores formed during mating, the abundance of PC was decreased relative to the rest of the cell body. Further studies concluded that the change in phospholipid distribution occurred after the cells had started to mate, hence the change in composition was caused by structural changes brought about by the mating process [6].

2.2.1 PHOSPHOLIPIDS

Phospholipids are amphiphilic molecules, meaning that the molecule has both a hydrophilic and a hydrophobic part. As shown in Figure 3 a phospholipid molecule is divided into three parts; a head group, a glycerol backbone and two fatty acid tail groups. The most common phospholipid head groups in the plasma membrane of eukaryotic cells are; PC, PE and PS.

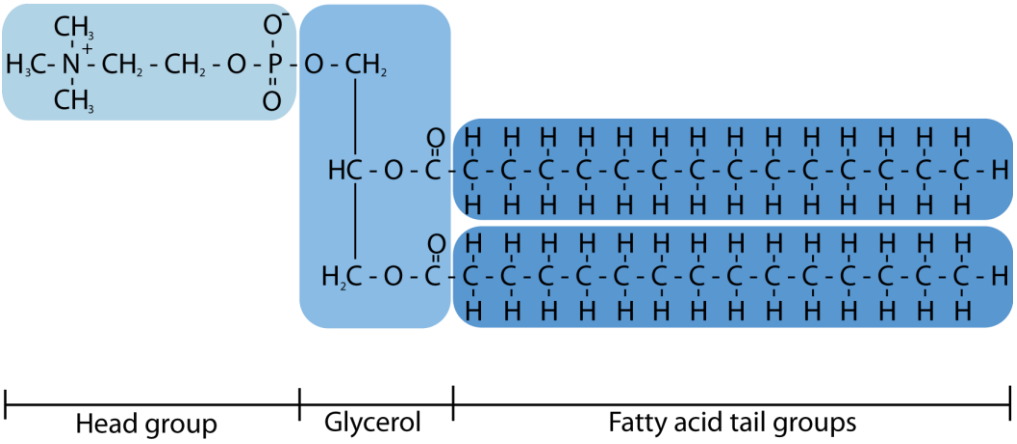

***Figure 3**. Schematic structure of the three parts in a phospholipid molecule, here shown as PC.*

The polar head group is the hydrophilic part of the molecule and the two hydrocarbon tail groups comprise the hydrophobic part. By combining tail groups, with a variety in length and number of unsaturated bonds, with different head groups, an almost endless number of phospholipid molecules with different properties can be formed. This diversity in phospholipids might be a way for the cell to regulate membrane shape, fluidity and permeability which can be important in specific cellular processes.

3 - Phospholipids in the plasma membrane

3.1 PC12 cells

Rat pheochromocytoma (PC12) cells originate from a tumor in the adrenal medulla of a rat [7]. The adrenal medulla is the core of the adrenal gland which is located on top of the kidney. The cells in the gland are in direct contact with the central nervous system and they release epinephrine and norepinephrine directly into the blood upon physiological, emotional or psychological stimulation [8]. The immortalized PC12 cells are easily cultured. They synthesize, store and release dopamine upon stimulation with potassium, but they lack the cellular machinery to make epinephrine and norepinephrine efficiently. The release of dopamine from single PC12 cells can be studied using amperometry [9]. In amperometry a carbon fiber electrode, which is held at a potential of 700 mV versus an Ag/AgCl reference electrode, is placed on top of a cell. When the cell releases the electroactive substance dopamine it is oxidized to orthoquinone at the carbon fiber electrode. This oxidation reaction results in two electrons being transferred for each molecule oxidized. The electrons create a current through the carbon fiber and as a result a peak will be seen in the amperometric trace. Due to the PC12 cells ability to release dopamine they are suitable to use as model cells to study the neurobiological process called exocytosis [10].

3.1.1 Phospholipids in exocytosis

Exocytosis is a process that enables chemical communication between neuronal cells. Figure 4A shows a schematic of an axon releasing dopamine to a receiving dendrite. This release is generally caused by a nerve impulse. Vesicles filled with dopamine, secretory vesicles, will fuse with the plasma membrane of the axon and release dopamine molecules into the junction between the axon and the dendrite. Receptors located on the dendrite will bind the dopamine molecules, resulting in a change of membrane potential and a modification of the signals in the cell. These can propagate the signal forward through the cell by depolarization of the cell membrane or can inhibit the cell from spontaneous or other stimulated signaling. The high content of phospholipids in both the plasma membrane (section 2.2) and in the secretory vesicles raises the question of the role phospholipids play in exocytosis. In Figure 4B a schematic is shown of the presumed orientation of phospholipids during vesicle fusion with the plasma membrane. During the fusion process regions of high curvature are being formed. Phospholipids can have different shapes depending on the size of the head group in relation to the size of the tail groups. Depending on the overall shape of the phospholipid molecule it might have a better or worse fit in these high curvature regions. Comparing PC and PE, PE has a more conical shape due to the smaller head group. PC can instead be said to have a more cylindrical shape, as shown in Figure 4C [3]. Considering the cylindrical shape of PC and the conical shape of PE it is likely that the conical shape would fit better into the high curvature regions of the membrane. In Figure 4D this is shown by the green and blue structures representing PC and PE respectively.

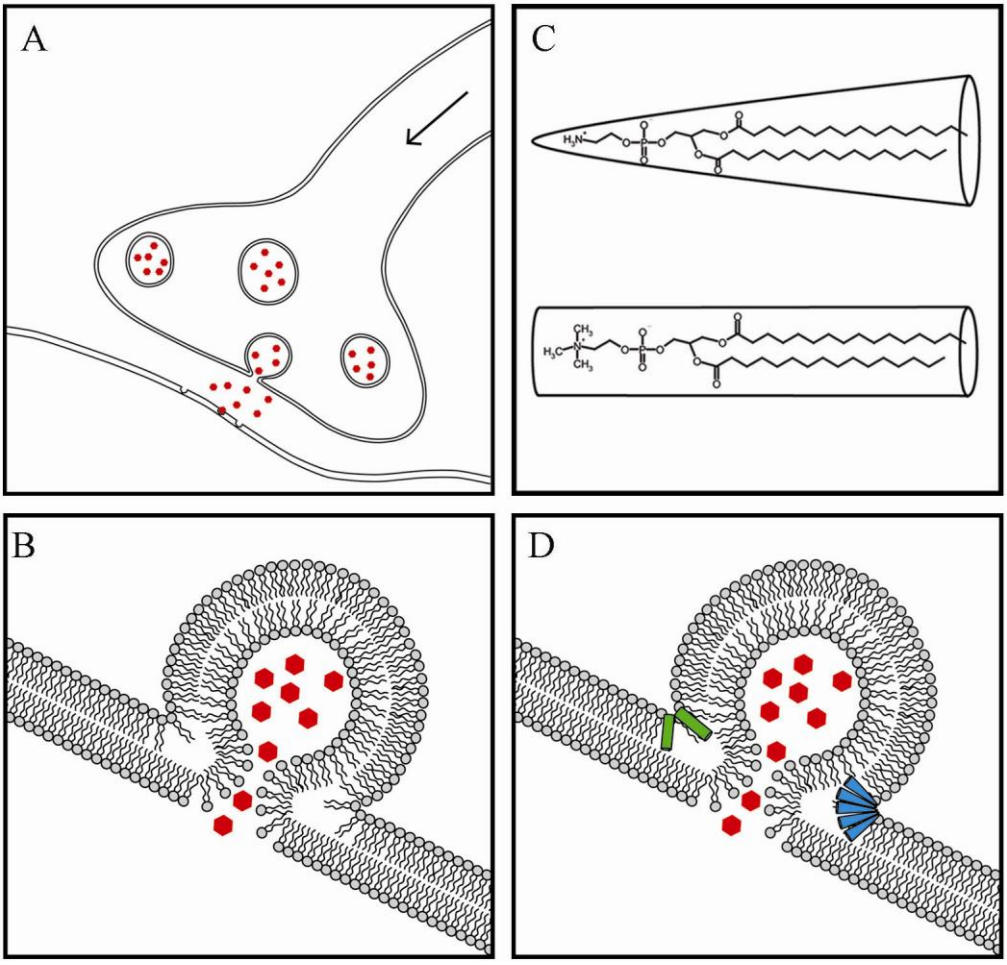

Figure 4*. The role of phospholipids in exocytosis.* ***A****) Schematic of an axon with secretory vesicle filled with dopamine and an exocytosis event releasing dopamine to be bound to receptors on the dendrite.* ***B****) The orientation of phospholipids during the exocytosis event.* ***C****) The molecular shape of PE is shown on top and PC on the bottom.* ***D****) The orientation of cylindrical and conical phospholipids during the event of exocytosis.*

3.1.2 Incubation studies

The importance of specific lipids present during exocytosis has been studied using PC12 cells [11] and chromaffin cells [12]. Amatore et al. incubated chromaffin cells with the inverted conically shaped lysophosphatidylcholine and the cylindrically shaped arachidonic acid prior to monitoring the exocytosis events using amperometry. Their study concluded that both the shape of the lipid molecule and its place in the membrane were of high importance for the dynamics of exocytosis [12]. Uchiyama et al. incubated PC12 cells with different phospholipids prior to monitoring the exocytosis events using amperometry. They concluded that the PC12 cells incubated with PE had a faster release of dopamine than non incubated cells and that PC12 cells incubated with PC had a slower release [11]. These results indicate that the conically shaped PE would have a better fit than PC in the high curvature regions formed during the exocytosis event. Both studies concluded that specific lipids are important in the membrane during exocytosis. However, the actual amount of exogenous lipid present in the plasma membrane of the cells after incubation was not known. For the purpose of better understanding the result of these studies it is important to know the concentration of exogenous lipid in the membrane after incubation. A high concentration in the membrane would indicate that the change in lipid composition in biological systems has to be extreme in order to change the dynamics of exocytosis. On the other hand a low concentration would indicate that the composition of phospholipids in the plasma membrane is of high importance for the process of exocytosis. A small change might be more biologically relevant and might indicate that the change in composition of phospholipids could be a way for the cell to control communication to other cells. It is therefore crucial to know the actual concentration of exogenous lipid in the plasma membrane post incubation. The quantification of exogenous phospholipids incorporated into the plasma membrane of PC12 cells using TOF-SIMS (section 3.2) is performed and discussed in Paper III. It shows that the accumulation of exogenous phospholipids in the plasma membrane of PC12 cells is small, only 0.5 % for PC and 1.3 % for PE at 100 µM concentration during incubation. This is the same concentration that was used in the studies by Uchiyama et al. and this suggests that the composition of phospholipids in the plasma membrane is of high importance to the exocytosis events.

