Phospholipid Bilayers: Mechanisms behind vesicle fusion as determined from computer simulations and experiments.

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Abstract:

In this article, the process of phospholipid bilayer fusion is discussed. As molecular dynamics simulations are a widely used method to obtain information on the behaviour of molecules, a short explanation is given of these techniques. Following this, the stalk-pore fusion theory is explained by discussing its various intermediary states. Attention will go towards explaining the dynamic processes that the system undergoes and how scientific experiments support these claims. After this, the stalk-pore fusion theory, which applies to pure phospholipid bilayer fusion, is tied to assisted fusion as it occurs in biological systems. First, the effects of ions on fusion are discussed. Then, the role of the SNARE protein in speeding up of fusion is explained. Next, DNA as a synthetic substitute for these SNARE proteins is evaluated. Lastly, the molecular machinery of viruses and the way they induce membrane fusion is considered. This article closes with a summary of the presented information and ends with a few remarks on the future of this field of study.

Introduction:

In biology, phospholipid bilayers play a major role. They form the membranes that cells use as barrier between their various internal and external environments[1]. The complete chemical and morphological organisation of a cell, the separation of lysozymes and other harmful compounds from other processes as well as the motions of cells, are dependent on dynamics of those membrane bilayers. For this reason, it is hardly a surprise that numerous research groups across the world dedicate themselves to understanding the underlying mechanisms that govern these membrane dynamics.

To even begin to understand the emergent dynamics of a whole membrane, we must first focus on its constituents. Biological membranes are comprised of molecules called phospholipids, cholesterol, various

transmembrane proteins and many other compounds. The phospholipids make up the largest portion, however, and as such are thought of as the driver of the bilayer membrane dynamics[2]. To complicate matters further, there exist many different phospholipid structures. Phospholipids consist of a head-group attached to a phosphate, which in turn is bonded to a glycerol backbone. Two fatty- acid chains are also attached to the glycerol backbone. The various different types arise from different head-group and tail combinations[3].

The fatty-acid chains are nonpolar and therefore hydrophobic whereas the head-group and phosphate are generally polar, if not zwitter-ionic, and are thus hydrophilic. This divide in hydrophilicity causes the tails to aggregate to minimize exposure to water molecules whereas the phospholipid heads do not seek this minimization of exposure to water so strongly. This results in interesting behaviour in aqueous solutions. The aggregation of the tails results in structures where the heads shield the tails from water[4]. One of these aggregation structures is that of the bilayer.

Phospholipid membranes have several characteristics that make them so functional as cellular barriers. They have a low permeability for many types of compounds, yet are capable of bending, stretching and compressing. Stretching of the membrane can occur in the membrane plane, so that the distances between the lipids in the same leaflet of the bilayer increases or decreases, and out of plane, so that the distance between the head groups of opposing leaflets increases or decreases. Bending can be considered a compression of one leaflet in the membrane plane and stretching of the other leaflet of the bilayer[5]. In membrane curving, one leaflet is positively curved and the other is negatively curved. With this in mind, each bilayer must have a bending modulus which arises from an in-plane stretching constant and also a stretching constant for the phospholipids in the out-of-plane direction[6].

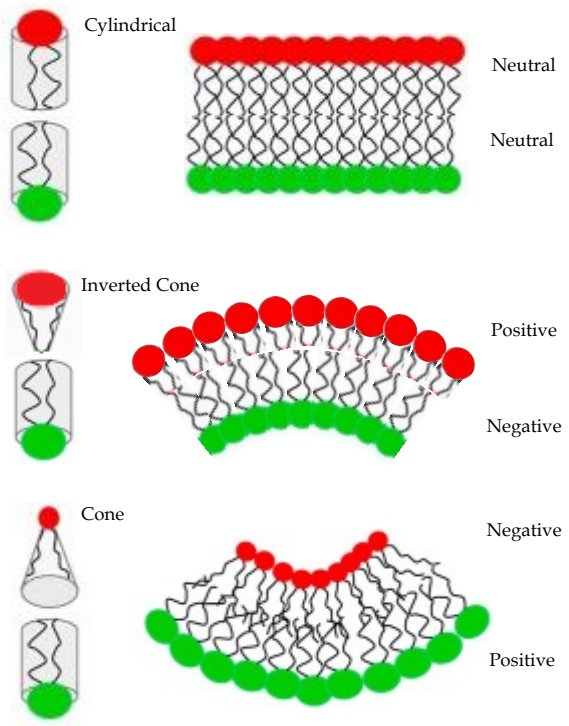

Figure 1 *(adapted from reference 74, Yandrapalli Muriaux & Favard, 2014):* The effect of cone shaped and inverted cone-shaped lipids in membrane leaflets, causing either positive or negative curvature in the leaflet of interest. The same effect can be achieved by adding the differently shaped lipid in the opposite leaflet.

