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Effects of lipid geometry on vesicle fusion kinetics in an artificial cell model for exocytosis.

Master of Science Thesis

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Abstract

The process of stimulated exocytosis is fundamental to neuronal communication. This involves fusion of neurotransmitter-filled vesicles with the plasma membrane of the presynaptic terminal, diffusive transport across the synaptic cleft and recognition of and binding to receptors on the postsynaptic membrane surface. This fusion is thought to go via the formation of a number of intermediates structures described by the stalk theory. It results in the formation of a fusion pore or nanotube, which then expands to incorporate the vesicular material in the surrounding plasma membrane. In this thesis we describe the use of a model system for studying the effect of lipid composition on the fusion dynamics in the final stages of exocytotic fusion. Surface-immobilized multilamellar liposomes created from soy bean lipid extract in a dehydration/rehydration process were manipulated into forming a tubular structure in the inside of a unilamellar liposome. This tube was then brought to inflate into a vesicle, which fused with the membrane and released the contained catechol solution. By varying the composition of lipids and by substitution with either 30%phosphatidylcholine or 30% phosphatidylethanolamine we wanted to investigate the effect of different lipid geometries on the vesicle fusion kinetics during exocytosis. Detection of release profiles and kinetics was done with amperometry. Although more experiments are needed to confirm the results presented here, the data so far suggest that a 30% increase of phosphatidylcholine into the membrane composition affect the kinetics during the final stages of exocytosis by increasing the rate of chemical release. However, by increasing the amount of phosphatidylethanolamine a slight increase in fusion speed was detected but was not concluded to be significantly different from the control measurements.

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1. Introduction

Neurons are the physical infrastructure that we rely on for both internal signaling (controlling the central nervous system) and external interaction with the surrounding including sensing, cognitive functions and communication with other individuals. Our brain is an amazingly advanced circuitry system consisting of billions of nerve cells and the average neuron has around 7,000 connections[2] to other neurons; a delicate and balanced system that when functioning fully is an extraordinary tool to help us make sense of the world. Unfortunately, this is not always the case and neurodegenerative diseases such as Parkinson's and Alzheimer's can severely impair the quality of life for affected individuals[3]. Thus, the value of a detailed understanding of neuronal communication cannot be understated.

Due to immense complexity of our nervous system, understanding and treating the arising problems of neurological dysfunction is indeed a tedious task. Although the challenge at times might seem too great, it is with the common effort as the scientific community that we move forward with every discovery, however minute it might seem. This is the importance of basic research, fundamental studies serve to guide work towards a new and improved of treatment strategies.

Exocytosis is the general process of chemical signaling between two synaptic neurons. Expulsion of intracellular material through the cell membrane by fusion of neurotransmitter-filled vesicles to the plasma membrane of the cell and resulting release of the neurotransmitter molecules to the extracellular space provides a chemical bridge over the synaptic gap for the electrical signals carried along the axon. The process is activated when an action potential is generated that depolarizes the cell plasma membrane and induces voltage gated Ca^{2+}-channels to open up. The flux of Ca^{2+}-ions into the cytoplasm trigger neurotransmitter-filled vesicles to dock and fuse with the plasma membrane and the neurotransmitter molecules bind to receptors on the postsynaptic membrane and the signal is carried on along the next axon[4].

It is known that depending on the molecular geometry of lipids they have different preferences for the formation and organization in bi-layer structures. Cylindrical lipids have a clear preference for packing into flat lipid bi-layers, whereas conical lipids prefer to form geometries with high curvature. Depending on the composition of lipids with various geometries in the bi-layers they exhibit different membrane dynamics as they undergo fusion[1].

Even though exocytosis has been widely studied it is a highly complex process and all the details of its functions are still not fully understood. In order to obtain richer and more precise explanatory models of our nervous system these secrets must be unraveled. A large amount of the fundamental research in this field has been performed *in vitro* to try to understand the underlying mechanisms of exocytosis. Many of these *in vitro* studies have been performed at single cells that release neurotransmitter molecules in a Ca^{+2} dependent fashion. Commonly used methods to investigate this process include electrochemical detection such as amperometry, cyclic voltammetry, patch clamp, fluorescence imaging and transmission electron microscopy (TEM).

In bottom-up approaches, artificial cell model systems have been developed where the minimal components necessary to mimic exocytotic events are studied. One of these model systems[5] uses liposome-lipid nanotube networks to study the lipidic contribution to membrane dynamics in the final stages of exocytosis preceding fusion pore formation. Surface-immobilized liposomes are prepared from soy lecithin and a micropipette is inserted through a unilamellar liposome by disruption of the membrane with a transient electric field. This enables formation of a nanotube, on the inside of the unilamellar liposome, which is inflated into a vesicle that fuses with surrounding membrane. By placing an amperometric electrode in close proximity to the artificial cell surface, detection of solution released by oxidation of electroactive substance as the molecules reach the electrode surface provides kinetic information about the exocytosis process.

In this master project, I have used the same artificial cell model system and used this to study vesicle release kinetics as a function of augmentation of lipids with various geometries. Three different liposome compositions were prepared from soybean lipid (SBL) extract into which two were supplemented with either cylindrical shaped lipids, or cone shaped lipids. These preparations were then used to study the effect of lipid geometry on membrane dynamics during the vesicle release process.

The major goal of this bottom-up approach of creating a protein-free artificial cell model is to greatly reduce the molecular complexity present in living cells and study the contribution of lipid composition alone on the membrane dynamics, during the final stages of exocytosis.

1.1 Scope

This project was limited to studying the release kinetics of the final stages of exocytotic fusion at three different artificial cell preparations where the lipid compositions are augmented with cylindrical and cone shaped lipids. Specifically, a 30% w/w substitution of soybean lipid extract (SBL) with phosphatidylethanolamine and phosphatidylcholine lipids extracted from the soybean will be performed. The augmented lipids will be added during initial liposome preparation, hence ending up in both the proximal and distal leaflets of the liposome membranes. The effect of lipid geometry on membrane dynamics at artificial exocytosis where then evaluated by comparing these two augmented preparations with the artificial cells made of 100% soy bean lipid extract.

2. Theoretical Background

2.1 Neuronal communication

The neuron (or nerve cell) is the basic building block of the nervous system. It creates a pathway for carrying information and its fundamental functions are receiving, conducting and transmitting electrical and chemical signals to other cells. Some of these cells communicate over long distance in the body they are usually extremely long and a single nerve cell in humans may be up to 1 meter[4].

The neurons are constructed of a cell body, dendrites and an axon. Dendrites are antennae-like branches extending from the cell body and are responsible for the process of receiving signal input from other neurons. The axon is a thread-like extension through which neuronal information is conducted to distant targets, where the axon branches out into axon terminals and delivers the signals to multiple target cells simultaneously. This is achieved by the connections of terminal axon branches of one cell to the dendrites or the cell body of the receiving target cells.

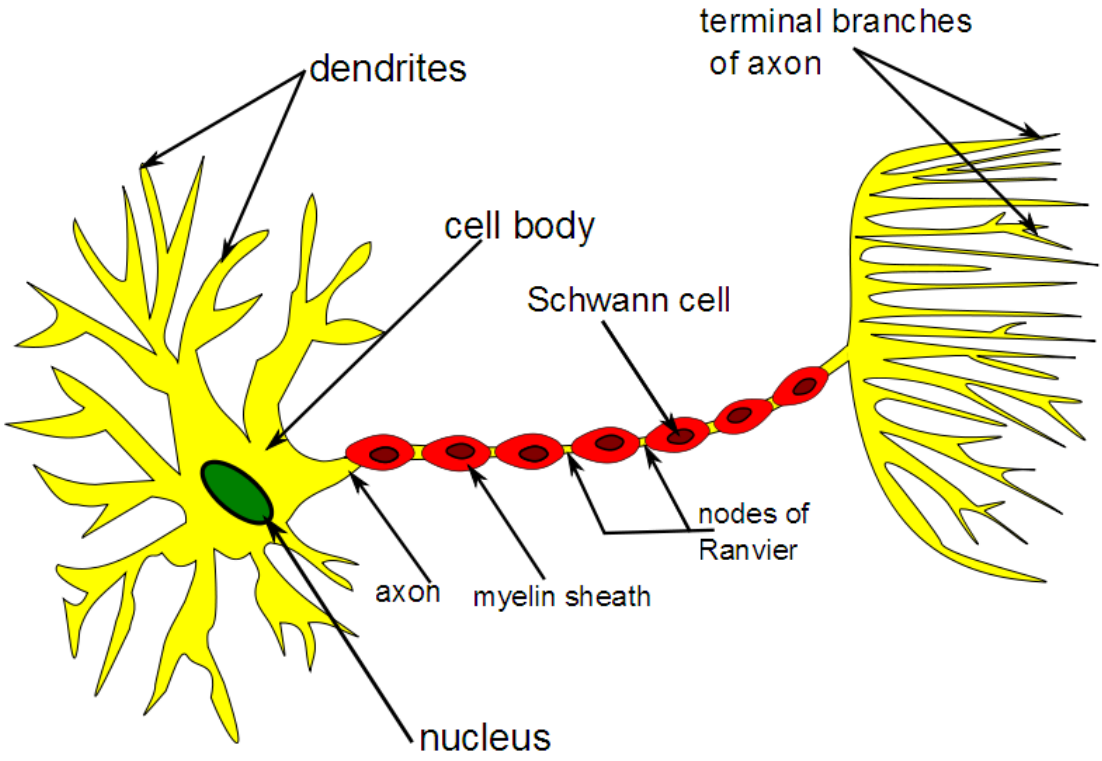


Figure 1. A schematic representation of a neuron showing the principal structure with the cell body surrounding the nucleus and branching out into the dendrites which act as the receptive part of the neuron. A single axon extends form the cell body; it is covered with myelin sheaths, which help to conduct the action potential, and ends in terminal axon branches that connect to multiple target cells. The Schwann cells are responsible for the formation of the myelin. [4]

The connecting interface between an axon terminal and the target dendrite is called a synapse. The axon terminal accounts for the presynaptic terminal and the dendrite for the postsynaptic terminal. These are separated by a small gap called the synaptic cleft. When the axon transmits an electrical signal to the pre-synaptic terminal, neurotransmitters are released into this synaptic cleft. From here these signaling molecules diffuse over to the postsynaptic membrane, were they bind to receptors on the postsynaptic terminal and the signal is carried on by the dendrite of the receiving cell.[4]

2.1.1 Neuronal signaling

The means by which signals are carried within and between neuronal cells are of both electrical and chemical nature. The electrical signals are generated by temporary differences in ionic current flux over the cell membrane that changes the electrical potential over the membrane,

known as membrane potential. The membrane potential arises from a gradient of ionic species between the outer and the inner membrane and is seen as in a thin layer of cations on the extracellular membrane surface and anions on the cytoplasmic membrane surface. The normal value in neuronal cells at rest is -70mV and is called the resting membrane potential.[6]

What maintains this ionic gradient and following membrane potential in a cell at rest is partly the fact that the lipid bilayer of the membrane is impermeable to diffusion of ions, which would otherwise balance out this difference in concentration, and partly due to the action of resting ion channels. Ion channels are transport proteins which allow specific ionic species to traverse the cell membrane. When the cell is at rest these channels are open. These two factors and the fact that different ionic species are present in different concentrations in cytoplasmic and extracellular fluids result in two opposite driving forces. The first is the chemical driving force that arises from the concentration gradient. This gradient is established by the Na^+/K^+ pump and makes the ion diffuse in the direction of a lower concentration. However, since the counter-ion of the moving ion cannot traverse the cell membrane, there will be a build-up of charge separation between the cytoplasmic and the extracellular side. This gives rise to an electrical driving force which will act on the moving ion in the opposite direction. As more ions flow out of the cell this force increases. At a certain point these two forces will balance each other out and the equilibrium value at which this happens is determined by the transport rates of the resting ion channels and how many of them are present in the membrane.