How the cells incorporate exogenous phospholipids from solution-phase liposomes has previously been investigated. Depending on the size and structure of the liposome there can be an exchange between the plasma membrane and the liposome or the liposome can fuse with the plasma membrane [13]. The uptake of phospholipids into the plasma membrane can also be facilitated by specific proteins in the cell membrane [14], which could provide insight into differences between phospholipid uptake in different cell lines. After incubation, exogenous phospholipids could also be directed to other parts within the cell due to the dynamic state of cell membranes [14, 15].

3.2 TOF-SIMS

Time of Flight Secondary Ion Mass Spectrometry (TOF-SIMS) enables the analysis of the upper most molecular layer of a solid sample in vacuum. TOF-SIMS involves bombarding the surface of the sample with a beam of primary ions, as shown in Figure 5A (red circles). As the primary ions hit the surface, secondary ions (shown as light red lines) will be created from the molecules present at the site of impact. The secondary ions are then electrostatically extracted into a TOF mass analyzer where they are detected based on their mass to charge (*m/z)* ratio. By rastering the primary ion beam over the sample surface, a mass spectrum (Figure 4B) is recorded from each impact, pixel. This results in the creation of ion images (Figure 4C) displaying the molecular distribution on the sample surface. In papers I-III a TOF-SIMS IV instrument from ION-TOF (GmbH) was used and the following sections will describe the technique in more detail.

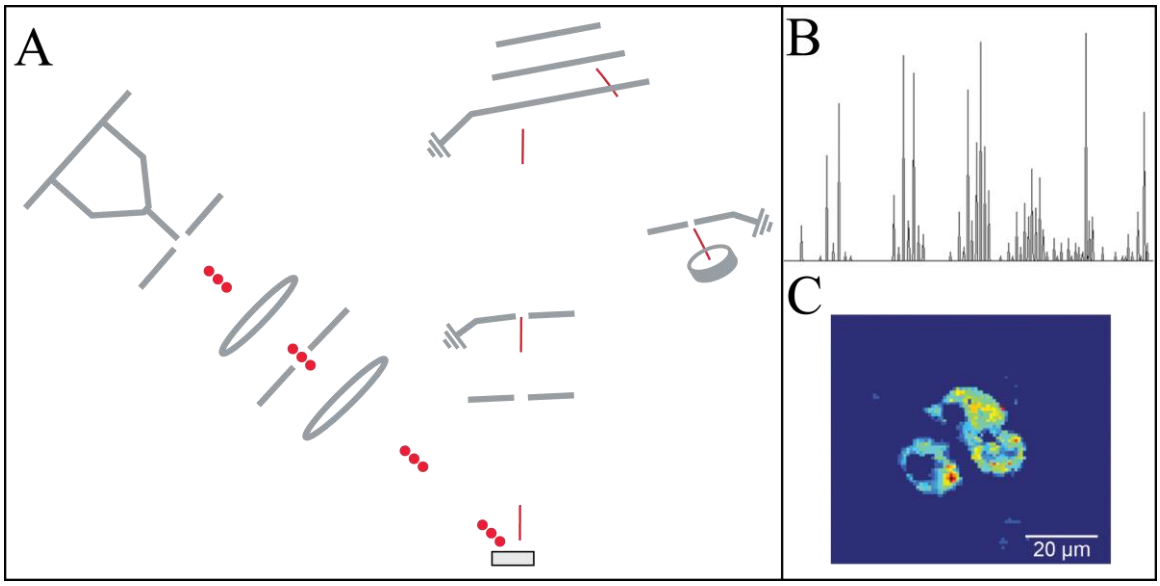

Figure 5*. **A**) The schematic process of TOF-SIMS. Primary ions (circles) are focused on to a solid sample. The secondary ions (lines) are extracted into a TOF mass analyzer. **B**) A mass spectrum from a PC standard, ranging from 10 to 105 Da. The y-axis displays intensity and the x-axis displays m/z. **C**) An ion image of m/z 184 (PC head group fragment ion) showing three freeze fractured PC12 cells.*

3.2.1 Secondary ion mass spectrometry

Secondary ion mass spectrometry (SIMS) utilizes the detection of secondary ions formed at a sample surface after bombardment with primary ions and can be operated in dynamic or static mode. Dynamic SIMS involves the analysis of the elemental composition of a sample as a function of depth and it has been widely used in the semiconductor industry [16]. In static SIMS the primary ion dose is low, less than 1 x 10^{-13} ions/cm^2, ensuring that less than 1 % of the sample surface is damaged during analysis [17]. With such a low primary ion dose the primary ions will only hit each spot on the sample once, resulting in the creation of secondary ions from the upper most molecular layer of the sample surface and not from a damaged spot. Static SIMS was first developed and named by Benninghoven in Münster [18, 19], even though the techniques enabling this development started in the 1950's [19-25]. The mass analyzer used in SIMS was originally a magnetic sector and later a quadrupole. It was not until 1981 that TOF-SIMS was first described [26]. The advantage of using a TOF as a mass analyzer is that it constantly detects and records all ions that enter the mass analyzer. Both the magnetic sector and the quadrupole focus on a selected *m/z* for detection, which means that the ions not detected are discarded leading to a decreased sensitivity. The quadrupole analyzer can, however, scan over a *m/z* interval, but will only spend milliseconds (ms) detecting each *m/z*. Another advantage of using the TOF mass analyzer in SIMS is that it is a pulsed technique, meaning that all the ions are collected and pushed through the flight tube at the same time. This is consistent with many primary ion guns used in SIMS as the primary ions are pulsed, creating a pulse of secondary ions from the sample.

3.2.2 Primary Ions

Several atomic species can be used as primary ions in TOF-SIMS. The choice of primary ion source directly correlates to; the amount of secondary ions being formed from the surface (secondary ion yield); the damage caused to the surface; the amount of fragment ions formed; the acquisition time and; the spatial resolution obtained. Cluster ions, such as Au_n^+, Bi_n^+, SF_n^+, and C_{60}^+ generally generate secondary ions at a higher yield than monoatomic ions such as Ar^+, Ga^+, Cs^+ etc. Cluster ions also cause less damage to the sample since the energy of the cluster will be divided among the atoms within the cluster, resulting in less direct energy to the surface upon impact. Monoatomic ions will therefore penetrate the sample deeper and give more fragment ions than the cluster ions.

In Papers I-III a 25 kV bismuth cluster primary ion source is used. The primary ions used are Bi_3^+ which enable a spatial resolution of about 200 nm. This makes it possible to image the molecular distribution in single PC12 cells that are only about 10 – 20 µm in diameter.

3.2.2.1 Bismuth primary ion source

Bismuth, together with gallium, indium and gold, belongs to the class of liquid metal ion sources (LMIG) and is set up as shown in the schematic in Figure 6 [17]. Liquid bismuth is created by warming the bismuth coated reservoir and needle (a and b in Figure 6). An extraction field (d in Figure 6) at the tip of the needle causes the ions to move forward and at the end of the tip they form a cone, known as a Taylor cone. Ions, Bi_n^{q+} (n= 1-7, q = 1-2 [27, 28]), are then emitted from the cone and form the primary ion beam. As the Bi_n^{q+} ions travel down the ion column they are focused, chopped up into pulses and mass selected through different electrostatic lenses.