The natural curvature of the bilayer is caused by the phospholipid composition. Due to the different possible combinations in head-group size and tail bending, the lipids can have a wide arrangement of shapes. Lipids with small head-groups and tails that form a wide v-structure are called cone-shaped. Lipids like this induce bending in the leaflet. Lipids with big head-groups and tails that are not protruding at a wide angle have an inverted cone-shape and induce bending in the opposite direction. The effects of the inverted cone-shape is illustrated in *figure 1.*

The lipids themselves are not bound to position and can move around in both the out-of-plane direction and the in-plane directions. Their motion is mostly caused by interactions with other lipids, the water molecules that collide with the bilayer and other such forces. In a sense, each lipid is busy in a constant dance to find its own energy

minimum in a system where the conditions are ever changing.

Dynamical processes of molecular systems cannot be visualised with microscopic or other imaging techniques as these methods rely on high energy particle irradiation and thereby affect the particles during their dynamical behaviour, irreparably altering the molecules and their dynamics[7]. Other methods rely on regular patterns in crystals to amplify the signal and are thus also not suitable for visualising dynamics[8]. The usage of computer simulation provides a solution to this problem.

Since the molecular structures of the relevant phospholipids are known and the influences that act on them can also be theorised, simulation of these complex and large molecular systems is possible. Simulations can provide insight into the complex and dynamic processes that form the foundations of a wide variety of lipid behaviours. Most interesting amongst these is the process of bilayer fusion as it forms the basis of cell division, vesicle separation and a lot of other cellular mechanisms of high importance.

In this article, the origin of membrane fusion is discussed as it is visualised through simulation with computer models. First, a short explanation of existing simulation tools will be given. Following that, the major theory on the process of bilayer fusion will be discussed. For each component thereof, it will be shown how the simulations relate to experimental data, if any is available. Then, the changes in these dynamics due to the presence of assisting complexes will be reviewed, to give a more elaborate insight into how the mechanism is manipulated in biological systems. In the end, we will have arrived at a description of the current views on the fusion process in biology.

Molecular Dynamics Simulations:

Many different types of force fields exist, each with their own specialties, strengths and weaknesses. Molecular Dynamics force fields are best suited for systems containing many molecules since the aim of simulating these systems is to show the dynamical changes in the bilayers that lead up to bilayer fusion. Molecular Dynamics force fields aim to reproduce realistic motion of molecules, either based on first principle derivation or based on experimental findings by adapting and parameterizing force definitions for all particles[9]. The fastest to calculate are the classical Newtonian equations of motion and are therefore most often used in these simulations[10].

Classical Newtonian Mechanics:

The idea behind classical molecular dynamics is that the atoms repel and attract each other and thus exert forces on each other. In addition, the atoms are bonded to other atoms in a molecule and so their motion is restrained[11].

In the classical approach, bonds with force constants are compared to springs with a certain stiffness which dictate how much energy a bond can store and which possible molecular vibrations this can result in. By using definitions for the different strengths of attraction and repulsion between different types of atoms, all relevant interactions can be defined[12]. The charged interactions also need to be taken into account as electrically charged ions differ from uncharged atoms. In addition, the electro-negativity of some particles can cause interactions such as hydrogen bonding or dipolar interactions. These various forces and interactions are often defined with Lennard-Jones and Coulomb potentials[13].

The strength of these interactions, and therefore the mathematical description of the potentials, is different for each set of particles and also depends on their surroundings[14]. This requires a careful parametrization of these forces, to which end two strategies are possible. In the first strategy, known as an empirical approach, the force and particle definitions are parameterized to reproduce experimental data of, for example, phase partitioning or other such characteristics. The

second strategy is known as the ab initio approach. It attempts to derive correct force and atom definitions from theoretical physics principles[15].

Most molecular dynamics simulations are run with a periodic boundary condition so that a realistic representation of the environment is simulated and all forces can be calculated from the system itself[16]. Other influences from the environment, such as temperature and pressure equilibration between the system and its surroundings, are recreated through the use of thermostat and barostat algorithms.