The starting point of electrical signal transduction within a neuron is when the dendrite receives the chemical messengers of a transmitting cell and binds to its receptors. Upon binding of a neurotransmitter, depending on its nature, it causes ion channels to either open or close and the permeability of the cell membrane to different ions is changed. Compounds that decrease the membrane potential (hypopolarization) are called excitatory neurotransmitters and the ones which increase the membrane potential (hyperpolarization) are called inhibitory neurotransmitters. When the membrane is hypopolarized and the potential reaches some threshold, typically ≈-65 mV, the axon is excited and an action potential is generated.[7]

The action potential is a depolarization of the cell that moves along the axon and includes activation/deactivation of a number of different ion channels. As the potential is lowered to the threshold of excitation voltage-gated Na^+ channels are opened. The positive sodium ions rush into the cell, resulting in a rapid decrease in membrane potential and subsequent opening of more Na^+ channels. Once the process has started it amplifies itself until all the channels are activated and within less than a millisecond the membrane potential has reached a value around +40-50mV.[4] With all the Na^+ channels open the cell would eventually stabilize in new “resting potential” if it was not for the coupled activation of K^+ channels, allowing potassium ions to leave the cell, that occur alongside this process. Furthermore, the Na^+ channels are inactivated and closed to prevent more influx of sodium. These two processes return the depolarized cell membrane to its original resting potential at -70mV, where the Na^+ channels are activated again (remaining closed) and the cell is ready to receive and transmit a second impulse.

A wave of depolarization moves along the axon with ion channels opening and closing rapidly. However, this is only possible at the nodes of Ranvier, the part of the axon which is not covered in myelin and has its membrane in proximity to the extracellular matrix. In the myelinated parts of the axon, which are highly insulated, current is carried rapidly from one node to the next and causes the ion channels of that node to open and the depolarization is propagated. This kind of jumping transmission of signals from node to node is called saltatory conduction[8] (from latin *salto* – jump).

As the action potential reaches the presynaptic bouton at the terminal axon, voltage gated Ca^{2+}channels open up and calcium ions flow into the cell. Inside the cell, the calcium ions promote the fusion of secretory vesicles with the cell membrane and neurotransmitters are released into the synaptic gap via a process called stimulated exocytosis. Once the molecules diffuse across the gap, they attach to receptors on the postsynaptic membrane on the dendrites of the receiving neuron. Na^{+} channels are opened up, an action potential is generated, and the process starts anew.

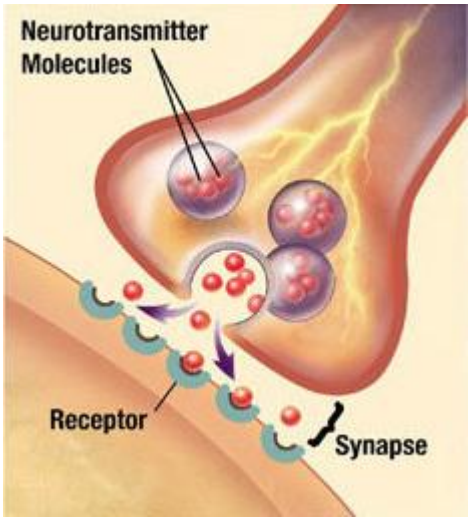


Figure 2. A picture showing the presynaptic bouton, the synaptic cleft and the postsynaptic membrane with receptors. The action potential triggers the release of neurotransmitter filled vesicles that merge with the plasma membrane of the presynaptic bouton, diffuses into and over the synaptic cleft and attach to receptors on the postsynaptic membrane surface. [From Wikimedia Commons Foundation]

2.2 Exocytosis

There are two alternative ways by which exocytosis can occur, namely constitutive exocytosis and regulated/stimulated exocytosis. Constitutive exocytosis is an ongoing process which takes place in all cells without the need for any external stimuli or signaling and is primarily responsible for maintaining a continuous incorporation of new membrane proteins and lipids into the plasma membranes. Stimulated exocytosis is a more specific and specialized process and is only found in more functionalized cells as for example neurons and it is the form of exocytosis that will be of relevance to this thesis. This process needs to be activated and remains passive until the cells receive some kind of stimuli, where an increase of Ca^{+2}-ion concentration is one of the most

frequently utilized ways of signaling and activation. Among the functions which are regulated by this process are release of for example neurotransmitters, enzymes and hormones etc. and also incorporation of functional compounds as receptors and trans-membrane transporters into the plasma membrane.[9]

The fundamental mechanism of exocytosis, or called vesicle fusion, is a process where the membrane of a synaptic vesicle merges with the plasma membrane, inducing mixing between the two lipid bilayers and finally forms a 2-5 nm fusion pore which creates a channel between the vesicular lumen and the extracellular space. The fusion pore is thought to further expand and finally collapses the vesicle into the cell membrane, extruding the contained material out into the extracellular space and this is called full exocytosis[9]. Alternatively, the fusion pore can release part of the vesicle content through the pore opening before re-closing, in what is called "kiss 'n run" exocytosis[10]. In either case this requires energy provided by protein machinery.

2.2.1 The SNARE Theory

Intracellular trafficking of secretory vesicles in nerve cells is a complex system. Different neurotransmitters have different effects depending on how and where they are released and biological systems contain a vast number of different membrane systems. Ensuring that each vesicle is delivered and fused at the intended membrane surface is key and the cells have developed a system of placing highly specific "tags" on each "cargo load" to increase the precision in this process.

The vesicle markers carries information as to identify what they contain, where they are supposed to end up and from where they originated. Each target site of delivery has a complementary receptor whereas to be able to recognize and help promote fusion when the right vesicle comes along. A proposed system for these functions is a group of proteins called SNARE-proteins (Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor)[4, 7, 11].

The proteins responsible for exocytosis of synaptic vesicles are synaptobravin, syntaxin and SNAP-25[4, 6]. Synaptobrevin is a vesicle-anchored membrane-protein and is therefore also called a vesicle (v)-SNARE protein. Syntaxin and SNAP-25 are membrane-proteins attached to the plasma membrane at the target site and are part hence called target (t)-SNARE proteins. The active parts of these proteins are helical regions that wind around each other to form a protein complex which draws the vesicle and membrane closer. This stage is known as vesicle docking and ensures that the vesicle stays locked to the target

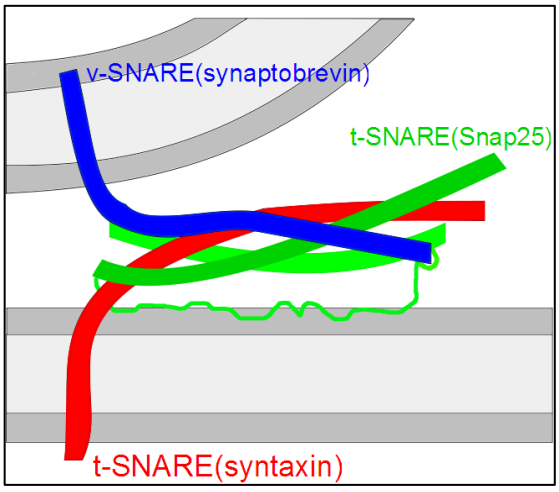


Figure 3. Synaptobrevin (blue), SNAP-25 (green) and syntaxin (red) forming a four-helix bundle of α-helices.

membrane and ready for release upon Ca^{+2}-influx.

The formation of the SNARE-protein complex in synaptic vesicle fusion is thought to start with the association of the two t-SNAREs syntaxin and SNAP-25 into a binary protein structure. This structure is responsible for the recognition and binding of the vesicle-anchored membrane protein synaptobrevin. Binding occurs as to position the membrane-bound parts of both the v- and t-SNARE on the same side (see figure 3). Together, the proteins form a four-helix bundle of α-helices where SNAP-25 contributes two and syntaxin and synaptobrevin contributes with one each[11].

However, docking of a vesicle in the vicinity of the target membrane does not always result in fusion. In order for the vesicle membrane to fuse with the plasma membrane they must come in close contact to each other and bridging the gap to form lipid-lipid continuity between the two lipid bilayers. For this to be possible water needs to be displaced from the hydrophilic membrane surfaces which is a highly energy-demanding process[4].

SNARE proteins are thought to help overcome this energy-barrier of surface hydration by using the energy that is freed when the hydrophobic helices are coiled to draw the vesicle closer to the membrane and subsequently push the water molecules away from the membrane interface as seen in Figure 4a. As the water is displaced (Fig 4b) the proximal leaflets of the two membranes come into contact and form a stalk of lipids joining the membranes (Fig 4c). When the SNARE-complex winds even tighter the distal leaflets join as well and an intermediate called hemifusion is formed (Fig 4d). Finally, the single bilayer in the hemifusion collapses into full fusion and the remaining channel is called a fusion pore (Fig 4e). The different steps from association of SNARE-protein to promoted synaptic fusion are shown in figure 4 below.

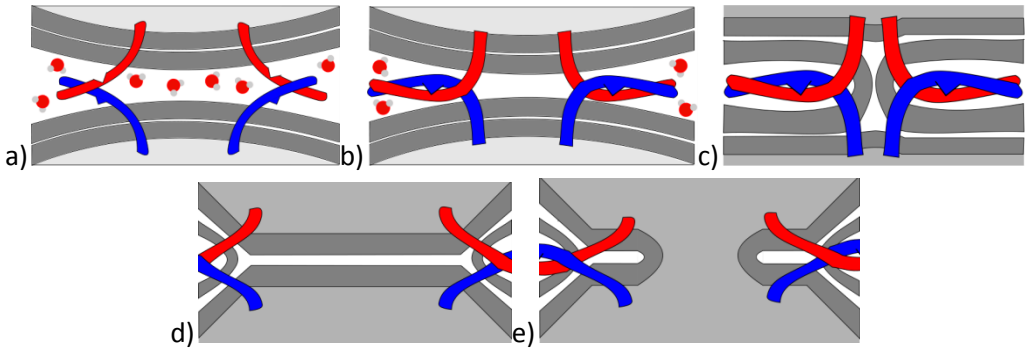


Figure 4. a) Association of v-SNARE and t-SNARE. b) Recognition and binding of complementary helices. c) Formation of lipid-lipid continuity. d) Establishing a single bilayer by joining the inner leaflets of the two membranes. Known as hemifusion. e) Hemifusion collapses into full fusion and a fusion pore, providing continuity between intra-vesicular and extra-cellular matrix, is established. Diffusion of neurotransmitters. (GREEN SNAP-25 have been removed in a-e for a simpler schematic representation)

2.2.2 Membranes composition and lipid geometry

Biological membranes are selective barriers, which serve as to divide and define boundaries of various intra- and extracellular environments. This structure with the main constituents being amphiphatic lipids and proteins maintains physiological conditions such as pH and ion concentrations. The lipids arrange into a 5 nm[4] thick, fluid bilayer with the hydrophobic tails facing each other and the hydrophilic head groups pointing towards the aqueous phases, thus providing a basic spatial matrix into which proteins and enzymes are incorporated as to provide different functionalities.