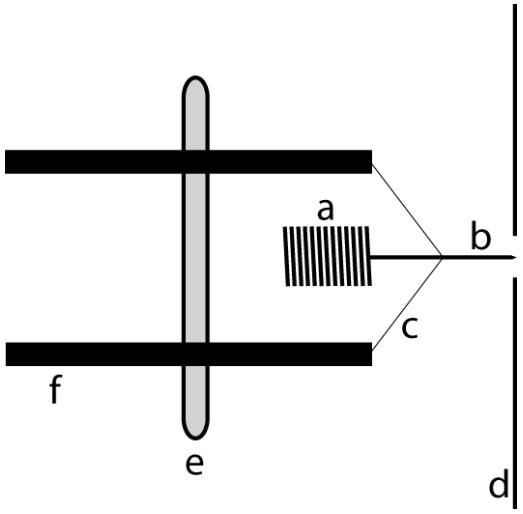

***Figure 6**. Schematic of a liquid metal ion source. Parts include: a) coated reservoir, b) coated needle, c) heater filament, d) extractor, e) insulating disc, f) support leg and electrical contact.*

The 25 kV bismuth primary ion source can be operated in two modes; burst alignment and bunched mode. In burst alignment mode the primary ion pulses are narrow but long (100 ns). This gives high spatial resolution but a severely limited mass resolution (m/Δm about 300). The bunched mode creates pulses that are short and wide, which will only give about 4-µm spatial resolution but a better mass resolution (m/Δm) of about 7000.

Studies comparing bismuth and gold clusters conclude that the bismuth ion emission is more intense which allows for better spatial and mass resolution [27, 28].

3.2.3 Secondary ions

Secondary ions are created by the impact of primary ions with the surface. The collision of primary ions with the surface transfers particle energy to the atoms within the solid sample. The atoms within the sample will collide until finally some molecules are sputtered into the gas phase [17]. It is believed that the transformation of molecules to ions takes place right at the solid phase surface. The surrounding molecules in the sample, the matrix, will have an impact on how many analyte molecules that become ions, the secondary ion yield. For example it has been shown that water has an enhanced effect of the secondary ion yield of hydrocarbons [29-31]. This is also seen and discussed in Papers I and II where PC12 cells are embedded in an ice matrix.

3.2.4 Time of Flight mass analyzer

In a TOF mass analyzer the ions are separated based on the time it takes them to fly through a flight tube, typically being 1-2 m long. Ions are simultaneously accelerated into the low pressure flight tube by an electrical field. This gives all of the ions the same kinetic energy but the velocity will depend on the *m/z* of the ion. The lighter ions fly faster than the heavier ions and will reach the detector first, giving them a shorter fly time. The *m/z* is then calculated by the velocity squared times twice the acceleration pulse potential. Even though all ions get the same acceleration pulse the spatial distribution between the ions will give them slightly different velocities. This causes broadening of the peaks in the mass spectrum which decreases the mass resolution. However this broadening can be minimized by the use of an electrostatic reflector called a reflectron. Figure 7 shows a schematic of a TOF mass analyzer with a reflectron.

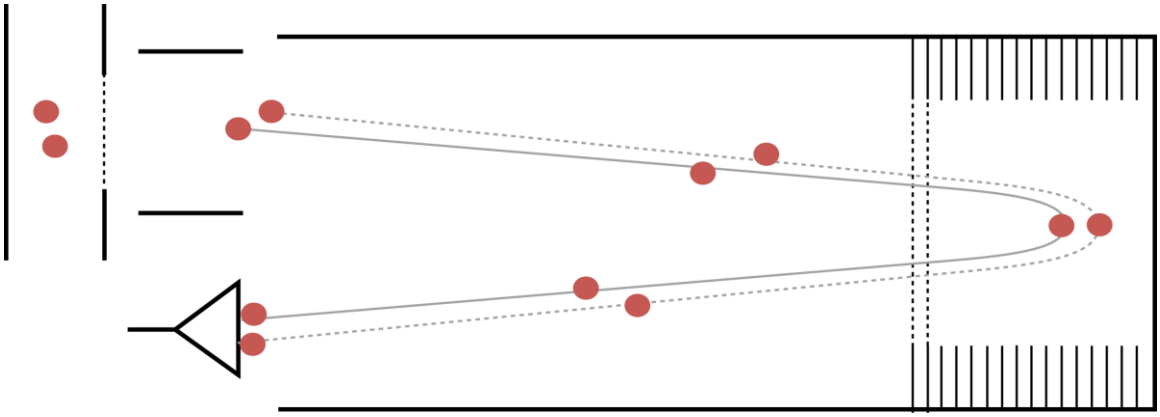

Figure 7. *Schematics of A TOF mass analyzer with a reflectron illustrating the path of two ions with the same m/z but slightly different velocity.*

A reflectron consists of several ring electrodes with increasing potentials. The ions will travel into the reflectron and their velocity will decrease the deeper they penetrate it, until they are repelled into the flight tube and to the detector. An ion with a higher velocity will penetrate the reflectron deeper giving it a longer flying path which equals a longer flight time. If two ions with the same *m/z* have different velocities the use of a reflectron will compensate for the differences in kinetic energy. This will cause them to end up at the detector closer to the same time, which increases the mass resolution.

3.2.5 Flood gun

When using a LMIG, a flood gun is necessary to provide surface charge stabilization for samples that are insulating, for example samples with an ice matrix. Every Bi_n^+ ion that hits the sample surface does not create a positively charged secondary ion. The sample will therefore end up with a higher surface charge during analysis which will affect the spatial resolution. This can be dealt with using a flood gun that emits low energy electrons over the sample to counter this charge build up.

3.3 Sample preparation for TOF-SIMS

TOF-SIMS is a high vacuum technique requiring transfer of samples into low pressure (10^{-6}-10^{-9} mbar). For single cell analysis in TOF-SIMS this can be accomplished in several ways. Samples can be freeze dried, freeze fractured, fixed, or imprinted on silver prior to analysis [32-37]. In Papers I, II and III single PC12 cells have been imaged after freeze fracture in order to analyze the cells frozen and hydrated. There are several advantages in analyzing freeze fractured single cells. These include; 1) the cell morphology stays preserved, 2) the low temperature limits the diffusion of molecules in the cell keeping the molecular distribution intact, 3) the risk for atmospheric contamination is low since the cells are fractured in the low pressure vacuum chamber, and 4) the water matrix has been shown to increase the secondary ion yield of organic fragments. The following sections will describe the in situ freeze fracture device that was developed to enable the analysis of freeze fractured single cells using the TOF-SIMS IV instrument (ION-TOF GmbH).

3.3.1 In situ freeze fracture device

To enable freeze fracture of cell samples inside the analysis chamber of the TOF-SIMS IV instrument a new strategy to create a sandwich of the cell sample that can be broken open in situ has been employed. This basic strategy has previously been successfully used to study single cells with TOF-SIMS. Cells were then placed between two silicon shards and the assembly was quickly frozen in liquid propane [33]. The sample package was then brought into vacuum and the freeze fracture was performed using a liquid nitrogen cooled cold knife. This removed the top shard and the cells on the bottom shard were analyzed. By performing the fracture in low pressure the risk of atmospheric contamination was limited since the surface for analysis was not exposed until it was in vacuum. The TOF-SIMS IV instrument does not have such a cold knife making it necessary to design a freeze fracture device to fit the instrument.

The freeze fracture device developed for the TOF SIMS IV instrument has been manufactured to fit the dimensions of the temperature controlled cold stage for the instrument. The ability to control the temperature of the cold stage enables monitoring and adjusting the temperature while the sample is inside the analysis chamber. Figure 8 shows the schematic of the freeze fracture device in open conformation. The device is made of copper which, being highly thermally conductive, allows rapid temperature control of the sample. The two silicon shards holding the cell sample are shown in black in Figure 8. These are held in place by stainless steel tabs. The device also features two mounting holes used to mount the device onto the temperature-controlled stage under liquid nitrogen (LN2). Further, a spring is attached to the device in order to increase the reproducibility of the fracture. Since both shards stay on the device after fracture, cells on both shards can be analyzed. This doubles the analysis area compared to the previous system [33]. This also enables mirror analysis since part of a fractured cell can be found on each side. This is discussed in Paper I.

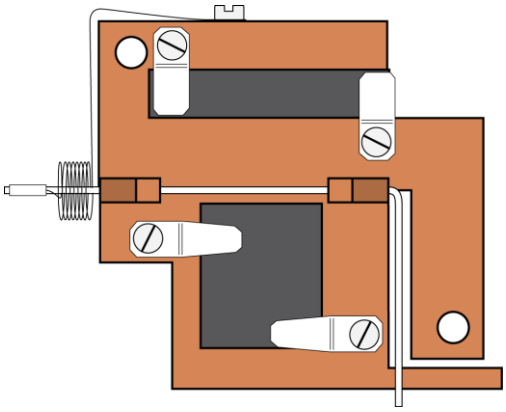

***Figure 8**. Schematic of the in situ freeze fracture device developed for the TOF SIMS IV instrument.*

To create a sandwich about 1-2 µL of the cell-solution is placed onto the smaller shard. The device is then closed, which sandwiches the cells in between the shards, and quickly frozen by plunging it into liquid propane at LN2 temperature (-196°C). When cells are frozen the crystallization of water within the cell can destroy cellular structures. In order to keep the cell water crystallization from being lethal to the cells the freezing rate must be very high. A rate of 1-10°C/s will destroy the cell while a rate of 1000°C/s will keep all structures within the cell completely intact [38].

Samples frozen in the freeze fracture device can be stored for a long period of time (weeks) in LN2. When it is time for analysis, the device is mounted onto the temperature controlled cold stage under LN2 and the assembly is then quickly entered into the load lock of the instrument. It is important that the device and cold stage are loaded into the LN2-cooled load-lock quickly. At these low temperatures the water in the air rapidly condenses onto the stage and inside the load lock. This makes it difficult to create the low pressure needed in the load lock and it will transfer ice into the analysis chamber increasing the pressure. Once the assembly has been entered into the analysis chamber the temperature is set to -110 degrees centigrade. The sample is then fractured manually using a fracturing device mounted onto the sample entry arm in the load lock. This is done by applying a moderate force to the top of the device which will force it to open, facilitated by the spring on the device. Once fractured the temperature of the assembly is raised to -105 degrees centigrade, which at this pressure is the temperature where the sample is in ice but there is no ice condensing onto the sample [38].