In this way, either from theory or from physical data, the aim of molecular dynamics is to reproduce realistic molecular interaction to predict dynamic behaviour in different systems. Even if an interaction distance cut-off is implemented, it requires the calculation of a large number of forces for every particle, a computationally demanding task.

Coarse Grained models:

To decrease this computational demand, a variety of system simplification strategies exist. Most effective of these is the Coarse Graining strategy. In Coarse Grained models, one particle represents multiple atoms. That way, the number of calculations that need to be performed is reduced. The decrease in structural detail leads to a decrease in conformational degrees of freedom for the modelled molecule which in turn causes a decrease in detail in the energy landscape[17]. The small energy maxima and minima are smoothened out, resulting in a model that gives a more active representation of the real system dynamics[18].

In reducing the number of particles in the simulation by making one particle represent several atoms, the forces and bonds need to be handled differently. Depending on how coarse the model actually is, the model motion needs to be properly transferred. This means that new bonds and new angles, each with their own force constants, need to be defined and parameterized in order for the model to capture the possible vibrations end bending modes of the molecule[19]. In addition, new bead types need to be defined for each combination of atoms. There are many possibilities and many levels of Coarse Graining, each with different mapping schemes, available[20]. Lastly, new forces and interactions need to be defined for the interactions of each of these bead types.

Again, this can be done through a theoretical approach, known as a bottom-up approach, which is based on mapping the force interactions and bonds from atomic simulations directly to the beads of the Coarse Grained model and by comparing and matching the resulting behaviour to that of atomic simulations[21]. Alternatively, it can be done through an empirical approach, known as a top-down approach, where the force definitions are parameterized in such a way that they reproduce experimental data[22]. In both cases, the aim is the construction of a model that reproduces realistic dynamics but is computationally cheaper to simulate than a full atomistic simulation. For this reason, it is a very useful tool for simulation of large systems.

The stalk-pore fusion theory:

Many simulations have been performed to gain insight into the processes of bilayer fusion. Most results support the stalk-pore fusion mechanism, which proposes that fusion occurs through the formation of a connection between the two bilayers where the outer leaflets of the bilayers are connected[23]. The full process of fusion is often described through a number of subordinate dynamics, each of which needs to occur for full fusion to happen.

In addition to the molecular dynamics simulations, in vitro experiments have been performed to study the membrane conformations in real systems. The results from these experiments either support or expand on the current theory. A number of experiments have been performed that shed light on lipid fluidity of a bilayer[24] and, more importantly, there have also been

experiments that used fluorescent vesicle content staining to visualise the processes through microscopy[25]. Though the current theory explains much of the experimental findings and matches the simulations, there are also some things that are not fully explained[26].

In the following sections, the major theory on phospholipid vesicle fusion, the modified stalk-pore fusion theory, will be discussed by going over the evolution to and from the major intermediary structures. For fusion, contact-site formation needs to occur first. The contact-site then needs to evolve into a fusion stalk. According to the stalk-pore theory, the stalk structure evolves into a hemi-fusion diaphragm[27]. This hemi-fusion diaphragm ruptures to allow for a pore to form. An alternative leaky pathway has also been proposed and will also be discussed.

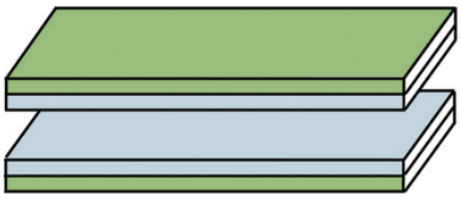

Figure 2 *(adapted from reference 49, Engel & Walter, 2008):* The beginning of bilayer fusion; the two bilayers are close to each-other but not yet in direct contact. A thin layer of water molecules, not depicted here, separates them.

Contact-site formation:

Initially, the two bilayers will be separate and not in contact, as depicted in *figure 2*. This means that their surface will only be in contact with the aqueous solution of the environment on the outside and the internal vesicle solution on the inside. When both membranes are close enough to each other, a local contact between the two bilayers may be formed. The formation of such a contact site consists of two steps. First, the bilayers must come into contact, and second, that initial point of contact must expand to form a patch where the bilayers are in direct contact and no longer separated by water molecules[28]. According to physical theory, precisely these water molecules are responsible for the energy barrier that needs to be traversed to reach the meta-stable contact point and also cause the energy barrier that must be overcome for this contact point to expand into an actual contact-site[29].