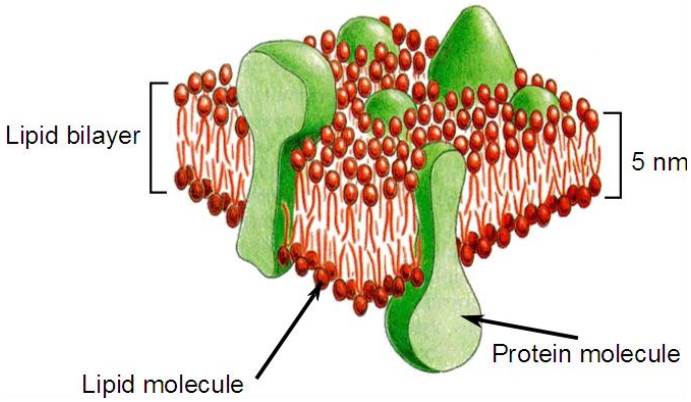


Figure 5. Schematic representation of a biological membrane. A 5 nm thick bilayer of phospholipids constitute the basic matrix into which membrane-embedded proteins and enzymes are incorporated whereas to provide various functionalities (Picture from http://math.lanl.gov/~yi/lipid.html)

The most common lipid in biological membranes are phospholipids[4]. They are large organic molecules, generally composed of two hydrophobic fatty-acid groups and a hydrophilic polar head-group including a phosphate moiety linked to a small organic molecule. Depending on which groups are present, each lipid will have different characteristics such as shape, size, head-group polarity and fatty-acid chain rigidity and these are determining for how a lipid bilayer is arranged and what dynamic properties it will possess.

As mentioned previously, the main focus of this thesis will be on lipid geometry and its effect on vesicle membrane fusion. Generally speaking, there are three different forms of lipid geometries and these are cone shape, cylindrical shape and inverted cone shape[1,12,13].

As seen in figure 6 the lipids will arrange themselves into monolayers with different curvature, depending on their inherent geometries[1, 13]. As biological membranes are composed of several different lipids[14] the dynamic properties and most energetically favorable geometry under unstrained conditions will be determined by the overall lipid composition. These properties are of key importance in regulating whether or not a vesicular membrane will fuse with the plasma membrane during exocytosis[12].

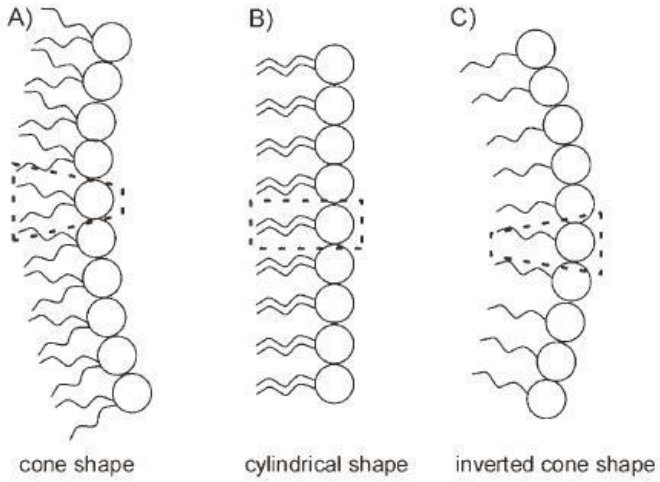


Figure 6. A picture showing the intrinsic curvature of different lipid geometries when forming non-stressed monolayers[1]. A) negative curvature, B) zero curvature , C) positive curvature[1]

There are two general opposing ideas proposed of how the fusion pore through the two joining phospholipid bilayer is achieved during exocytosis[15,16,17]. One states that the initial formation of the fusion pore is brought about by proteins that enter and span both bilayers and upon a conformational change creates an aqueous channel followed by an expansion. The other model claims that the initiation of the fusion pore is purely lipidic at its core and that proteins only act as to draw the fusing bilayers into close proximity of each other and that the fusion occurs spontaneously as they come into contact. As the model system used in this thesis is purely lipidic[5], no emphasis will be put on proposed proteinaceous models of fusion pore formation but focus will instead be directed on the prevailing lipidic model "stalk theory" [18 ,19].

As illustrated in Figure 7, the stalk fusion model is initiated in membranes in close proximity by a point-like protrusion[20], which is a local highpoint in membrane topology that lowers the energy of hydration repulsion between the two contacting leaflets[13]. Upon contact, the two monolayers of the proximal leaflets fuse (1→2) to form a stalk that allows

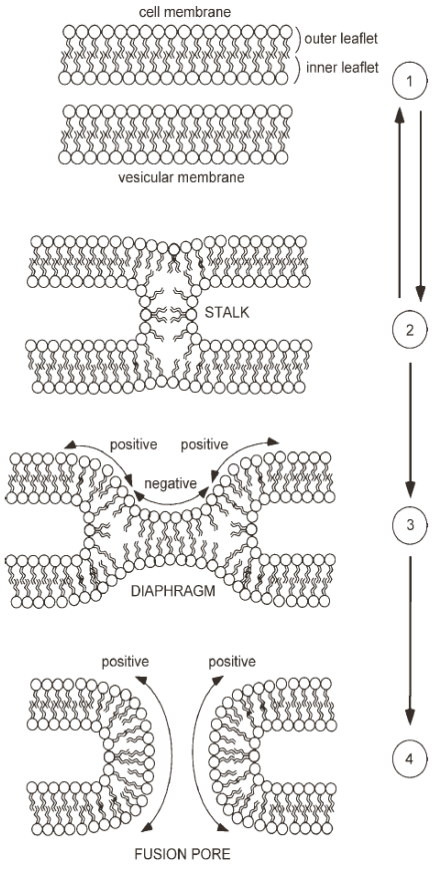


Figure 7. Schematic representation of the different intermediate steps of fusion through stalk theory[1]

for lipid-exchange between the proximal but not the distal leaflets. As an increase of the curvature of the inner monolayers are energetically favorable[21,18] the stalk is expanded further to form (2→3) what is usually called the hemifusion diaphragm. The hemifusion diaphragm ruptures (3→4) into the fusion pore.

Live cell studies of stimulated exocytosis of cathecholamines in adrenal chromaffin cells have shown that incubation of micromolar quantities of lysophosphatidylcholine (LPC) and arachidonic acid (AA) to the outer leaflet of the plasma membrane altered the release kinetics[1]. LPC, which has inverted cone shape geometry, promoted fusion rate, frequency and charge released whereas AA, with cone shape geometry, inhibited the fusion process.

A study of fusion of two phospolipid bilayer performed by Chanturiya et al 1995[22], showed a similar effects of fusogenic promotion alternatively inhibition upon insertion of LPC or AA. LPC inserted into the contacting monolayers in fusion (corresponding to inner leaflet in cell) showed an inhibitory effect and promotion when supplemented to the distal monolayer (outer leaflet). Insertion of AA to the contacting monolayers resulted in promotion of fusion and when added to the distal leaflets it was inhibitory.

A more recent article by Ewing et al 2007[23] shows alteration in vesicle fusion kinetics from incubation of various phospholipids in PC12 cells. Augmentation of phosphatidylcholine (cylindrical shape) tended to slow down the expulsion and reduce the amount of released neurotransmitters whereas phosphatidylethanolamine (cone shaped) accelerated the release process.

2.3 Methods for investigating membrane fusion

There are a number of methods available and utilized in the studies of membrane fusion and exocytosis. The most commonly used methods are either various forms of electrochemical detection or optical microscopy methods for visualization. In most setups an electrochemical method is coupled with microscopy to ease manipulation of electrodes and micropipettes.

2.3.1 Amperometry

Amperometry is a quantitative electrochemical technique that has been widely used in the study of exocytosis. The basic principle is that a detection electrode held at a constant potential that is above the oxidation potential versus a reference electrode for the oxidation potential of the neurotransmitter molecule that will is to be detected. The electrode is usually a microelectrode and is placed in close proximity to the surface of the cell plasma membrane[23] of a cell or the outer membrane of an artificial cell model[5,24]. When a cell is stimulated to exocytosis, the release of neurotransmitters can be detected as a transient current as the neurotransmitter molecules are

oxidized at the surface of the electrode. Key advantages of this method are high temporal resolution and precise quantification of discharged intra-cellular material[24].

By analyzing the shape and size of each transient one can obtain information about several different aspects of the exocytotic event. The area of a peak, which corresponds to the charge transferred at the electrode surface due to the oxidization of neurotransmitter molecules from a single vesicle released. By utilizing Faradays law (equation 1) one can therefore calculate the number of moles of neurotransmitter released during each single vesicle release event. For example, the oxidation reaction of catechol, which is the electro active molecule used in the artificial cell model is shown in figure 9. Integration of a current peak (figure 8) with respect to time will give us the total charge transferred (Q) and since it is two-electron (n=2) process Faraday's law tells us that the amount of neurotransmitter molecules released from a single fusing vesicle can be calculated by taking half the value of Q and dividing it by the Faraday's constant.

Faradays law: $N = Q/nF$

$Q = c\square arge\ transferred$
$N = number\ of\ moles$
$n = number\ of\ electrons\ transferred$
$F = Faradays\ constant = 96485\ Coulomb/mol$

Other peak parameters can provide kinetic information about vesicle fusion and neurochemical release. The amplitude of the peak gives the maximum flow of neurotransmitter, the half width which is the width of the peak at half of the maximum amplitude, gives the duration of the exocytotic release, the rise time of the peak is dependent of how fast the fusion pore opens and the decay time is thought to correspond to how fast the fusion terminates[25]. Definitions of these parameters are visualized in figure 8. The mass-transport of the transmitter consists of two parts, flow and hemi-spherical diffusion[26] and because of this diffusive contribution it is important to keep the distance between the fusion pore and the measuring electrode to a minimum, whereas otherwise one would get a diffusive broadening of the peak and following distortion of the acquired data.