The fracture of the device produces a clean surface for analysis where the cells are embedded in the sample solution. Some cells are fractured through the cytoplasm, some expose the plasma membrane, and some are hidden underneath a layer of ice. Figure 9 shows a Scanning Electron Microscopy (SEM) image of HeLa cells (human cervical cancer cells) that have been dislodged, centrifuged, placed in 10 mM HEPES solution, and freeze fractured using the freeze fracture device. Even though these cells originally were embedded in an ice matrix frozen and hydrated, the sample was dried (down to -90°C) in situ prior to SEM analysis. Freeze fracture of cells produces several different fracture planes as shown in Figure 9. Some cells are completely intact while others loose part of their plasma membrane. The smaller image in Figure 9 shows cells that have been fractured through the interior of the cell and exposing intracellular structures such as the nucleus. These fracture planes are also observed with TOF SIMS. In Figure 10 a TOF SIMS image of a cluster of cells that have been fractured through different planes of the cell is shown. This has been determined by the use of diagnostic ions. High potassium (K^+), shown in red, reveals the cells that have been fractured through the cytoplasm. High levels of PC, shown in green, reveals cells where the outer leaflet of the plasma membrane has been exposed and high levels of PE, shown in blue, reveals the exposure of the inner leaflet of the plasma membrane [39].

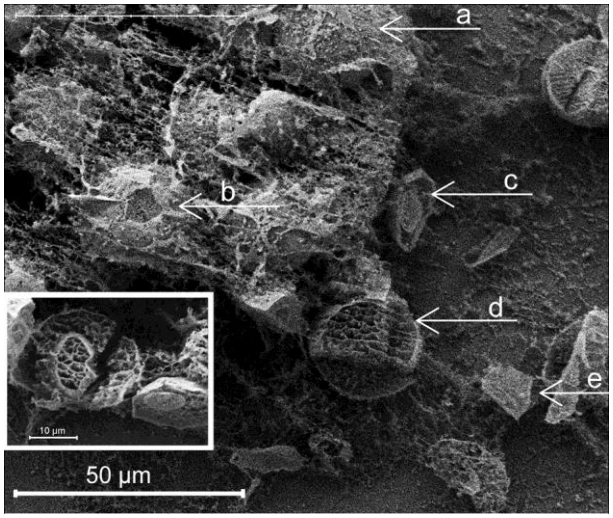

***Figure 9**. SEM image of dried freeze fractured HeLa cells: a) an intact cell with exposed plasma membrane, b) a cell with partially ruptured plasma membrane, c) a cell that has been fractured exposing the nucleus, d) a cell that has been fractured not exposing the nucleus, e) part of the plasma membrane from a cell on the opposite shard. Inset shows two cells that have been fractured exposing the nucleus.*

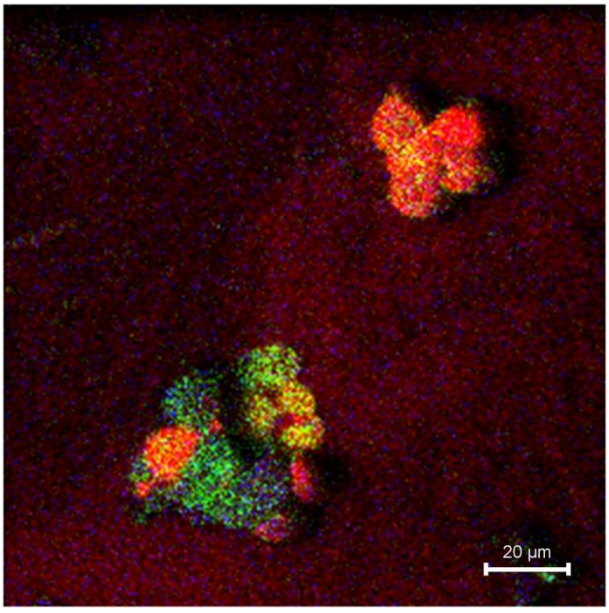

***Figure 10**. A TOF SIMS image of two clusters of PC12 cells showing K^+ in red, PC in green, and PE in blue.*

3.3.1.1 The matrix is important for a successful freeze fracture

The matrix embedding the cells is highly important for a successful freeze fracture using the freeze fracture device. A successful freeze fracture splits the sample in two, leaving part of the sample on either shard as shown in Figure 11A. An unsuccessful fracture will instead fracture between the shard and the sample as shown in Figure 11B and C.

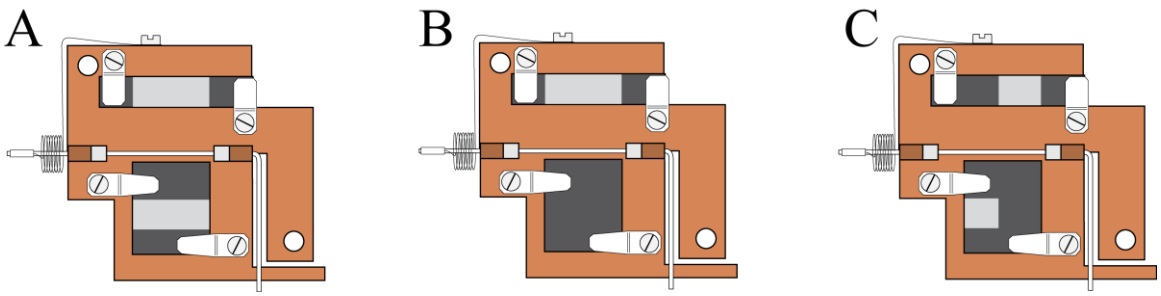

***Figure 11**. Different ways of fracturing the sample: **A**) the sample is fractured through the ice, successful fracture, **B**) the sample is fractured by the shard and the ice, **C**) the sample is fractured by the shard and the ice.*

A solution containing 10 mM HEPES at pH 7.4 (adjusted with ammonium hydroxide) produces a successful fracture when clean shards that have been kept in normal atmosphere are used. However, a shard that has become contaminated making it more hydrophobic produces unsuccessful fractures. Upon visual observation of the shape of the drop loaded onto the shard, the fractures success rate can be predicted by the contact angle. Figure 12 shows a schematic of a drop on a shard with a desired contact angle (Θ). In the case of a hydrophobic shard the contact angle will be larger and the solution will not wet the shard to the same degree. When the shards are treated with ozone, making them very hydrophilic, there is no visual contact angle when loading the sample. This results in solution all over the shard and underneath the stainless steel tabs making the device unable to freeze and store in a closed configuration.

That the contact angle is of high importance can be seen in Young's equation, equation 1. According to Young's equation a more hydrophilic surface will have a higher surface energy.

$$\gamma^{sv} = \gamma^{sl} + \gamma^{lv} \cos \theta \qquad (1)$$

The equation states that the surface free energy of the solid equals the free energy of the solid/liquid interface plus the surface free energy of the liquid times cosine of the contact angle.

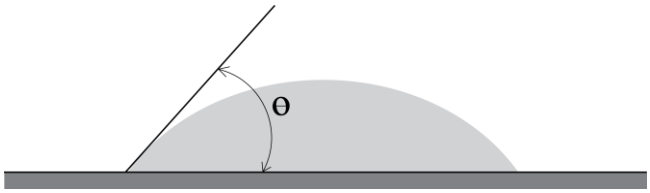

Figure 12. *Contact angle.*

The amount of sample loaded onto the shard is also important. If too much is loaded, the solution will spill over to the copper surface and the spring when the device is closed. This will cause ice formation both on the shard and on the copper. In these instances the opening of the device will not create a successful fracture. Instead, all the ice will be on the side where it is in contact with the copper surface. In order to get a successful fracture it is also important to keep the cell solution as clean as possible. When the cell sample is prepared it is important to remove any lipids or other contaminants that might be in the solution before loading the sample. This is preferentially done by repeated centrifugations and resuspensions of the cells. Too much lipid in the sample solution will counteract a successful fracture.

4 - Phospholipids as biomarkers

4.1 Anammox bacteria

ANaerobic AMMonium OXidizing (Anammox) bacteria were first found in waste water in the 1990's and have since then been found in lakes, ocean water and ocean sediments [40-44]. They are about 1 µm in diameter and have a doubling rate of approximately 2 weeks, which is extremely long comparing to *E. coli* bacteria with a doubling rate of 20 min. Anammox bacteria have specialized in performing anaerobic denitrification. In this process biologically available nitrogen, such as ammonia and nitrate, is converted into dinitrogen gas (N2) which is returned to the atmosphere. The overall reaction of this conversion is shown in Formula 1 [45]. This process is environmentally advantageous since too high levels of biologically available nitrogen in oceans or lakes can cause eutrophication (over fertilization).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

Formula 1. *The conversion of ammonia and nitrite to dinitrogen gas and water.*

Since the discovery of anammox bacteria, scientists have been trying to understand the role anammox bacteria play in the global nitrogen cycle. It is estimated that anammox bacteria are responsible for 25 - 50 % of the total global marine N2 production [46, 47], indicating that the bacteria are crucial for the environment. This has led to anammox research in two branches; 1) use of anammox bacteria in treatment of human waste water and 2) further exploration of the anammox bacteria's whereabouts in the environment.