Water molecules form a hydration shell around most chemical species that are in solution. This hydration shell is an ordered layer of water molecules. The hydration shell is more ordered around apolar molecules than around slightly polar molecules. Entropy requires the hydration shell to favour a less ordered state. This effect gives rise to the behaviour of phospholipids to orient themselves so that the head-groups are directed towards the solvent as the head-groups require a less ordered hydration shell[30]. This layer of ordered water molecules resists being broken and thus enacts a restrictive force on the lipids. This force must be overcome for the lipid bilayers to come in contact with each other. The smaller the disruption of the hydration shell, the smaller the related energy requirement.

For this reason, contact between the bilayers is commonly initially formed by a small point-like protrusion from one bilayer to the other[31]. The lipid head-groups are then kept in proximity of each-other by van der Waals forces. The reconstruction of the hydration shells for both head-groups corresponds to a reduction of entropy for the water molecules as well, which can only occur at the expense of energy from the system. So, in short, the formation of contact between the outer leaflets of two opposing bilayers are governed by attractive van der Waals forces and other such interactions counteracted by the rigidity of the hydration shell, the so called hydration force[32].

This point-like protrusion, where one phospholipid makes contact with another from the opposing leaflet, elastically drags the phospholipids that neighbour the contact point towards the opposing leaflet. The neighbouring lipids now have a higher probability of breaking their hydration shells as well, so they too can form a contact point

with the opposing leaflet of the other bilayer. Ultimately, a larger patch of contact points has been formed where no water molecules are present between the two bilayers. This contact-site is theorised to be an intermediate meta-stable situation that may either lead to detachment or to the formation of a stalk[33].

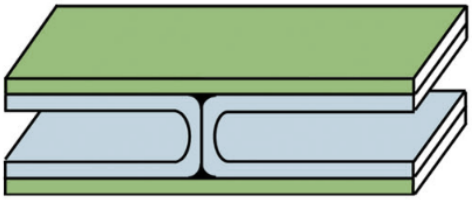

Figure 3 *(adapted from reference 49, Engel & Walter, 2008):* The formation of a stalk structure between the two bilayers as followed from contact-site formation..

Stalk formation:

Once this contact-site has formed, the lipids in the outer leaflets of both bilayers can undergo tail flipping. Tail flipping occurs when one of the tails of a lipid crosses the barrier of the two layers of head-groups. This process is also often called tail splaying. When this happens, one of the tails of the molecule comes to lie in the opposing leaflet so that one phospholipid is shared between the two opposing leaflets[34].

The phospholipids with the splayed tails are in an unfavourable conformation and will decrease the strain that they undergo. If enough phospholipids are shared between the two leaflets, the optimal conformation is that of a stalk. This stalk-structure can best be described as a short micellar column with its ends bent to connect to the outer leaflets of both bilayers[35]. *Figure 3* illustrates this stalk-structure.

In theory, the exchange of lipids can occur through the stalk, so the two vesicles can equilibrate the packing of their outer leaflets. It has been experimentally confirmed that this mixing and equilibrating behaviour occurs[36]. An experiment involving a multilayer system where x-ray diffraction imaging was used to track the shapes of the bilayers showed that the hourglass shape indeed follows dehydration and approach of the bilayers[37]. Together, these results support the realism of this simulated state as a meta-stable intermediate of bilayer fusion.

The likelihood of progression from this state onward towards full vesicle fusion has been linked to the lipid compositions of both the inner and outer leaflets of the two bilayers. The lipid composition is important as every type of phospholipid has a different natural curvature. If one of the leaflets of the bilayer is made up of phospholipids with a cone-shape, then the natural curvature of that leaflet will be negative and if the shape of the phospholipid is that of an inverted cone, the natural curvature is positive.

In the case of cone-shaped phospholipids, this means that the leaflet will be in an energetically more relaxed state if it is curved negatively[38]. This affects the conformational energy of the formation of the stalk. If the outer leaflets of the vesicles have a negative natural curvature, they will readily form the hourglass shape of the stalk as it is a tight negative curve. Consequently, a leaflet composition that strictly favours negative curvature initially benefits stalk formation. If the natural curvature is positive instead, the stalk will be unstable and be very short-lived[39], likely too short to form a hemi-fusion diaphragm.