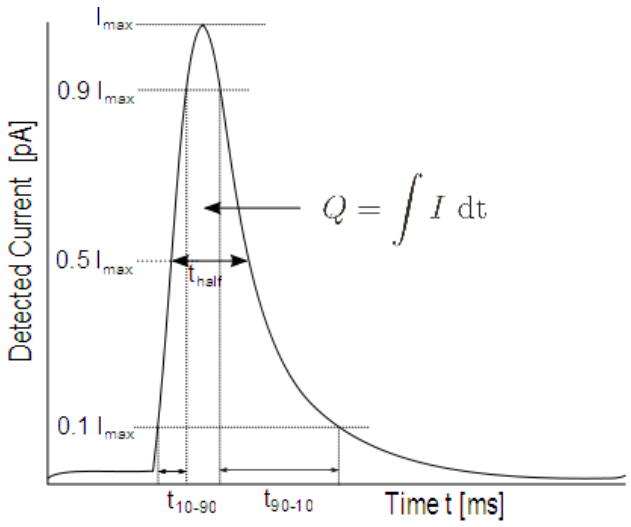


Figure 8. Definition of peak parameters for a stylized electrochemical trace for an exocytotic event. Q is the area of the detected peak, t_{half} is the half-width, t_{10-90} is the rise time, t_{90-10} is the decay time and I_{max} is the maximum peak height.

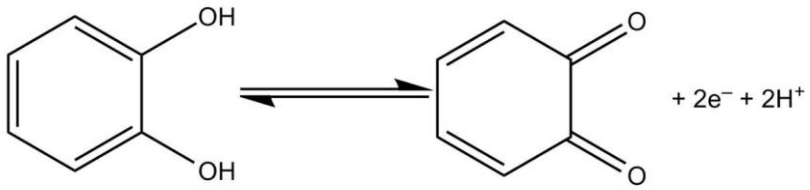


Figure 9 9. Catechol being oxidized to 1,2-benzoquinone resulting in the loss of two protons and two electrons.

2.3.2 Cyclic voltammetry

Cyclic voltammetry (CV) is a qualitative technique that, as amperometry, also has been of great use in the exploration of exocytosis. The basis of CV is that, as opposed to amperometry, the potential of the working electrode is ramped linearly with time and when the maximum set potential is reached the potential ramp is reversed. Scanning the working potential of the electrode results in what is known as a cyclic voltammogram, which is characteristic for the analyte in solution. Since these voltammogram shows the electrochemical characteristics of the analyte it is possible to distinguish between different neurotransmitters[25]. To reiterate, the main difference is that amperometry is suitable for quantitative detection of neurotransmitter however it lacks the ability to discern between different chemical compounds which cyclic voltammetry has.

2.3.3 Patch-clamp

Another technique that has been used to investigate both exocytosis in general and the fusion pore formation is the patch-clamp technique. In patch-clamp, a micropipette is pressed against the proximal side of the plasma membrane to form what is called a "giga-seal" (10^9 ohm > electrical isolation)[25,27]. Once this chemical and electrical isolation has been established, the small membrane-patch covering the opening of the micropipette may be removed by applying a small negative pressure and this results in a continuity between the intracellular environment and the inside of the pipette. This mode of using patch-clamp is commonly known as "whole-cell". Connecting the pipette to instrumentation for current amplification and placing a reference electrode in the extracellular environment finalizes the setup and one is able to measure ionic currents[25].

In observing a plasma membrane with the patch-clamp technique two types of ionic currents can be measured. The first is a transmembrane current which arises from the permeation of ions through ion channels. The other is a capacitive current which is due to the ionic flow which is required to maintain a desired membrane potential. Since biological membranes have a specific charge per membrane-area, changes in the capacitive currents can be used to measure changes in membrane area associated with incorporation of membrane material from secretory vesicles. In the case of the transmembrane current, what is detected is an actual flow of ions across the membrane and changes in this current can be used to measure opening/closure or incorporation of ion channels to the membrane[25,28].

2.3.4 Fluorescence microscopy imaging

It is possible to use fluorescent moieties as means to detect and observe vesicle fusion. For example multilamellar vesicles containing fluorescent dye have been utilized as an optical indicator of vesicle fusion with a planar lipid-bilayer membrane[29]. Fluorescence microscopy is a powerful tool for observing phenomenon occurring in real time, provided a suitable fluorophore is available.

FM1-43 is an activity-dependent flourescent dye[30] from a family of styryl compounds that has been utilized in studies of exocytosis[31]. What distinguishes the FM dyes from other fluorescent dyes and makes them highly suitable for assays regarding exocytosis and exchange of membrane-material is that they are virtually non-fluorescent in water but when introduced into a hydrophobic environment their quantum yield increases approximately 350 fold. Carrying a permanent 2+-charge on the head-group inhibits diffusion through the stained membrane and also prevents so called "flip-flop" which is when a molecule in the proximal leaflet of a lipid-lipid bi-layer jumps to the distal leaflet or vice versa. These properties enable selective labeling of secretory vesicles formed in an environment with the dye present.

FM1-43 is but one of the fluorescent dyes in the FM dye family. Another compound is for example the FM2-10 dye which has ethyl-groups linked to the nitrogen in the tail-group instead of butyl-groups whereas to reduce its hydrophobic affinity. By modifying the length of the

conjugated π-system in the linker between the head- and tail-group one can tune the fluorescent wavelength and this opens up for the possibility of several distinct markers being used simultaneously.

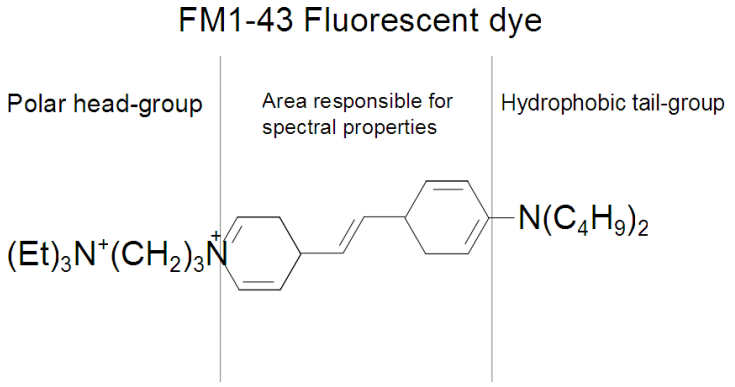


Figure 10 A schematic division of the functioning groups of the FM1-43 fluorescent dye. The polar head-group carries a permanent 2+-charge and ensures that the dye does not diffuse through the stained membrane. The structure of the hydrocarbon linker between the head- and tail-group is responsible for fluorescent properties and the length of the hydrocarbon groups attached to the terminal nitrogen in the tail-group governs the hydrophobic affinity of the dye.[30]

2.3.5 TIRF microscopy (Total Internal Reflection Fluorescence)

TIRF microscopy is a fluorescence technique based on the optical phenomenon of total internal reflection[32]. When a beam of lights hits the interface between two optical media with different refractive indices and the interface will act like mirror and reflect the beam if two criterion are met.

These criterions are that $n_1 > n_2$ and that the angle θ_i of the incident beam, relative to the interface of the two media, is larger than the critical angle θ_c , determined from $\theta_c = \arcsin(\frac{n_2}{n_1})$

Due to interference between the reflected and the incident light an evanescent wave (standing wave of light) is generated and as we move away from the interface the intensity of this evanescent wave will decay exponentially.

$$E(\psi, z) = E_0(\psi)e^{\left(-\frac{z}{d_p}\right)}$$

$$E(\psi, z) = intensity\ of\ wave\ at\ distance\ z$$
$$E_0(\psi) = intensity\ of\ wave\ at\ medium\ interface$$
$$d_p = \frac{\lambda_0}{2\pi\sqrt{sin^2\theta_i - (\frac{n_2}{n_1})^2}} = dept\square\ of\ penetration$$

Utilizing the phenomenon of TIR (Total Internal Reflection) and combining this with normal fluorescent spectroscopy, TIRF microscopy results in a technique that allows for fluorescent excitation of a dye in a limited distance from the microscope glass slide into the aqueous layer

situated on top of it. When placing a cell on the glass slide, secretory vesicles stained with dye will be visible only a few hundreds of nanometers above the surface as the exponential decay of the evanescent wave's intensity will drop too much to excite any of the labeling molecules found above this cut-off region.

As most of the steps preceding actual fusion of a secretory vesicle take place at a nanometer distance from the plasma membrane this selective excitation of fluorescent markers is very suitable for studying exocytotic events. Instead of seeing all of the stained vesicles, TIRF microscopy limits detection to vesicles near to or undergoing fusion with the plasma membrane and a fusion event can be detected as diffusion of released fluorescent content[32].

2.4 Cell model studies

2.4.1 Vesicle fusion with a planar lipid-bilayer membranes

One of the first successful experiments showing vesicle-membrane fusion in a purely lipidic system was done by Zimmerberg et al. in 1980[29,28]. Using a system of multilamellar phospholipid liposomes with an aqueous fluorescent dye in the aqueous compartments of the liposome and membrane-associated marker in the form of a voltage-dependent anion channel (VDAC) they managed to show fusion of the liposomes with a planar lipid bilayer.

Introducing the fluorescent stained liposomes in the *cis* compartment of a dual-chamber system, once the liposomes fused with and traversed the separating lipid bilayer a single fluorescent particle would emerge in the *trans* compartment[29]. Incorporation of the membrane-markers were detected as stepwise increase in transmembrane capacitance and the simultaneous (<200µs) insertion of several VDACs ruled out non-fusion transfer as a mechanisms[28].

2.4.2 Artificial synapse – insights into exocytosis

The basic artificial cell model used in this thesis is based on a model that was developed by Cans et al using a purely lipidic system that simulates membrane fusion during the final stages of exocytosis[5].

Here, surface-immobilized unilamellar liposomes were prepared by a dehydration/rehydration method from soy lecithin. These liposomes were manipulated with an electrified glass micropipette and an electroporation electrode to create an artificial synaptic vesicle filled with electroactive substance connected to the artificial cell membrane by an elongated fusion pore consisting of a lipid nanotube.

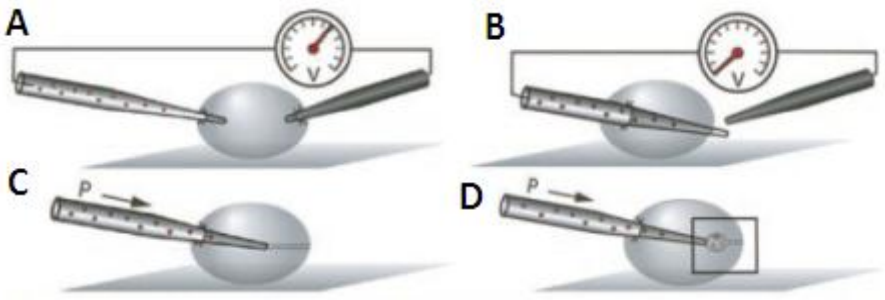


Figure 11. Schematic represenatation of micromanipulation of unilamellar liposomes. A) A conducting glass-micropipette and an electroporation electrode is used to disrupt the membrane by applying a transient electrical field. B) Glass pipette is inserted to and throughout the liposome. C) Pulling back of the pipette results in the formation of a tube. D) Increasing the pressure inflates the tube and initiates vesicle formation[5].