Two waste water treatment plants in Sweden are currently using anammox bacteria for removal of biologically active nitrogen; Himmerfjärdsverket in Stockholm and Ryaverket in Göteborg. The anammox bacteria are kept in big tanks growing in biofilms on small, about 1 cm in diameter, plastic wheels called caldnes. As waste water passes through the tanks biologically active nitrogen is removed, converted into N2 and returned to the atmosphere by the anammox bacteria. This purification process prevents the biologically active nitrogen from being released into oceans and lakes.

To explore the whereabouts of anammox bacteria in oceans and lakes many methods have been developed and used. Examples are; Fluorescent In Situ Hybridization (FISH), real time quantitative Polymerase Chain Reaction (qPCR), Gas Chromatography (GC) coupled to FID or MS, and LCMS [40, 41, 43, 47-50]. Unfortunately, neither one of these methods specifically detects viable anammox bacteria. This makes it hard to distinguish between live bacteria and residues from dead bacteria during data analysis. In order to reflect viable bacteria phospholipids used as biomarkers need to be intact, not degraded or derivatized in any way [51-57]. Phospholipids used as biomarkers also need to have unique molecular structures that will separate them from phospholipids commonly found in cells. Such phospholipids are found in the membrane of an organelle inside the anammox bacteria, called the anammoxosome.

4.1.1 Anammoxosome

The anammox bacteria have a unique inner organelle quite contrary to normal bacterial cells, Figure 13. The organelle is called the anammoxosome and its membrane is believed to be the site where the anammox process takes place. This hypothesis is supported by the finding of specific membrane proteins involved in the process [58]. During the anammox process a proton gradient is thought to be formed across the membrane and toxic intermediates, including hydrazine, are believed to be enclosed inside the anammoxosome. This puts high demands on the anammoxosome membrane to maintain the proton gradient and to protect the cell's DNA from contact with the toxic intermediates. The membrane therefore needs to be extremely dense. This could be the reason why it is built up by phospholipids with unique tail group structures, called ladderane lipids.

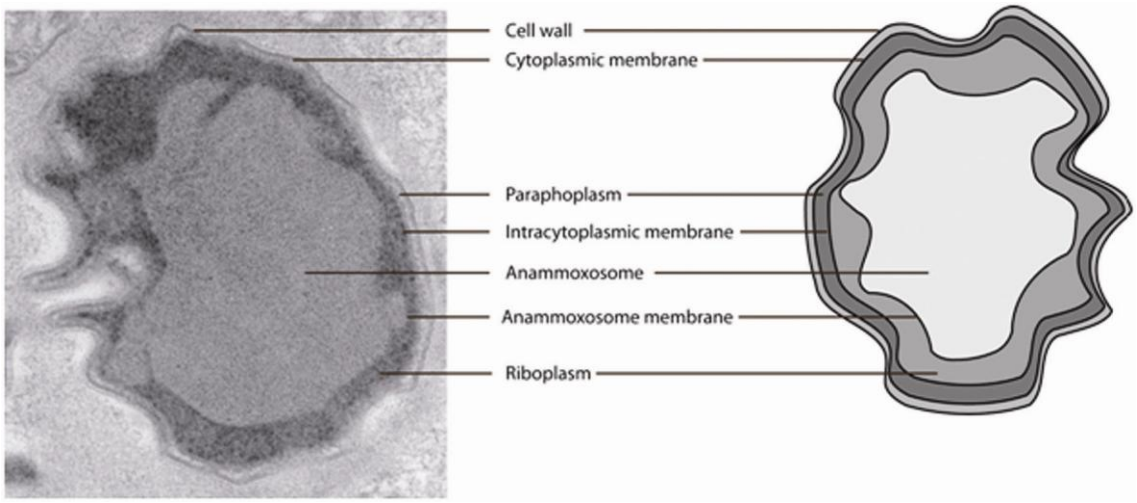

Figure 13. *Electron microscopy image of an anammox bacterium to the left and a schematic to the right. The different parts of the cell are marked.*

4.1.2 LADDERANE LIPIDS

The head groups of ladderane lipids can consist of PC, PE, or phosphatidylglycerol (PG) but it is the tail groups that are special [59-61]. At the end of the tail group there is either five cyclobutanes or three cyclobutanes and one cyclohexane connected to each other as shown in Figure 14. The linked cyclic hydrocarbons make the tail groups extremely hydrophobic and cause the ladderane lipids to pack tightly, which leads to a very dense membrane [60]. The ladderane tail groups are connected to the glycerol back-bone either by an ester bond or an ether bond.

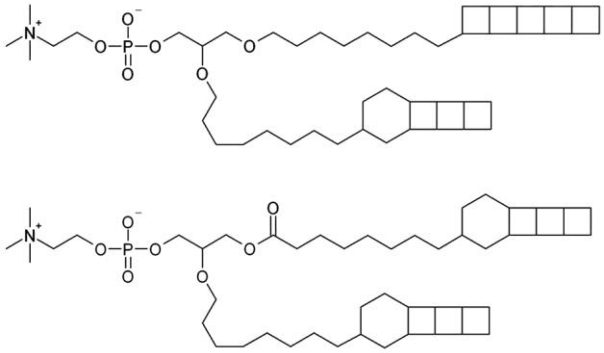

Figure 14. *Two examples of ladderane PC lipids, the top example has two ether bonds to the glycerol backbone and the bottom has both an ether and an ester bond.*

To date, the ladderane lipids have only been found in anammox bacteria and because of their unique molecular structure they can be used as biomarkers for anammox bacteria. This means that it is highly likely that where-ever ladderane lipids are found there are anammox bacteria. However, this statement has to be modified a little bit. In order to conclude that anammox bacteria are alive at the sampling site, the ladderane lipids have to be intact phospholipids [51-57]. If only a part of the ladderane phospholipid is found, such as the ladderane fatty acids or the head group and one tail group (lysophospholipid), it is probable that the molecule has been degraded after cell death.

4.2 Sediment Sample Clean Up

Finding a specific molecule, such as a ladderane lipid, in ocean sediment is not an easy task. There are plenty of molecules in ocean sediment which can originate from nature, be manmade, or be degradation products from either of the two. The sediment sample therefore has to be subjected to an extensive clean up procedure to remove unwanted molecules but to still keep the ladderane lipids. In Paper IV several methods have been explored in order to get the cleanest ladderane lipid extract with the highest yield.

Cultured anammox bacteria have been shown to grow in clusters most likely covered with a protein polysaccharide matrix [62]. It is also believed that anammox bacteria in sediment grow in similar clusters. The first step in the sample clean up procedure therefore involves lysing the cells in order to get the ladderane lipids in solution. In Paper IV the sediment samples are treated with 5 % sodium (meta) periodate to break up large clusters of cells making it possible to extract the ladderane lipids. In order to investigate the result of the periodate treatment, samples of cultured anammox were imaged before (Figure 15A) and after (Figure 15B) treatment with periodate. The fluorescence images in Figure 15 have been taken using DAPI to stain bacteria DNA. In Figure 15A the DNA is visible as half moons, because the anammox DNA is outside the anammoxosome in the cell, whereas the DNA in Figure 15B is spread out, indicating that the cells are lysed.

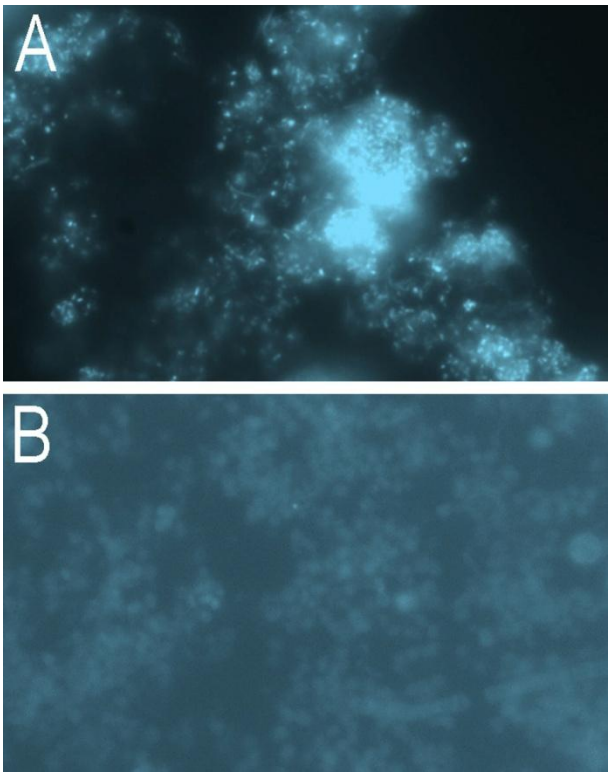

Figure 15*. Fluorescence image of cultured anammox bacteria where the DNA is dyed with DAPI.* ***A****) cultured anammox bacteria in clusters.* ***B****) cultured anammox bacteria in clusters after treatment with periodate.*

After lysing the cells the ladderane lipids have to be extracted from the sediment into solution. Repeated extraction procedures ensure that the majority of the ladderane lipids will be removed from the sediment to the solution. However, the extraction procedure is not specific and many unwanted compounds are also extracted to the solution. In Paper IV the sample is further cleaned up by liquid-liquid extraction (LLE) and solid phase extraction (SPE).