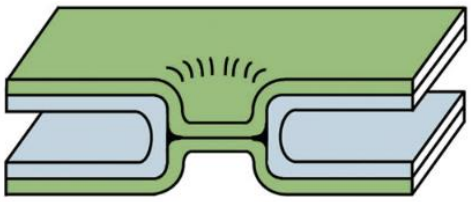

Figure 4 *(adapted from reference 49, Engel & Walter, 2008):* The hemi-fusion diaphragm formed from the opening of the stalk.

The hemi-fusion diaphragm:

Now that a stalk is present, the next step in the stalk-pore fusion theory is the formation of a hemi-fusion diaphragm from the stalk structure[40]. In the stalk, the outer leaflets have merged and form a bridge between the two vesicles. If the two bilayers drift closer or if

some kind of tension is applied to the outer leaflets in the stalk, the inner vesicle leaflets are pulled closer to each-other.

When the inner vesicle leaflets come in contact, they form a single bilayer that now separates the contents of the two compartments from each other, as shown in *figure 4*. The stalk becomes a circular zone where the inner leaflets of the vesicles form a singular bilayer but which separate at the edges into the old bilayers with their corresponding outer leaflet[41].

The outer leaflets have effectively become a single outer leaflet for both vesicle compartments. It must be noted that no diaphragm is formed without assistance from forces such as vesicle tension to pull the diaphragm rim apart in systems with biologically realistic lipid compositions, so the assistance of protein machinery is essential in biological systems[42]. How this machinery would induce such tension is as of yet not fully understood.

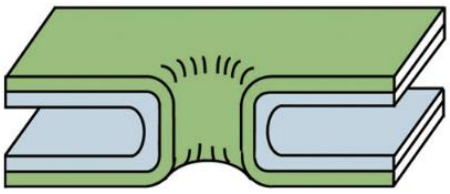

Figure 5 *(adapted from reference 49, Engel & Walter, 2008):* The hemi-fusion diaphragm ruptures and forms a fusion pore.

Pore formation:

The stalk-pore fusion mechanism theory states that the hemi-fusion diaphragm ruptures and that this allows the contents of the vesicles to mix. Molecular Dynamics simulations showed that fusion occurs through rapid expansion of hemi-fusion diaphragm rims and not in fully widened hemi-fusion diaphragms, unless their lipid composition is unrealistically unstable[43].

Due to tension from the expanding of the diaphragm rim, holes begin to form in the hemi-fusion diaphragm bilayer. These holes will expand and the single bilayer will rupture. The lipids at the edges of these holes will form round edges so that a connection between the two separate leaflets is formed[44]. If the forces acting on the diaphragm are of the same order of magnitude as the forces that keep the lipids in their bilayer conformation, the fluidic dynamics of the lipids will keep closing the holes, causing rapid opening and closing of the diaphragm. Tension induced electro-physiological experiments show that small pore formation occurs in the absence of protein and also shows a rapid opening and closing of the pore, which fits with the stalk-pore theory and hemi-fusion diaphragm fluidity[45].

The inner leaflet lipids that now are able to mix and mingle retract back to the outer leaflets and arrange in a single leaflet again to form one single bilayer as seen in *figure 5*. The new bilayer is composed of an outer leaflet with lipids from the outer leaflets of the two vesicles and an inner leaflet with lipids that previously formed the inner leaflets of both vesicles[46]. The stalk-pore fusion theory thus predicts that no mixing occurred between inner and outer leaflets. If this theory is correct, no inner and outer leaflet mixing should be found in experiments. Yet, experiments that show mixing of inner and outer leaflet lipids during the fusion process have been performed[47]. In most of these cases, the mixing is explained by natural processes of lipid exchange between the inner and outer leaflets.

In simulations, an unrealistic vesicle size or an incomplete equilibration of the bilayers may also cause unrealistic tensions. This unrealistic tension can cause the lipids to flip from the inner to the outer leaflet in the simulation. Such exaggerated strain on the leaflets could also cause pore formation in the vesicles, so that exchange from the inner to outer leaflet occurs[48].

In addition, there have been simulations that showed the possibility of formation of a small temporary pore near the stalk. These pores are often thought to be a result of an instable leaflet composition or due to unrealistic sizes of the vesicles. These short-lived pores near the stalk allow for a small

period of mixing between the inner and outer leaflets of one of the two vesicles. They would quickly close again, but allow for a small amount of the vesicle contents to leak out of the compartment[49].