This is illustrated in Figure 11, where injection of glass micropipette through the bilayers of a unilamellar liposome (A-B) was achieved by briefly applying an electric field and thus disrupting the membrane integrity. The pipette was pulled back (C) to form a tube which was then inflated to form a vesicle (D) containing fluorescent dye and catechol.

Upon fusion, the diffusion profile was monitored with fluorescence microscopy coupled with amperometric detection to define the different stages in vesicle content release. Reference experiments of stimulated dopamine release from PC12 cells showed that even though the release events from the artificial model occur on a longer time-scale, they were qualitatively similar and thus proved the validity of the artificial synapse as model system for membrane fusion during exocytosis.

3. Methods and material

3.1 Cleaning of glassware

Round-bottom flasks used for liposome preparation were cleaned to make sure that no contaminants were present during synthesis. The flasks were submerged in water in a beaker, filled with a 1% Deconex solution and placed on a heating plate at 60°C. After 20 minutes the flask was emptied out, refilled with fresh Deconex solution and kept at 60°C for an additional 20 minutes. Furthermore, the flasks were rinsed five times with mQ-water, left at 100°C in an oven for 2 hours to dry out excess water and finally sealed with saran wrap to keep fresh.

3.2 Potassium phosphate buffer

In liposome synthesis a potassium buffer (KPi-buffer) of 5mM Trizma base, 29 mM KH_2PO_4, 11 mM K_2HPO_4, 15 mM K_3PO_4, 0.5 mM EDTA and 1mM $MgSO_4$-$7H_20$ in mQ-water was used to mimic physiological conditions. The buffer was prepared for a pH=7.4.

3.3 Solution with Neurotransmitter analogue

The artificial synaptic vesicles were filled with an electroactive neurotransmitter analogue solution consisting of a 5 mM catechol stimulation. This solution was prepared fresh before each experiment by adding 50µl of 0.1 M catechol in $HClO_4$ to 950 µl of KPi buffer. In time for the experiment the solution was injected into a glass micropipette using a 1ml syringe fitted with a flexible micro-needle.

3.4 Lipsosome preparations

The liposomes preparation consisting of 100% soy lecithin where prepared by adding 40 µl of 25µg/µl soybean lipid extract (Avanti Lipids) and 100µl of chloroform to a 10 ml round-bottom flask. In the liposome preparations consisting of soy lecithin with a lipid augmentation of either 30% PE or 30% PC, 28 µl of 25µg/µl soy bean lipid extract was mixed with 30µl of 10µl/µg soy PE (Avanti Lipids) or 30 µl of 10 µl/µg of soy PC (Avanti Lipids) respectively and added to 100µl of chloroform in a 10 ml round-bottom flask.

The flask with lipids added in chloroform was were then placed under rotary evaporation at room temperature for 1-2 hours to create a thin dried lipid film at the surface of the glass and ensure all the chloroform was evaporated.

1 ml of filtered KPi-buffer (pH 7.4) was added to the dried lipid film and the sample was left to swell for 3 days. This method of gentle swelling generates large giant vesicles in solution. The vesicles were ultra-sonicated for 15 minutes to generate small unilamellar vesicles and were refrigerated until usage. If the liposome preparation had been left standing for more than a couple of days, the sample was ultrasonicated once more for 15 min to achieve sample homogeneity. Due to the results of experiments regarding storage protocol and sample quality, the samples were later batched into aliquots of 30µl and stored in a -80°C freezer until use to ensure sample integrity and working with fresh samples.

Upon usage, an aliquot was thawed and a of 5µl of small unilamellar liposome suspension was placed on top of a glass cover slip and was placed in a vacuum chamber to dehydrate into a lipid film. This was followed by rehydration by adding ~2ml KPi-buffer once the glass slide was positioned under the microscope resulting in spontaneous formation of surface-immobilized multilamellar liposomes from which unilamellar vesicles were budding. The rehydration was monitored via the microscope and spherical unilamellar vesicles budding from symmetric multilamellar liposome were selected for use in the artificial cell model experiments.

3.5 Carbon fiber electrodes

Two types of carbon fiber electrodes were used in this project. In this section the methods used to prepare amperometric detection electrodes and electroporation electrodes will be presented.

3.5.1 Amperometric detection electrodes

A 5 µm carbon fiber was placed in a glass capillary and pulled with a micro pipette puller (Model P-1000 Flaming/Brown, Shutter Instruments, USA or Model PE-21.Narashige, Japan). The micropipette was subjected to ocular inspection under microscope and imperfections were corrected by cutting excess glass/carbon fiber of with a scalpel. The outmost tip of the pipette was lowered into an epoxy solution (2 ml part A, 8 ml part B , EPO-TEK, Epoxy Technology, MA, USA) and kept there for 2 minutes, allowing capillary forces draw the epoxy into the pipette and seal any hollow spaces. The pipettes where left at 100°C in an oven over night to cure the epoxy.

Once more the pipettes were inspected in a microscope and cut off to make the carbon fiber as long as the pulled glass covering it.

Before use in the measuring device the electrode was mounted in the holder of a K.T. Brown Type Micro-Pipette Beveler (Shutter Instruments, USA). The beveling angle was set to 45° and the upper part of the glass was marked with a black pen whereas to later provide information of the orientation of the beveled tip. The pedestal was fitted with a 104D fine grinding stone and 3-4 drops of pedestal oil was added for lubrication. The top of the grinding stone was covered with mQ-water to ensure any particles formed during the beveling were washed away from the electrode tip. The pipette tip was carefully lowered to the rotating beveling stone and kept slightly pressed towards the beveling surface for several (5-20) minutes.

To ensure that the beveled tip of the electrode was well functioning, it was submerged in a 10 mM solution of catechol in KPi-buffer, bubbled with N_2 to prevent oxidation of the catechol, together with a reference electrode. This electrochemical cell was setup in a Faraday cage to minimize disturbances from surrounding electromagnetic fields.

The electrode potential was ramped linearly from -0,2 to 0.8 V and back again and the resulting CV-diagram was used to determine if the noise levels and hysteresis was between acceptable limits.

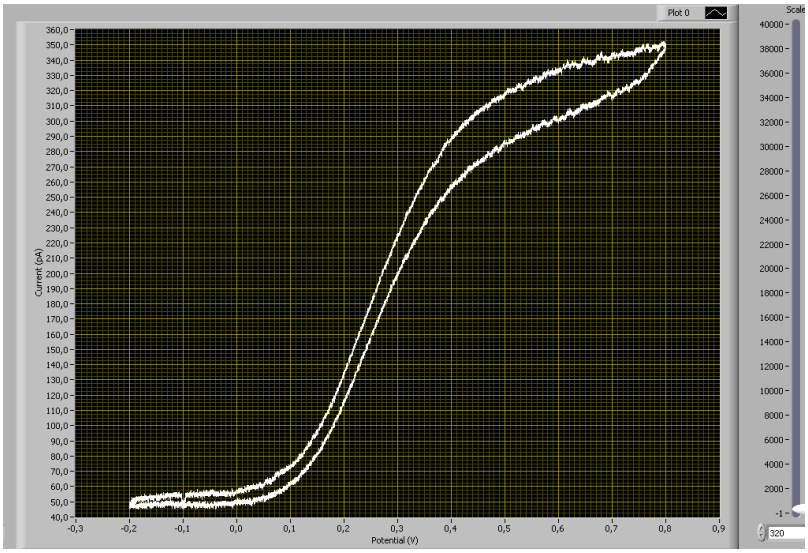


Figure 12. Cyclic voltammetry diagram for a 5µm carbon fiber detection electrode acquired with LABVIEW software. Y-axis shows current (pA) and X-axis potential(V).

3.5.2 Electroporation electrodes

Electroporation electrodes were prepared using three different methods during the project. Initially the electroporation electrode was prepared as the measuring electrodes mentioned previously with the exception that a 30µm carbon fiber was used instead.

Later during the project pre-made carbon fiber electrodes (CFEs) mounted in a pipette tip where used. The tips where filled with a conductive silver paste, which solidified upon evaporation of the solvent, and fit with a glass-tube with a silver wire inside.

Alternatively, if the last of the prepared electrodes broke during an experiment a new one was made by simply filling a CFE-tip with KPi-buffer, fitting it with a glass-tube with a silver wire and securing it by enveloping in parafilm.

3.6 Bright-field microscopy

Bright-field imaging was performed using a Sony Exwave HAD charge-coupled device camera (Sony Medical Systems, Park Ridge, NJ) on an Olympus IX-70 DIC Microscope (Olympus America, Melville, NY) through a 40x oil objective.

3.7 Liposome manipulation and amperometeric detection

Figure 13 illustrates the set-up used, where a micropipette filled with 5mM catechol solution was mounted in the left micromanipulator device (A), a 30µm carbon fiber electroporation electrode was mounted in the right micromanipulator device (B) and 5µm detection electrode held a potential X vs a calomel reference electrode (C) was mounted in the 45° tilted micromanipulator (D). The micropipette was connected to a FemtoJet^{TM} micropump, which supplied the pressure to

inject the catechol solution during experiments and also for manipulating the unilamellar vesicles with the force of flow. The parameter PC (pressure constant) was calibrated to counteract the capillary forces (10-300 hPa overpressure depending on tip size) and keep lipid material and microvesicles from entering and clogging the tip of the pipette.

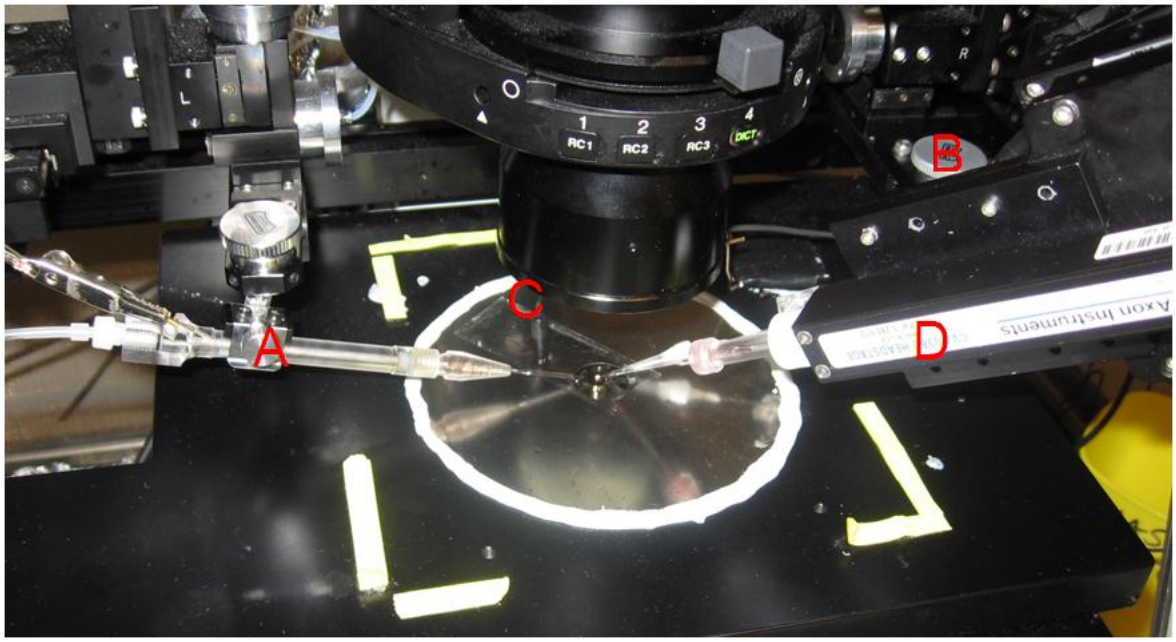


Figure 13. The picture shows the complete setup for manipulation and measuring on the lipidic model. A glass slide containing the sample seen in the middle and surrounded by: A) Micropipette with catechol solution fitted in holder mounted in left-hand micromanipulator B) Electroporation carbon fiber electrode mounted in right-hand micromanipulator C) Calomel reference electrode submerged in the buffer solution covering the lipid sample D) Detection electrode mounted in 45° tilted micromanipulator

Upon finding a unilamellar vesicle with suitable shape and size an initial test of membrane rigidity was performed by positioning the micropipette in proximity to the lipid bilayer and subjecting it to a "pulse" of stimulation solution by pressing PI (pressure inject) in the FemtoJet and observing the resulting temporary bending/dislocation. The parameter PI was usually set to 2-3 times that of the PC (30-900 hPa depending of tip size).