4.2.1 Liquid liquid extraction

In LLE at least two liquids that are immiscible are mixed with the sample, as shown in Figure 16, left. The molecules within the sample will then partition into the solvent where they are soluble, separating the sample in two phases as shown in Figure 16, right. The phase that contains the molecule of interest is saved and the other phase is re-extracted to increase the yield. By changing the properties of the solvents used in LLE the molecule of interest can be recovered with small amounts of contaminations. In Paper IV a mixture of DCM:MeOH: NH4Ac (10 mM) (2:1:0.8) was used to extract lipids out from the sediment sample. In order to enable a two phase separation the LLE was performed using DCM:MeOH:NH4Ac (10 mM) (2:1:0.9) [63]. With this procedure the phospholipids partition into the lower phase, mostly containing DCM, and the more hydrophilic molecules stay in the upper phase. The lower phase was kept and most of the liquid was evaporated in order to perform the next step in the sample clean up procedure – SPE.

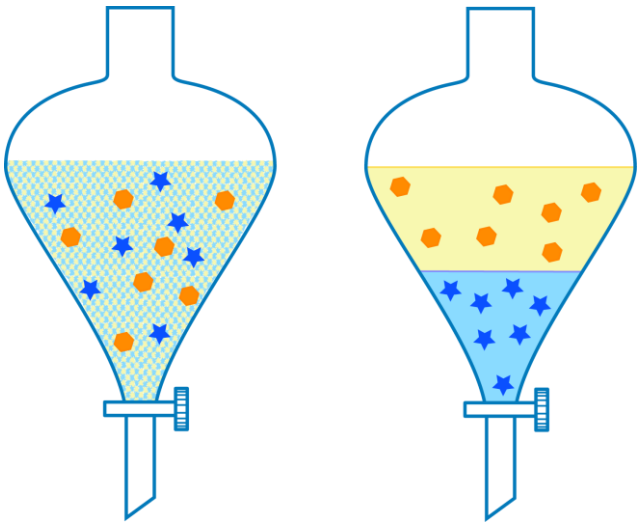

Figure 16. *Schematic of liquid liquid extraction, left – immisciable liquids are mixed with the sample, right – the two liquids and the molecules in the sample have separated into two phases.*

4.2.2 Solid phase extraction

SPE is performed with a packed dried stationary phase in a plastic column. The columns are typically used only once and have a low efficiency, meaning that the analytes are either adsorbed to the column or not. In Figure 17 the procedure for SPE clean up is shown. From left to right; the dry column has to be activated in order for the functional groups to open up and contamination from manufacturing to be removed. The next step is to condition the column, which is performed to remove the activating agent and get the column ready for the sample. The sample is then loaded onto the column. The analytes of interest together with other contaminants will be adsorbed while some contaminants will pass right through the column. After loading the sample one or several washing steps will remove unwanted adsorbed contaminants. Finally the analyte is eluted. Some remaining contaminants will stay adsorbed in the column. By carefully choosing solvents to use and functional groups on the column, the analyte of interest will be eluted without too much contamination at a high yield. Unfortunately there are no commercial standards available for ladderane lipids, so the yield after sample clean up using LLE and SPE in Paper IV is unknown. The lack of standards is unfortunate since it makes the development of the sample preparation difficult and the final results cannot be related to the actual amount in the sediment. However, to overcome this issue during sample clean up development, triplicate samples of carefully weighted cultured anammox bacteria, even though not a pure culture, were used.

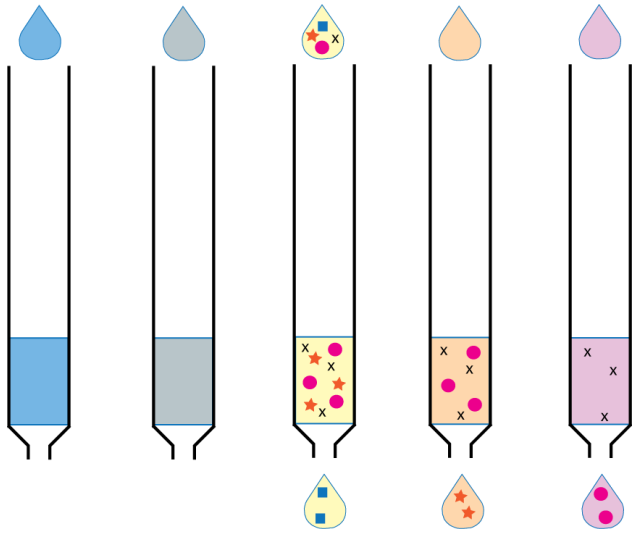

Figure 17. *Schematic of SPE. From left to right: Activation of functional groups, conditioning of the column, sample loading, washing, analyte elution.*

4.3 Liquid Chromatography Mass Spectrometry

The separation technique used in Paper IV is liquid chromatography (LC) coupled to a triple quadrupole (QqQ) mass analyzer with an electrospray (ESI) interface or coupled to a quadrupole – time of flight (QTOF) mass analyzer with an ESI interface. These techniques are discussed in more detail below.

4.3.1 Liquid Chromatography

The principle of LC is based on the interaction of the analytes with the stationary phase and the mobile phase. Due to discrimination effects (section 4.3.4) it is important that the analyte of interest is separated from the other analytes. LC can be run in different modes that are classified depending on the functional groups on the stationary phase (polarity) and the mobile phase. Paper IV uses reversed phase (RP) LC, which has a hydrophobic stationary phase that retards the analytes based on hydrophobicity. This means that the most hydrophobic analyte will have the longest retention time. The column used is a C18 column for which the stationary phase functional groups consist of chains with 18 hydrocarbons attached to the silica beads as support. The mobile phase used to migrate the analytes through this jungle consists of 4 % H_2O in MeOH, which has a high elution power. The elution power of isopropanol (IPA) is even stronger and it is therefore used to clean the column from any residual contaminants after sample analysis.

Figure 18A shows five PC molecules with different tail groups. All PC molecules, but #3, have two ester bonds linking the hydrocarbon tail groups to the glycerol backbone. By instead having two ether bonds #3 is more hydrophobic than #2 even though they have the same tail length. This can be seen in the chromatogram in Figure 18B where #3 has a longer retention time than #2. The longer tail group in #4 and the substituted tail group in #5, however, have a larger impact on the retention causing both of them to elute after #3. Phospholipid #1 with the shortest tailgroup is the least hydrophobic of the five molecules and elutes first in the beginning of the chromatogram.

Figure 18C shows a chromatogram of the PC molecules from cultured anammox bacteria, analyzed using LCQqQ scanning for parents of *m/z* 184. Intact ladderane lipids coelute in pairs and are found in the two last peaks designated III, IV and in I, II. The molecular structures of I, II, III and IV are shown in Figure 18D. The long retention time, which is due to the extremely hydrophobic tail groups of the

ladderane lipids, separates the ladderane phospholipids from other phospholipids in the sample.

An interesting feature that is evident when comparing the two chromatograms in Figure 18B and C is the lower signal from PC molecules linked with two ether bonds to the glycerol backbone. In Figure 18B the concentration of the five molecules is equal, however, the peak from #3 much less intense. This is most likely due to a lower proton affinity in the ether-ether molecules compared to the ether-ester molecules, resulting in a higher detection limit.