Other simulations that also reproduced the pore formation near the fusion stalk reported that the stalk would expand to surround the pore in a zipper-like dynamical mechanism, resulting in bilayer fusion through an alternative mechanism. This mechanism is called the 'leaky' bilayer fusion mechanism[50].

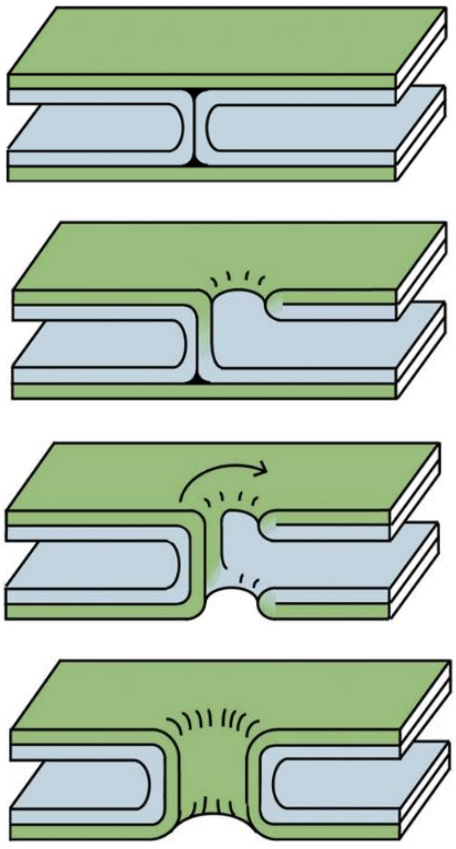

Figure 6 *(adapted from reference 49, Engel & Walter, 2008):* A rupture forms near the stalk. The stalk expands around the pore to close it, resulting in a fusion pore. This is the 'leaky' fusion mechanism.

'Leaky' bilayer fusion mechanism:

Original simulations in molecular dynamics with unrealistically small vesicles show that instead of a monolayer fusion diaphragm a pore appears in the vicinity of the stalk. The edges of this pore would then close by merging with the bilayer of the opposing vesicle[51]. The entire process is illustrated step by step in *figure 6*. It is debated whether this is a valid alternative method to fusion, or an artefact rising from the unrealistic vesicle sizes. However, in a Monte-Carlo simulation of bilayers of rod-like amphiphilic molecules, this mechanism is also reproduced[52].

Not only simulations show the leaking of vesicular content. Some experiments also show the occurrence of leaky vesicle fusion[53]. These experiments are, however, always of specific enzyme or protein activity with little indication of whether these mechanisms are more tightly regulated by other protein mechanisms in actual biological systems. As such, the realism of the leaky mechanism is still very much under debate where there has been more support for the hemi-fusion diaphragm mechanism.

Assisted fusion:

In vivo, the fusion process is assisted by proteins and other complexes by changing the energy barriers for specific steps. This can be done by stabilizing certain intermediary conformations, by providing forces that direct the progression of the system towards the following intermediate or by lowering the energy barriers between two states by altering the local interactions[54].

In organisms, fusion is strictly regulated by proteins. Many different structures exist but the best studied amongst these are the SNARE proteins, a family of proteins that consists of a membrane anchor and a unique amino-acid sequence for recognition. These proteins connect through their recognition sequences and thereby anchor two bilayers together. The SNARE protein mechanism is widely conserved and can be found throughout a wide range of species and is thus of enormous importance for biology[55].

The fusion proteins that viruses employ to infect hosts have also been studied widely in the hopes of finding a way to prevent viral

infections. As a wide array of virus types exist, a wide range of viral fusion proteins has also been reported, each with different characteristics[56]. Some attention has also gone out to the understanding of vesicle fusion and secretory mechanisms for the role they play in the functioning of neurons[57].

Various methods of fusion assistance will be discussed. First, we will discuss the simplest form of fusion assistance, that caused by the presence of ions. Then, we will discuss the common fusion regulating SNARE proteins. This will be followed by a review of DNA-mediated fusion, which is of little relevance from a biological viewpoint but is of interest from a chemical design and medical drug delivery perspective. Lastly, we will discuss the mechanisms that viruses employ to facilitate fusion.