If the bilayer demonstrated a sufficient flexibility but at the same time remain anchored to the same position the experiment continued to the next step. The micropipette and the electroporation electrode was positioned perpendicular to the tangent plane on each side of the vesicle, shortly (4-20 ms) subjecting the vesicle to an applied electrical field (60-99 V) to disrupt the bilayer and simultaneously inserting the micropipette into the vesicle. Once inside, a small pulse of stimulation solution was injected to see whether or not the pipette had gone through the bilayer. If successful, this could be observed as slight increase in vesicle size due to the added volume and if not, the bilayer would be covering the pipette and expand into a elongated bubble upon injection. In the latter case, once the pipette had been covered by a lipid bilayer it usually

got contaminated by lipid residues which later would obstruct manipulation of the vesicles and thus was withdrawn and replaced by a new pipette.

Once the micropipette had been confirmed to be inside the vesicle and free of contamination, the micropipette was pushed through the interior of the vesicle to the opposing side of the liposome. Here the micropipette tip was pushing on the membrane from inside and out on the membrane and by applying an electric field over the bilayer on the opposing face of the vesicle, the pipette tip was penetrated through the second membrane. To ensure this was successful, solution was ejected from the micropipette tip by pressing inject and observing the solution flow occurred on the outside of the vesicle.

Upon penetration of both bilayer faces of the vesicle, the tip of the micropipette was subsequently slowly pulled backwards into the vesicle. If successful, this maneuver resulted in the formation of a tubular structure adhering to the tip of the pipette and the lipid membrane material being drawn from the lipid bilayer of the unilamellar vesicle to the inside. Initially, this was an almost impossible task to perform but it was discovered that increasing the compensation pressure on the Femtojet pump slightly (roughly 50% increase) before pulling it backwards greatly increased the probability of establishing a tube. Without this increase the pulling back of the pipette usually only resulted in the formation of a funnel-like structure in the bilayer which collapsed back into the membrane upon further extension. As the tube was highly dynamic and strived to connect to the closest point within the hemispherical unilamellar vesicle great care had to be taken maintain the tube parallel with both the X-Y plane and the X-Z plane and in height with the focal plane of the microscope.

When a stable tube had been established and pulled to a length of about 2-4 micrometers, the compensation pressure of the pump was lowered and this resulted in the inflation of the tube to form a small, spherical vesicle (A-D in figure 14). The inflation started at the tip of the pipette and as it traversed along the tube the vesicle increase in size until it came in close proximity with the membrane of the unilamellar vesicle. If the conditions were ideal, the vesicle formed from the tube collapsed into the larger vesicle surrounding it and the volume contained within was expulsed through the transient opening formed during the fusion (E in figure 14). This was follow by continuous regeneration of the tube (F) and inflation of a vesicles resulting in fusion. However, in several cases the inflated vesicle kept expanding and did not integrate with bilayer of the unilamellar vesicle. Sometimes this could be solved by disrupting the membrane by applying a transient electric field, resulting in the regeneration of the tubular state but in most instances this behavior indicated a permanent reluctance to fusion of a formed vesicle and a new unilamellar vesicle had to be chosen to continue the experiment.

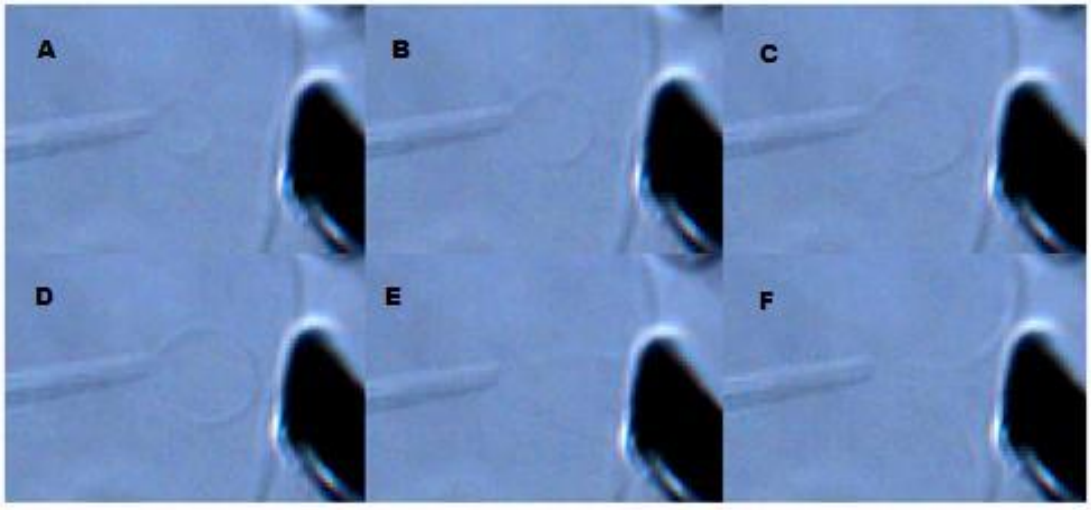


Figure 14. Time-lapse series of vesicle inflation. (A-D) Inflation of tubular structure into vesicle. (E) Fusion of vesicle and maximum release of catechol. (F) Starting point for regeneration of tubular structure.

Once continuous vesicle generation and fusion had been established, the detection electrode was brought into close contact with the membrane and the position of the micropipette as well as the detection electrode was calibrated spatially to achieve the highest possible signal. The expansion and vesicle fusion was video recorded in a DIC mode with a camera and as an amperometric current trace with an AxoScope digital oscilloscope. Size of vesicle was varied and the goal was to achieve sampling of n=5 for each vesicle size and recordings of at least three different diameters of fusing vesicles.

3.8 Data analysis

The data acquired from both the microscope camera and the detection electrode needed to be processed and analyzed in order to obtain numerical values to relate to the vesicle fusion.

3.8.1 Analysis of ampeometric trace

The recorded electrotrace was exported from the AxoScope software into the program MiniAnalysis. MiniAnalyis was used for analysis of the data from the amperometric traces. During the acquisition of data a 500Hz Elliptic Low Pass filter was applied to reduce noise and thus increase the signal-to-noise ratio.

3.8.2 Analysis of video data

Recorded video data was imported to VLC (Video Lan Client) media player, process in frame-by-frame mode and the last frame before the vesicles fused with the unilammelar bilayer membrane was captured for vesicle size measurements. The recording of video and amperometric detection had to be started manually and resulting in a 1-2 second time-gap between the two recordings and first few vesicles captured in each data set was matched with the corresponding spikes, in order to create a common time-frame, before proceeding to analyzing the whole data set.

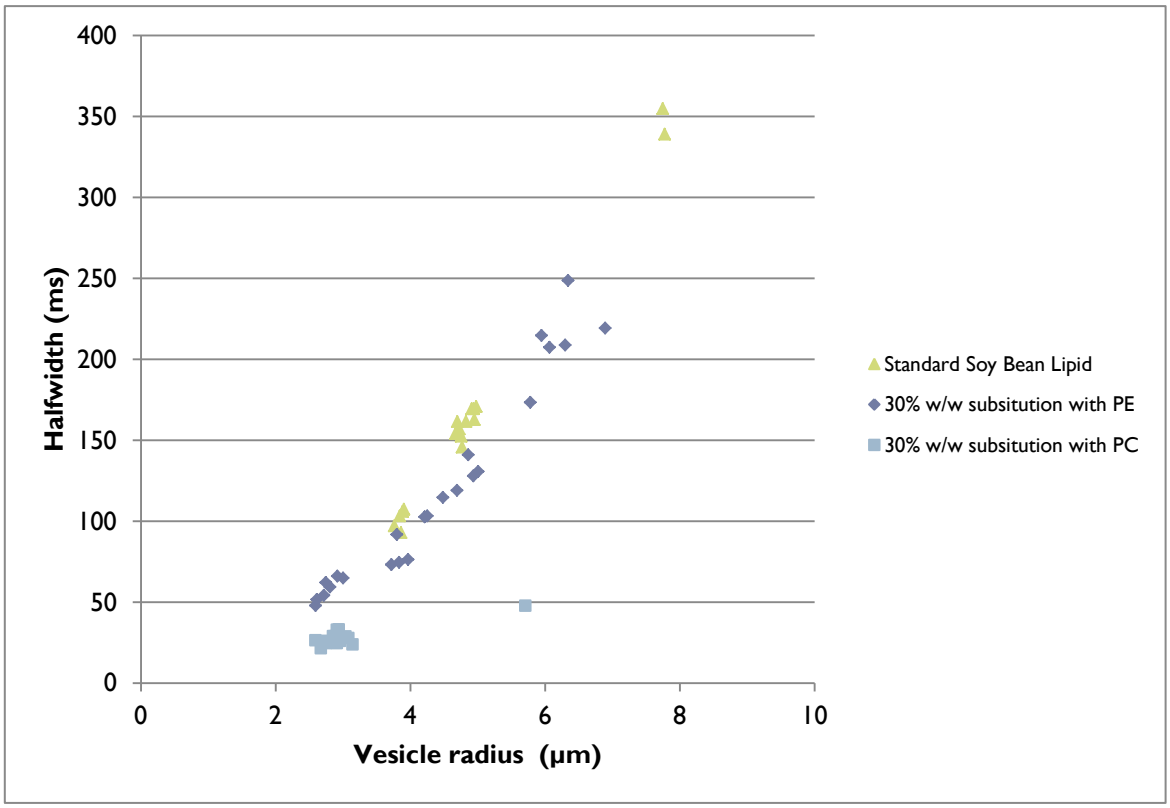
The captured frames of the vesicles were imported into Photoshop CS3 Extended and the diameter for each vesicle fusion event was determined by using the built-in ruler in the Analysis section of the program and was compared to a calibrated imaged length scale. The diameter was measured both top-to-bottom and from side-to-side as the vesicles not always were perfectly spherical and based upon these values a mean radius for every vesicle was determined.