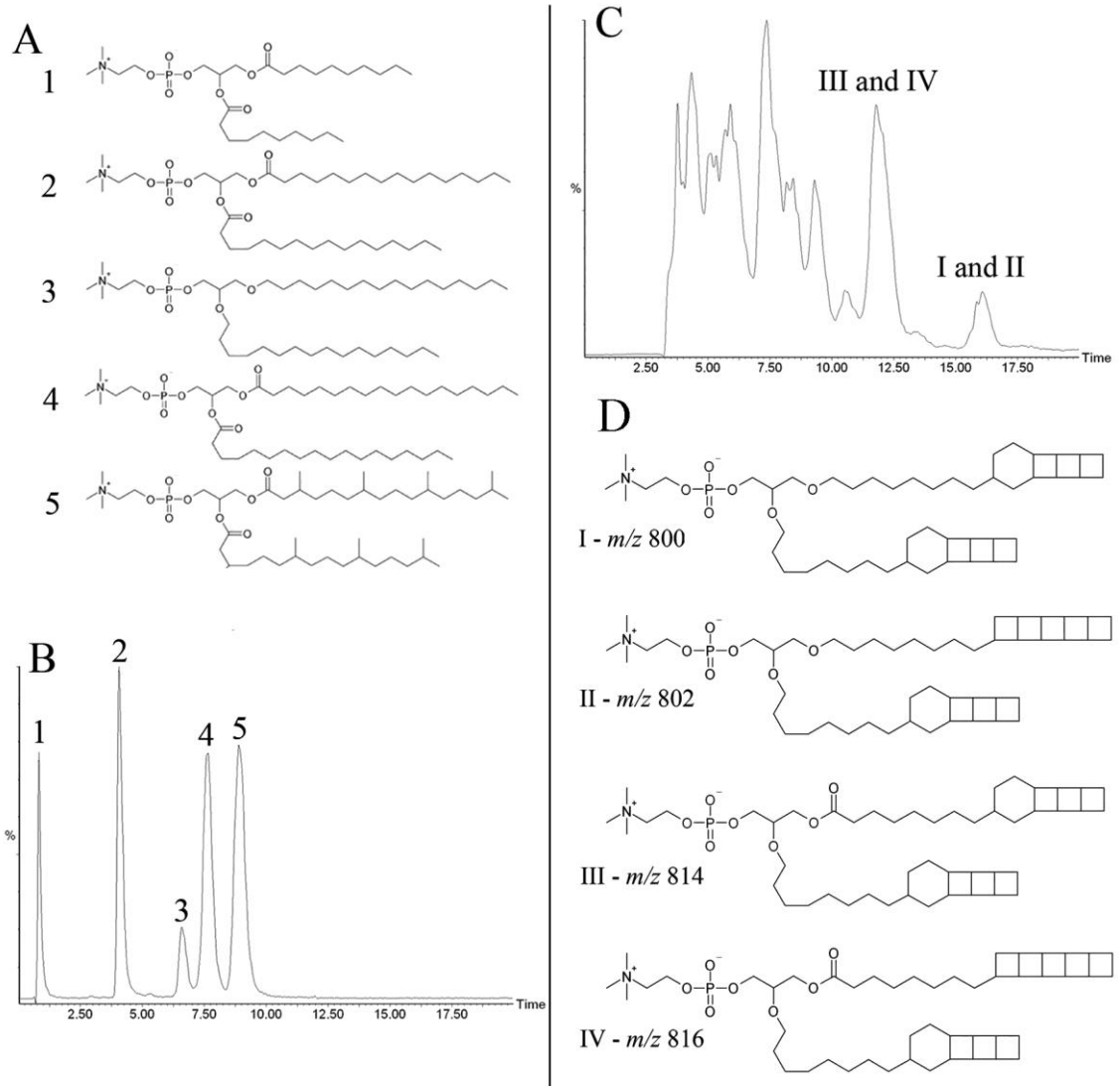

***Figure 18**. **A**) Molecular structures of four PC species, **B**) LC trace of molecules in A, **C**) LC trace of phospholipids extracted from cultured anammox bacteria, **D**) Molecular structures of ladderane PC species detected in C.*

4.3.2 Quadrupole mass analyzer

A quadrupole is set up with four metal rods that are operated in pairs at low pressure. By altering the polarity of the potential applied to the rods, ions passing through can be separated based on their *m/z*. The direct potential and the amplitude of the frequency applied to the rods determine the *m/z* that will have a stable trajectory through the quadrupole. An ion that does not have a stable trajectory will hit a rod and become discharged, which will keep it from being detected [64]. A quadrupole can be operated in both negative and positive mode and can be used to either select a single *m/z* or scan over a mass interval with a scan speed of up to 1000 Da/s, stopping at each *m/z* for a short period of time (ms). A quadrupole runs continuously, which makes it suitable to use in combination with separation techniques like LC, GC or capillary electrophoresis (CE). However, the stepwise scanning of the instrument limits the use of separation techniques having high separation efficiency, with narrow peaks. Further, quadrupoles are limited by having only unit mass resolution and can therefore not be used in applications that need high mass resolution or high mass accuracy.

A quadrupole can be coupled to another mass analyzer, for example another quadrupole or a TOF to enable MSMS analysis. Figure 19 shows the schematics of a QqQ operated in single reaction monitoring (srm) mode. From left to right; several ions are approaching Q1, which only allows ions with a specific *m/z* to pass through. The selected ions are subjected to collision-induced dissociation (CID), which takes place in an rf only hexapole (not shown in the schematics) by the addition of an inert gas (Ar or N2) to the ions. The ions will collide with the gas, which will cause them to fragment and produce fragment ions (daughter ions). In the hexapole all ions are kept in a stable trajectory since no direct potential is applied. The fragments will then enter Q3, which is set to keep a specific *m/z* stable passing through the quadrupole. The fragment ions with a stable trajectory in Q3 are then detected at the detector (not shown in schematics). By performing an srm experiment the selectivity of an analyte is high since both the molecular ion and the fragment ion are singled out.

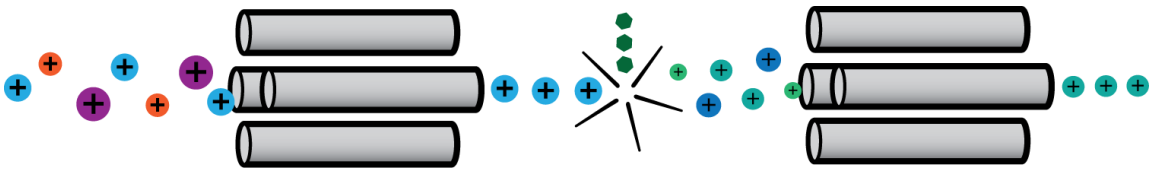

***Figure 19**. Schematics of a triple quadrupole operated in positive SRM mode, Q1 and Q3 are visible.*

There are also other modes to operate a triple quadrupole in MSMS mode and all are summarized in Table 1.

	Q1	Q3
Selected reaction monitoring	Select	Select
Parent ion scanning	Scan	Select
Daughter ion scanning	Select	Scan
Neutral loss	Scan	Scan minus loss

***Table 1.** Summary of MSMS modes in QqQ.*

During method development in Paper IV, PC species were analyzed using parent ion scanning. PC species are easily recognized in MSMS mode by monitoring the parent ions of the head group fragment at *m/z* 184, which is stable and readily formed.

4.3.3 Time of flight mass analyzer

Due to limited mass resolution and higher detection limits in the QqQ a QTOF was used for sample analysis in Paper IV. The molecular ion of the biomarker for anammox bacteria was formed at *m/z* 816 at relatively high intensity. The retention time of this biomarker in the LC method described in Paper IV was very specific and the peak is well separated from other non-ladderane peaks in the chromatogram. The additional selectivity of MSMS analysis was therefore not needed and the QTOF was used in positive MS mode. TOF uses a pulsed technique to accelerate the ions and therefore a pusher, which collects ion packages, must be used when coupling TOF to a quadrupole (Section 3.2.4).

4.3.4 Electrospray ionization

In order to separate and detect molecules in a sample using mass spectrometry the molecules must be ionized and brought into gas phase. There are several ways of doing this depending on the sample introduction method and the mass analyzer that will be used. Both instruments used in Paper IV are equipped with an electrospray ionization (ESI) source, which is a good choice when coupling LC to MS. In ESI the molecules in the sample are ionized in the liquid phase and then brought into the gas phase. Figure 20 shows a schematic of ESI in positive mode.

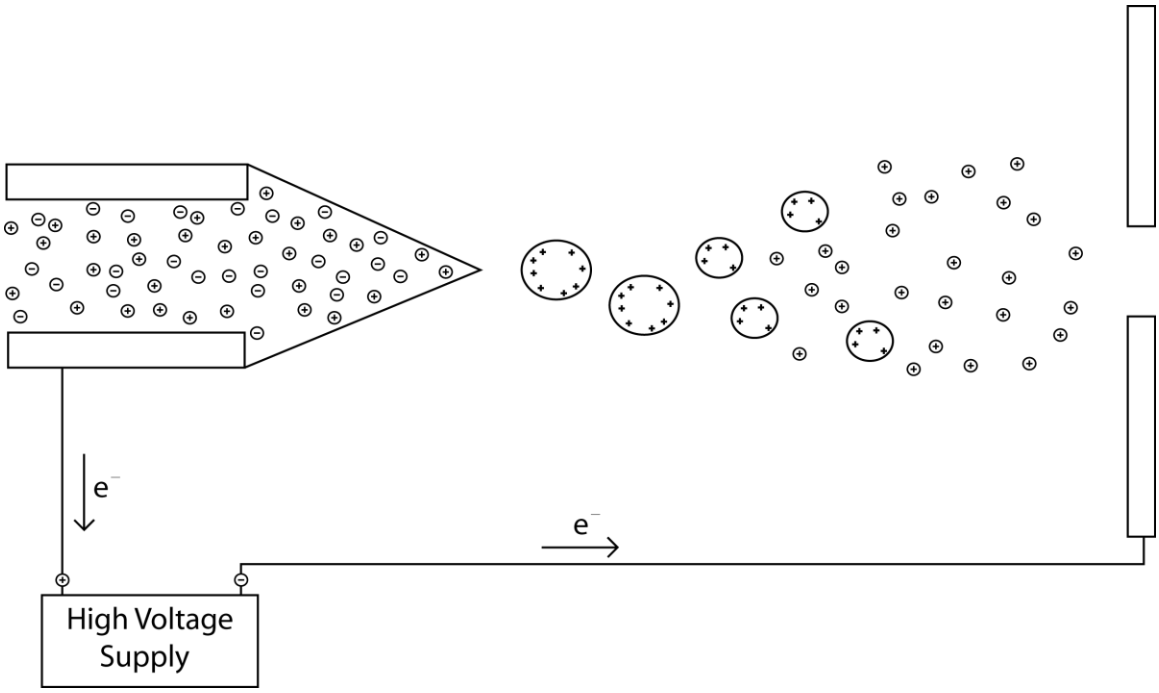

Figure 20. *Schematic of electrospray ionization in the positive mode, not scaled to size.*

In order to create ions a high potential (2-4 kV) is place over the capillary. This causes oxidation processes to occur in the liquid and positive ions are created. N2 assists the formation of a Taylor cone at the end of the capillary and a fine mist with droplets is sprayed into the chamber at atmospheric pressure. One theory of the formation of single ions in gas phase is that the droplets shrink due to solvent evaporation and concentrate the ions within them. When the ions then get close enough they repel each other causing smaller droplets and single ions to be formed in the gas phase.