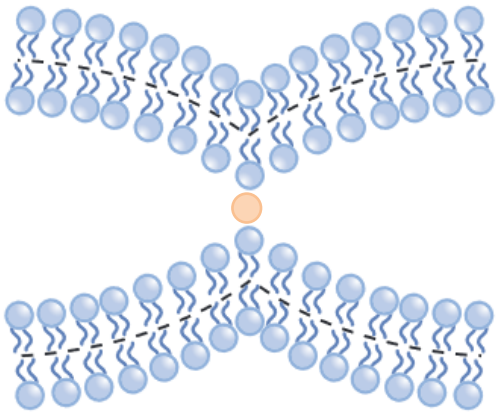

Figure 7 *(adapted from reference 75, Chernomordik & Kozlov, 2008):* The trans-complex of one Ca^{2+} ion and two zwitter-ionic lipids from opposing bilayers.

Ion assistance:

The presence of ions in biological systems is very common. Especially the presence of calcium ions is common and occurs in the synapses of neurons. Naturally, it was quickly suspected to play an important role in signal transduction. Many of the various ways for Ca^{2+} to impact the numerous signal transduction mechanisms are still a mystery, but the influence of Ca^{2+} on secretion and vesicle fusion has been studied extensively.

The chemical simplicity of Ca^{2+} ions indicates that it can have a wide range of effects on its environment. The particle is charged, which means that it will undergo attractive interaction with negatively charged molecules and repulsive interaction with positively charged molecules. Molecules with electron delocalisation and polar components will also respond to the presence of the ion. Water molecules also respond to the presence of ions by hydrating them.

The electronegative oxygen atom is attracted to the positive charge of the ion, and this causes the water molecules to arrange themselves around the ion in a regular pattern[58]. The presence of Ca^{2+} can therefore result in local dehydration.

Ca^{2+} was experimentally shown to interact with the phospholipids that had a zwitter-ionic head-group. Due to the relatively long range of charge interactions, Ca^{2+} was capable of forming trans-complexes with two lipids from opposing bilayers, as is depicted in *figure 7*. As such, it lowered the energy barriers of point-protrusion and the forming of the initial contact between the two bilayers. In addition, the ions induced disorganization in the hydration shell around the lipids, so that further contacting would also be easier[59].

So far, these interactions have only been observed in calcium ions and not in any other type of ion. Studies have been performed where the fusion of vesicles at low pH was tracked, to see whether the positively charged hydrogen ion could have a similar effect but it turned out that the proton actually must be inducing stalk formation as it causes lipid mixing but not content mixing[60].

The effect of Ca^{2+} has also been compared to that of Mg^{2+}, the magnesium ion. It was suspected that the magnesium ion would affect the bilayers in a similar way due to its similar electric charge. In truth, however, the magnesium ions had a less strong interaction overall and did not induce fusion as strongly as calcium ions seem to do[61].

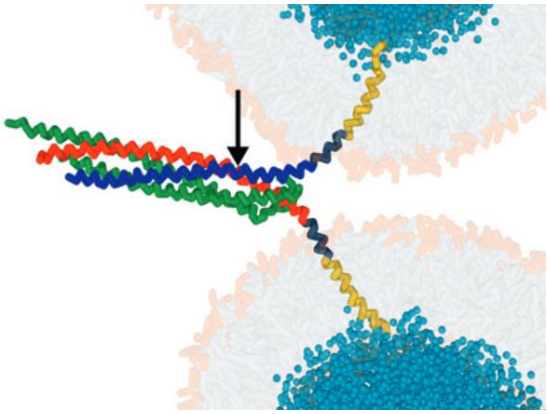

Figure 8 *(adapted from reference 64, Risselada & Grubmüller, 2012):* A SNARE complex, consisting of four coils, two of which are anchored in lipid bilayers. The arrow indicates the point where the binding of the coils ends.

SNARE proteins:

The most important and widely studied protein fusion machinery is that of the soluble N-ethyl-maleimide-sensitive fusion protein attachment receptor, SNARE for short. This protein complex has been proposed to mediate all intracellular membrane fusion events. There are more than thirty different types of SNARE proteins in mammalian organisms alone, each specific to a cellular or organelle membrane[62]. This wide-spread family of proteins is strongly conserved throughout evolution. They consist of a transmembrane domain at the c-terminal end, shown in yellow in *figure 8*, that is connected to a variable recognition motif, depicted in red and blue in the same picture. Usually, there are coils present to assist the binding, depicted in green in the figure, as well. Depending on the type of SNARE, there can be a variety of regulating groups attached at the n-terminal[63].