4. Results

4.1 Lipid augmentation

To investigate how lipid composition and geometrical shape of the constituent lipids affect the vesicle fusion kinetics in exocytosis, a lipidic model system was used to electrochemically measure changes in membrane dynamic behavior for fusion of a small spherical vesicle, connected via a nanosized pore, with a larger unilamellar vesicle. A liposome preparation constructed solely on the natural composition of phosholipids extracted from soy beans was chosen as a control to which variations of different kinetics parameters brought about by liposome preparation made from soy bean lipids with a 30 % w/w substitution of either cylindrical (soy PC) or cone-shaped (soy PE) phospholipids were compared.

The electrochemical recorded amperometric spikes detected from catachol releasing vesicles in the artificial cell model were evaluated in terms of spike rise time, half width and decay time. These data were then correlated with the size of fusing vesicles as determined from the simultaneous video microscopy imaging recordings. In graph 1, the halfwidth of the detected amperometric trace for the oxidized catechol stimulation solution is shown as a function of vesicle radius for the three different liposome preparations SBL (soy bean lipid), PC (30% w/w substation of phoshatidylcholine) and PE (30% w/w substitution of phosphatidylethanolamine).

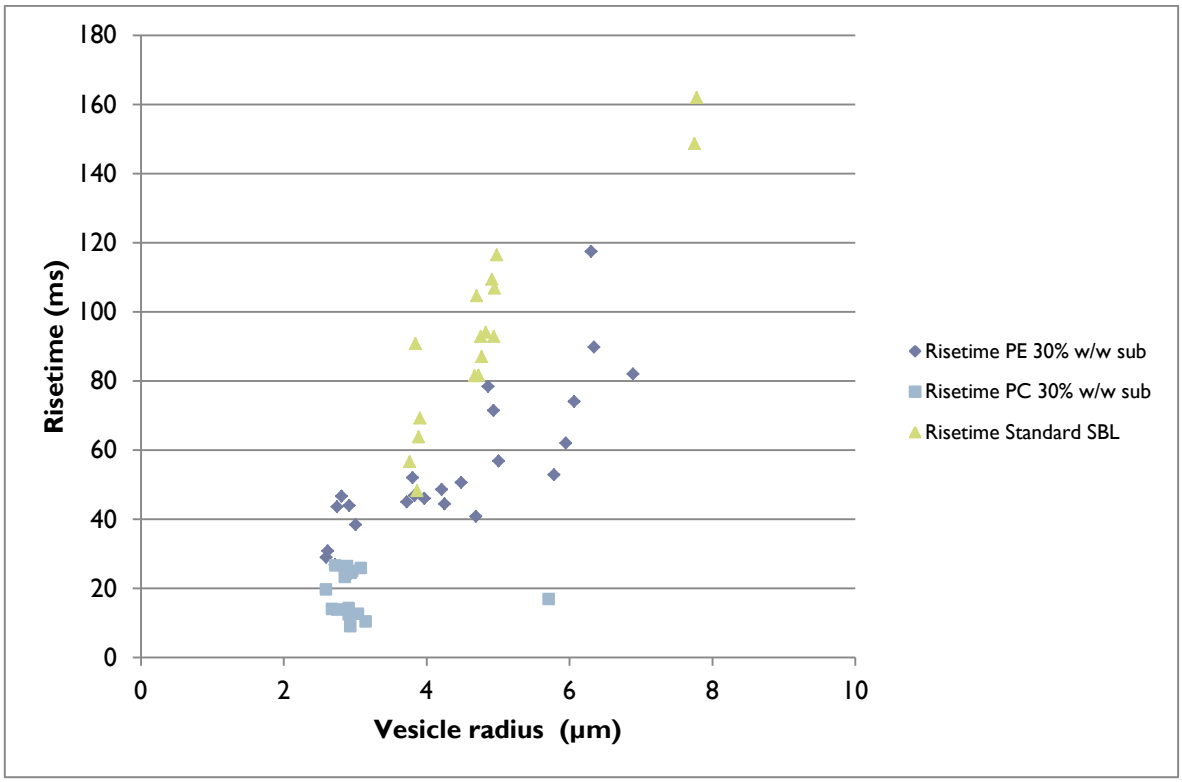


Graph 1. The vesicle fusion kinetics in terms of spike halfwidth as a function of fusing vesicle size for the three different kinds of lipid composition. These data are collected from SBL-control, PC-augmented and PE-augmented liposomes.

The artificial cells constructed with elevated concentrations of PC lipids in the membrane were the only preparations that seemed to exhibit any noticeable change of the halfwidth of the detected amperometric trace. Although the data is scarce, this is also supported by the observation that during attempts to initiate a lipid tube and inflate it to a vesicle in PC-augmented liposomes, the tube showed a much higher resistance to formation than other liposome preparations and required higher solution flow from the in order to promote vesicle fusion. This is indicative of a greater resistance towards formation of high-curvature structures and thus a comparatively higher membrane rigidity and increased resistance to membrane bending once the formation of vesicles was brought about.

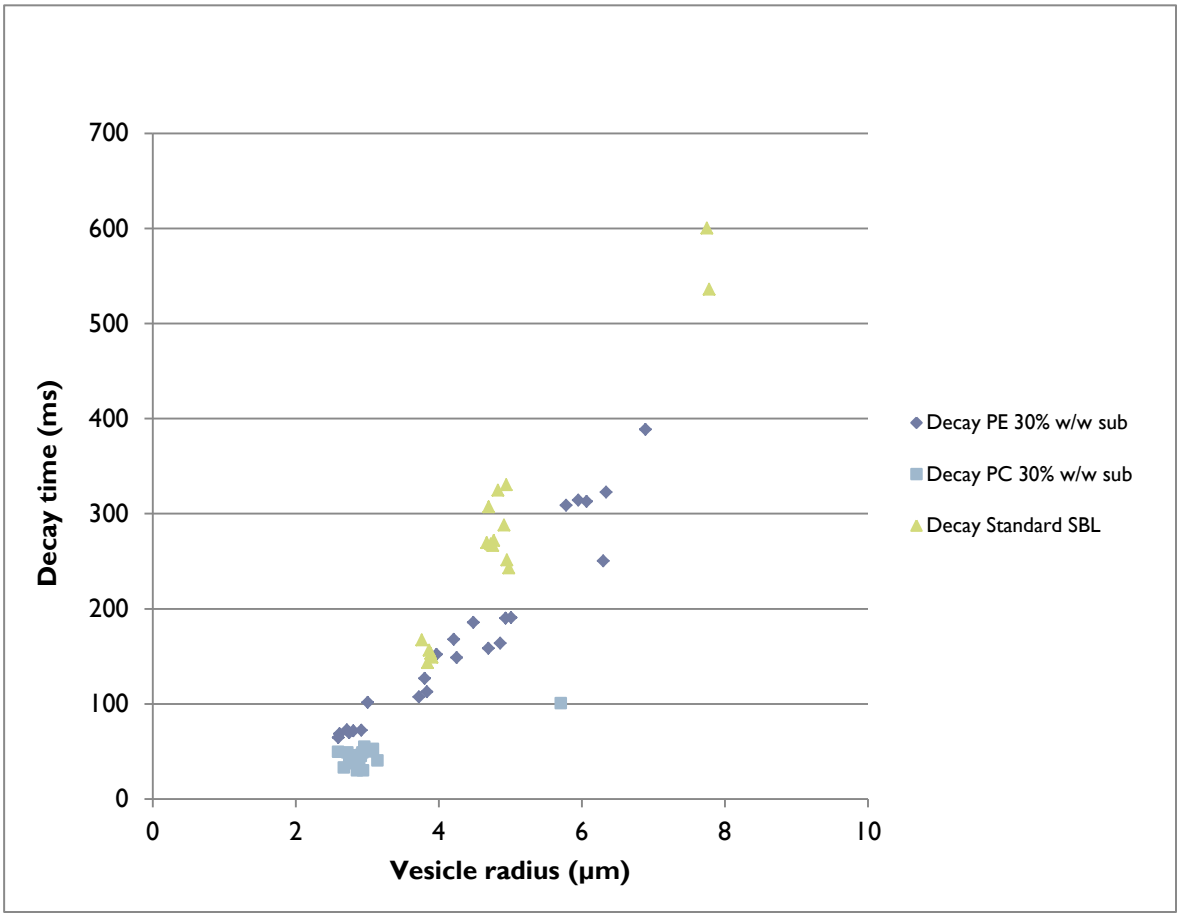
In the case of PE augmented liposomes only a selected set of the available data is shown in the figure above. The data provided is from experiments performed in the early stages (<1 hour) of a presumed maturation of the lipidic model and are thus to be most representative of corresponding experiments in the SBL-standard liposomes and the PC-augmented liposomes, as these also were performed within the first hour of sample rehydration. Based solely upon the data provided in figure 1, there is a slight indication to a decrease of the halfwidth with the PE-

augmented liposomes compared to the SBL-standard but with the uncertainties associated with the measurements taken into account; this decrease is too small to be significant.



Graph 2. The vesicle fusion kinetics in terms of spike rise time as a function of fusing vesicle size for the three different kinds of lipid composition. These data are collected from SBL-control, PC-augmented and PE-augmented liposomes.

The rise times of the amperometric spikes of SBL, PC and PE can be seen in graph 2. The rise time provides information about how rapid the membrane-spanning pore expands during the vesicle release event. Although again, with reservation for the very limited amount of data points the PC-augmented lipsomes that show a large increase and the PE-augmented liposomes show a smaller increase in fusion pore expansion kinetics in comparison with the SBL-control. This could point towards an increase in the ease of pore expansion prior to full fusion of the vesicle. In general, the obtained data for the rise times show a larger difference between the three liposome preparations compared to the halfwidth of the corresponding membrane compositions. This might suggest that kinetics for pore expansion might have a stronger dependence on lipid composition in comparison to the duration of the fusion event. However, this large variation in rise times for a given lipid composition and vesicle size makes it hard to say anything about the effect on pore expansion kinetics with any certainty.