Ions formed from high mass molecules with several ionization sites, like proteins, are created with multiple charges. This is advantageous since it enables analysis of large compounds without the need of high range instruments since the mass analyzer detects ions based on their *m/z*.

Not all molecules are ionized to the same degree in electrospray ionization. There are some discrimination effects that can take place if several molecules are ionized simultaneously. The molecule with the higher proton affinity (in positive mode) will be ionized to a higher degree. There is also a discrimination effect which depends on the hydrophobicity of the ion. A more hydrophobic ion will partition to the surface of the ESI droplet whereas the more hydrophilic molecule will more easily be dissolved inside the droplet. During the evaporation process the ions on the surface of the droplet are more easily transformed into the gas phase causing them to enter the mass analyzer to a larger extent.

5 SUMMARY

Mass spectrometry is a good method to choose for analysis of complex biological samples since it can be used to separate similar ions based on their mass to charge ratio. There are, however, some limitations to the technique. The basics of the strengths and weaknesses of this method for phospholipid investigations has been presented here.

In TOF SIMS the sample must be brought into vacuum before it can be analyzed. Even though the sample does not need any specific sample pre treatment it can be tricky to bring it into vacuum without changing the molecular distribution within the sample. In TOF SIMS there is also a concern regarding the secondary ion yield of molecules in the sample. In order for the technique to be successful for a large variety of analytes and samples these issues must be dealt with. The development of the in situ freeze fracture device addresses both the issue of sample entry and secondary ion yield. By entering the cells frozen in a sandwich configuration the sample can easily be placed into vacuum and the in situ fracture produces a clean surface for analysis. The ice matrix around the molecules in the sample facilitates the formation of secondary ions to some degree, which has made it possible to image low abundant species like PE. The rapid freezing of the cells, followed by analysis of them frozen and hydrated, has enabled the quantification of exogenous phospholipids in the plasma membrane. The results show that when PC12 cells were incubated with different concentrations of PC and PE the exogenous phospholipid was accumulated in the plasma membrane. The accumulation, relative to endogenous phosphocholine, was between 0.5 and 2.1 % for exogenous PC and between 1.3 and 9.4 % for exogenous PE. This clearly shows that the accumulation of exogenous phospholipids in PC12 cells is dependent on the concentration in the surrounding media. In combination with previous studies on exocytosis from incubated PC12 cells these results suggests that the process of exocytosis in PC12 cells is influenced by the composition of phospholipids in the plasma membrane. In future research this “plasticity” in neuron model cells might be shown to be important in brain functions such as learning and memory.

When very complex samples, such as marine sediment samples, are analyzed it is extremely useful to use LC before MS analysis. Under the right conditions, LC can be used to separate analytes from contaminants. This provides a cleaner sample with less discrimination effects for MS analysis. In this thesis sample clean up combined with LCMS enables the detection of ladderane lipids in ocean sediment. The results concerning the location of anammox bacteria are in agreement with previous findings.

The work that has been presented in this thesis clearly shows the capacity of mass spectrometry to analyze phospholipids in complex biological samples. It also provides some new methods. These methods are; 1) the ability to introduce a frozen cell sample into the analysis chamber for imaging mass spectrometry and 2) the extensive sample clean up combined with a LC method for ladderane lipid analysis in sediment samples with ESI-MS.

6 FUTURE APPLICATIONS

To my knowledge, there is no such thing as a perfect technique for analysis of complex biological samples. All techniques have limitations. However, in order to understand the importance of chemistry in biological and environmental processes we need to develop new methods and techniques to overcome these limitations. I believe that this can be achieved by combining several analytical techniques which each can provide a piece of the puzzle. Some research using mass spectromety that would be interesting to do to follow up the results presented in this thesis include the following.

A) To further understand what happens in the cell when it is incubated with exogenous phospholipids there are some questions that come to mind. What happens with the deuterated phospholipids that are accumulated in the cell, do they only stay at the plasma membrane or does the increase in a certain phospholipid in the plasma membrane cause some of them to be transported to other places in the cell? Is the observed accumulation due to an exchange of phospholipids or only an uptake of additional lipids? Is it the changed phospholipid composition in the plasma membrane that affects exocytosis or is there also a change in the composition of phospholipids in the synaptic vesicles?

   These questions can be answered by combining TOF-SIMS and LC-ESI-MS. For TOF-SIMS, the freeze fracture device can be used to reveal the localization and accumulation of deuterated phospholipids in the cytoplasm of single cells. For LC-ESI-MS, all phospholipid species in different parts of the cell can be analyzed revealing an increase or decrease of certain species after incubation.

B) To increase the knowledge of anammox bacteria, ladderane lipid analysis can be performed together with complementary anammox bacteria and nutrition analysis. Samples can be collected at different water and sediment depths on a yearly time dependent basis. Such studies will contribute to a greater understanding of the role of anammox bacteria in the nitrogen cycle on the ocean floor.

C) Another thing that would be interesting to study is the accumulation and distribution of other substances within single cells. These substances could be drugs, peptides, signaling molecules or metabolites which can be analyzed in single cells using the freeze fracture device and TOF-SIMS. To make this possible, the molecule of interest needs to have a high secondary ion yield and a unique *m/z* without overlap in the mass spectrum. If there is no unique *m/z* isotopic labeling could be used.

D) Single cell analysis with imaging mass spectrometry can also be used to find molecular changes in a cell over its life-time. It has for example been noted that PC12 cells that have been cultured for several generations (one generation typically lasts one week) have a decreased exocytosis activity. If there is a molecular reason behind this, it could be detected by imaging mass spectrometry. Unfortunately TOF-SIMS causes fragmentation of most molecules during ionization, which makes it hard to know the molecule of origin. However, there are ways to limit this fragmentation and get more molecular ions. By using a C60 ion beam instead of a Bi cluster ion beam there is less fragmentation. The loss of spatial resolution that comes with that change will not be so important in this case since it is an eventual molecular change in the cell that is monitored.

There are also other methods for imaging mass spectrometry that can help in finding answers to biological questions. There are mass analyzers that trap the ion giving high mass resolution and high mass accuracy, which enables molecular assignment of unknown peaks. The assignment of unknown peaks can further be utilized by the ability to do MS^n, which provides additional structural information based on the fragmentation pattern. By switching to other ion sources for imaging, such as MALDI and DESI, high molecular mass ions can be detected. However, as of today, these sources have limited spatial resolution for imaging making them unsuitable for single cell analysis. Another interesting aspect in imaging mass spectrometry using a C60 ion source is that it has the ability to remove molecules on the surface of the sample. This makes it possible to get molecular information from layers down through the sample. By doing so, molecular 3D images of the sample, for example a single cell, can be created.

7 SUMMARY OF PAPERS

7.1 PAPERS I AND II

These papers describe and characterize the freeze fracture device that was developed to enable mass spectrometry imaging of frozen hydrated single cells using the TOF-SIMS IV instrument. The papers discuss how the device functions and show ion images of freeze fractured single PC12 cells. The plane of fracture through the cells as well as an increased secondary ion yield due to the water matrix is also discussed. In Paper I ion images of a frozen hydrated cell before and after in situ freeze drying are shown indicating that the phospholipids can be spread out when the temperature of the sample is rapidly increased. Paper II shows the ability to do mirror images of freeze fractured PC12 cells with the device as well as some results from sub cellular localization of phospholipids in a single PC12 cell. The new freeze fracture device increases the ease by which one can analyze single cells in the frozen and hydrated state with imaging mass spectrometry instruments since no special sample entry chamber is necessary using the device.

7.2 PAPER III

This paper presents the relative quantification of incorporated exogenous phospholipids into the membrane of single PC12 cells. Deuterated phospholipids, which create fragment ions with a specific *m/z*, are used to incubate the cells. The counts from the deuterated phospholipids are then related to the endogenous phospholipid counts in the cell, which gives the relative amount of exogenous phospholipid that is accumulated in the plasma membrane. The results show that after an overnight incubation with a 100 µM deuterated phospholipid the amount of deuterated phospholipids present in the plasma membrane of PC12 cells are 0.5 % for PC and 1.3 % for PE. In combination with previous studies on exocytosis in PC12 cells these results indicate that the phospholipid composition of the plasma membrane is of high importance for the process of exocytosis.

7.3 PAPER IV

This paper describes a new method to detect and relatively quantify the abundance of anammox bacteria in sediment samples using an intact ladderane phospholipid as a biomarker. Besides evaluating different incubation and extraction methods the paper provides a RPLC method to separate PC species based on tail group hydrophobicity. Finally the paper provides information on anammox abundance in a sediment sample depending on depth. The selected phospholipid biomarker was found in the upper sediment layer in agreement with previous nutrition profiles and expectations, whereas another ladderane phospholipid is not that specific. The development of this method can potentially contribute highly to research of anammox bacteria in natural environments. In the future this can lead to an increased understanding of the importance and vulnerability of these bacteria on earth.

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