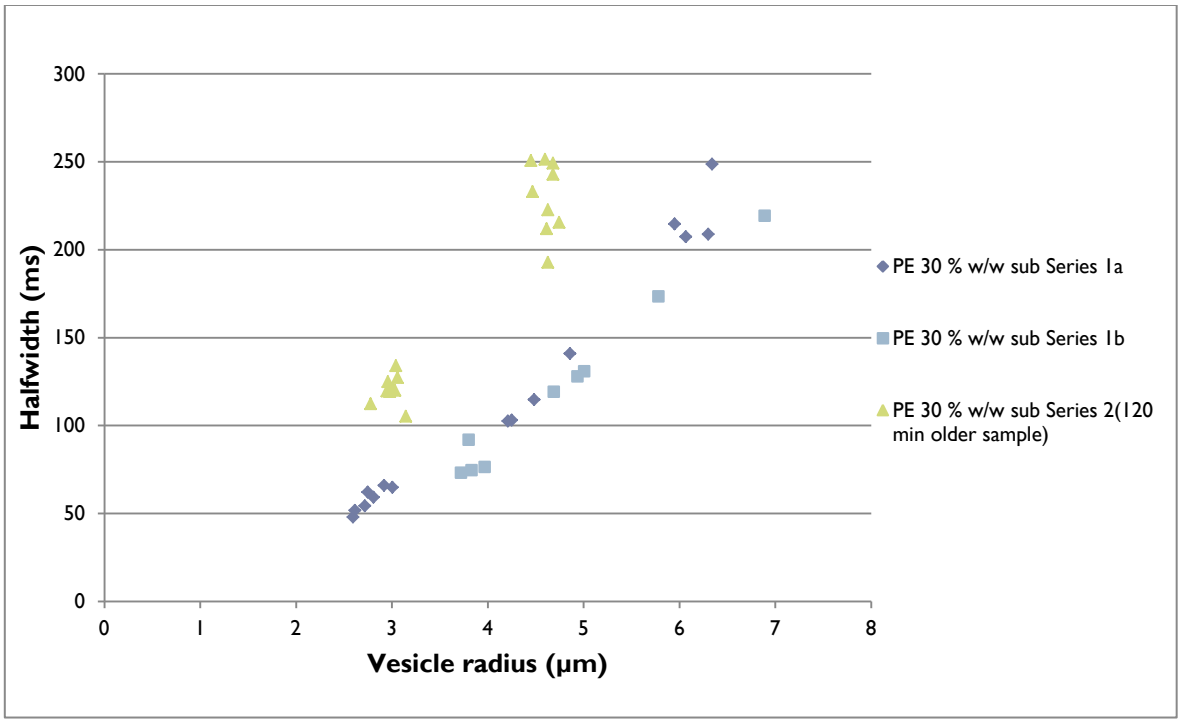


Graph 3. The vesicle fusion kinetics in terms of spike decay time as a function of fusing vesicle size for the three different kinds of lipid composition. These data are collected from SBL-control, PC-augmented and PE-augmented liposomes

Membranes augmented with PE show no significant difference in spike decay time. However, even though the major difference in trend relies mainly on a small cluster of data and one single data point, the liposomes augmented with PC lipids demonstrate the largest effect with significantly reduced decay times in comparison to the control and PE-augmented lipid composition. To ensure this trend more experiments would be needed.

4.2 Time dependent measurements.

Through the course of these experiments, it was observed that the time that passed following rehydration correlated to increased difficulty with lipid manipulation. To investigate the importance of the duration of the experiment, a set of measurements were done at two separate points in time (2 hours difference) for the same 30% PE-augmented liposome preparation. The results from this experiment are shown in graph 4.



Graph 4. Halfwidth (ms) versus vesicle radius (µm) for two different points in time after rehydration of PE-augmented liposomes. Series 1 are recorded <1 hour after rehydration and series 2 is recorded 2 hours later than serie 1. These data are collected from n=2 liposomes <1 hour after rehydration and n=1 liposomes after additionally 2 hours after rehydration in comparison to series 1.

Series 1a and 1b are two consecutive (<10 min) measurements performed within the first hour of rehydration on the same unilamellar vesicle, interrupted only by the collapse and re-establishing of the tubular structure. The series 2 was obtained from the same sample 2 hours later and a significant increase in halfwidth was observed. It was also observed that the amount of buffer present on top of the sample glass slide had decreased due to evaporation of the water, indicating a correlation of higher extra-vesicular ion concentrations and slower fusion kinetics in the lipidic model system.

5. Discussion

5.1 Variations in fusion kinetic as a function of lipid composition

Comparing the three key kinetics parameters halfwidth, rise time and decay time for the PE- alternatively PC-augmented liposomes with that of the SBL control liposomes, the substitution of PC lipids showed the most significant change in fusion kinetics for all three spike parameters evaluated in this work, whereas PE substitution only demonstrated a slight change in rise time in

comparison to the SBL – control liposomes. Although the uncertainty of this trend due to very few data points in measurement, the insertion of PC lipids increased the overall speed of fusion of the inflated vesicles with the unilamellar vesicle. This finding is in opposition of the inhibitory effect of PC incubation of PC12 cells showed by Ewing et al[23] in 2007, This is interesting as the major difference in these two experiments is that in our artificial cell model we are increasing the PC lipid content in both leaflet of the membrane, whereas in the incubations of lipids in the plasma membrane of the living cells, only the outer leaflet of the membrane is affected. However, the increase in vesicle fusion kinetics induced by PE augmentation is in part correlation with the results for the PC12 experiments. However, in cell incubations significant increase in half width and decay time and no significant increase in rise time was observed. In our artificial cell model the only slight increase in spike characteristic was seen in rise time.

One possible explanation of the difference in fusion kinetics as a function of increased PC content in the artificial model as opposed to the decrease showed in the PC12 experiments is that the PC-lipids are only incubated in the outer leaflets of the cell membranes and in the artificial model the PC-lipids are presumed to be evenly distributed between both the both leaflets. Thus, in the artificial cell model where a thin lipid nanotube is pulled and a small vesicle is inflated, the membrane is experiencing a very large increase in high curvature membrane bending. In the case of PC lipids where the intrinsic curvature equals close to one and the membrane bending of PC in both leaflet is close to zero, the bending modulus will be very high and result in high-energy cost. Thus, this bilayer of spontaneous low-curvature is forced into a high-curvature structure and is more eager to revert back to its previous, more energetically favorable state.

In the case of PE-lipid augmentation, the artificial cell show no significant changes for halfwidth and decay times, but the spike rise time seem to be significantly different in comparison to the SBL- control liposomes.

Looking at the rise times for SBL- liposomes, and liposomed augmented with both PC and PE (graph 2), the data demonstrates a generally higher variability than the data for halfwitdh and decay time. This phenomenon might arise from a strong physical dependence on lipid composition in pore during expansion and therefore a high sensitivity towards local transient variations in the composition of the lipid flow that provides the material during inflation. This idea is based on an explanatory model for fusion pore flickering proposed in by Chernomordik et al in 1988 that states the different lipid geometries promote or inhibit pore expansion and that the flow of lipid through a pore is enough to replace it every millisecond[22]. Most importantly, to be able to draw solid conclusions from these amperometric kinetic data more experiments would need to be performed.

5.2 Time-dependence and stability of the lipidic model system

During the experiments with all the different compositions of the lipid model a tendency of time-dependence of vesicle fusion was observed. The membranes tended to become tenacious to micropipette penetration and tube formation with time. The most pronounced indication of this is the increase in halfwidth demonstrated for the PE-augmented liposomes in graph 4, where two

data sets from the same preparation obtained two hours apart basically resulted in double the halfwidth for the older sample.
A simple explanation for the change of fusion kinetics with time is that the experiments are performed in an open volume of 1-2 mi of buffer solution placed on top of a glass cover slip that is positioned on a microscope stage. The liquid film of buffer solution which covers the sample is heated by the microscope lamp and evaporates with time. This results in an increase of extra-vesicular ion concentration with following osmotic pressure acting on the surface of the unilamellar liposomes. The osmotic pressure from the vesicle exterior is known to be compensated for by the vesicle in form of water release from vesicle interior and the increase in salt concentration in the media can have a significant effect on the membrane dynamics.

5.3 Low success rate in amperometric detection

Achieving the disruption of the lipid bilayer in both entering and exiting the unilamellar liposome in order to insert the micropipette, followed by establishing the tubular structure and finally inflate the tube into a vesicle and maintaining a continuous vesicle generation/fusion turned out to be a tedious task. This was achieved several times but unfortunately a majority of the experiments that reached this staged were unsuccessful due to a too low signal-to-noise ratio of the amperometric trace or even complete inactivation of the detection electrode.

The amperometric detection was carried out with 5mM catechol solution that was oxidized with a 5µm CFE detection electrode positioned where the vesicle release occurred. In hindsight, the chosen size of the detection electrode should have been larger and/or the concentration of the catechol solution should have been higher. Increasing the size of the electrode surface would have increased the surface area of detection and increased the coulometric efficiency as well as increased the likelihood of electrodes positioning at the artificial fusion pore during fusion events, A higher catechol solution would have enabled detection with a much higher signal-to-noise ratio thus increasing the total number of successful experiments.

6. Conclusion

The outcome of the experiments of augmenting lipids with different geometries into the bilayer membrane of the artificial synapse model concludes that lipid composition and geometry possibly do alter the release kinetics in the final stages of vesicle fusion.

The 30% wt/wt substitution of phosphatidylcholine (PC) demonstrated an increase in spike rise time, halfwidth and spike decay time in comparison to the other two liposome preparations, indicating that an increase in PC-lipids in both leaflets of a bilayer destabilizes the high curvature membrane in terms of membrane pore formation and small lipid vesicle formation in comparison to membrane composition with a lower concentration of cylindrical shaped lipids. Augmentation of 30% phosphatidylethanolamine (PE) showed tendencies for increased fusion pore expansion kinetics in comparison to the soy bean lipid (SBL) reference sample. more data is needed to conclude whether these kinetic results increase are significant. Furthermore, the obtained rise

time data for all three samples showed significantly larger internal differences than the other measured parameters halfwidth and decaytime, indicating that pore expansion is more sensitive to variations in lipid composition.

The release kinetics of vesicle fusion in this model system is dependent on the amount time passed from rehydration of the lipid sample. In the case of substitution of 30% wt/wt phosphatidylethanolamine (PE) this phenomenon was seen as a decrease in fusion kinetics.

7. Future Work

Primarily, more experiments on fusion release kinetics with augmentation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are needed. Increasing the number of data will serve to establish the statistical significance of the results. Additionally, including more samples with e.g. a 10% stepwise additions (10%, 20%, 30% etc.) of both PC and PE will provide information on concentration dependent behavior of the effects discussed within this thesis.

Another interesting experiment would be to modify the compositions of solely the distal leaflet of the unilamellar liposome by incubation of specific lipids in the bulk solution or the proximal leaflet the by microinjection and modification of the interior of the vesicle. Hence the obtained data would be possible to compare with cell studies where insertion has been performed in only one of the two monolayers of a membrane[1] and lipid models[22] showing that that the same lipid has opposite effect if added to the proximal or distal leaflet. Furthermore, a model with a non-homogenous distribution between the two leaflets would be of larger impact as cells have an asymmetric distribution between distal and proximal leaflets in both secretory vesicles and plasma membranes[14].

The amount of lipids in the unilamellar vesicle is minute in comparison to the whole multilamellar liposomes. There might be a non-homogenous distribution of lipids between the unilamellar and the multilamellar liposomes and thus the actually lipid composition in the fusing membranes could be different from the assumed homogenous distribution. Utilizing amphiphatic fluorescent probes of various geometries and with different spectral properties could answer this question by observing movements of lipid and lipid distribution during manipulation of the model.

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