Two SNARE proteins, one from each of the two bilayers that are to undergo fusion, bind together with their recognition motifs. The various regulating groups can either promote or suppress this binding, but the mechanisms thereof lie outside the scope of this article and thus will not be discussed. It suffices to say that these regulation mechanisms vary from organism to organism and differ per cellular compartment[64]. The recognition motifs will form a coil and thereby draw the two trans-membrane anchors closer together. With the anchors, the bilayers themselves are also pulled closer together. By increasing the proximity between the two bilayers, the probability of the first steps of fusion to occur is drastically increased. In addition, if contact has been formed between the two bilayers, the coiling of the SNAREs induces curvature in the two bilayers, which in turn promotes the phospholipid tail-flipping and stalk formation processes[65].

There has been some indication that the presence of the protein actually promotes the tail-flipping process in another manner as well. With the presence of the bilayer anchors, a region may be formed where the tail of a lipid can more easily cross the head-group barrier, as the connection between the two bilayer anchors may provide a less polar environment[66].

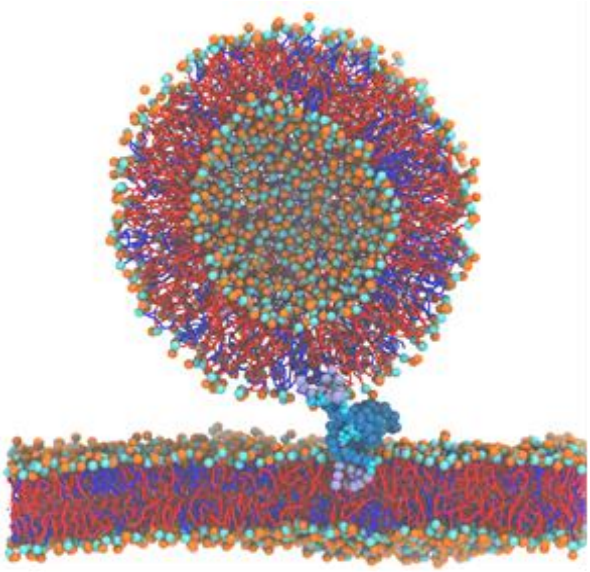

Figure 9: A vesicle anchored to a bilayer through two bonded strands of DNA, each with a lipid anchor inserted in one an outer leaflet of the two bilayers.

DNA mediated fusion:

In biological systems, DNA plays a key role. That role, however, is not the mediation of bilayer fusion. Nevertheless, due to the specificity of DNA molecules in binding and the possibilities for regulation thereof, the mediation of fusion through DNA molecules

is of major importance for chemical and general engineering purposes. If the system can be reliably implemented, it will provide scientists and chemists with a method of selectively fusing two vesicles, allowing for nano-chemical experiments to be performed, involving very small concentrations of chemicals[67].

Regulation of fusion through DNA is possible by limiting the number of strands of the molecule that are present on the outside of the vesicle, as it was shown that this has an important influence on the rate of fusion. It is also possible to predetermine which vesicles fuse with which by using specific nucleotide sequences, so that fusion will only occur between vesicles with complementary DNA strands[68].

The fusion process with DNA is very similar to that of SNARE proteins, but allows for easier control over the factors that influence the DNA binding and the strength with which this occurs. DNA binding also draws the bilayers closer together and thereby increases the probability of fusion[69]. The conjoining force that the DNA binding enacts on the bilayers affects fusion through the exact same way, but simply allows for more control.

An illustration of the method of binding between two DNA-labeled vesicles is given in *figure 9* where the DNA is given by a light-blue colour and the bilayers are given in red and blue.

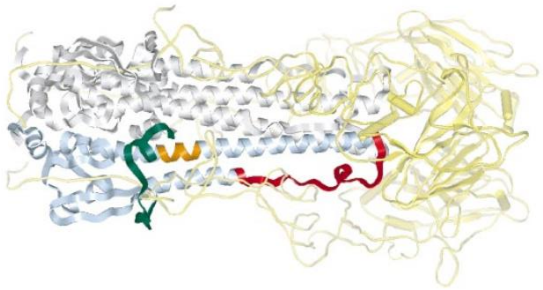

Figure 10 *(adapted from reference 72, Eckert & Kim, 2001):* The pre-fusion structure of the Influenza protein Hemagglutinin. The three yellow HA1 monomers flip to the side when exposed to low pH. The grey and blue arms then unfold, injecting their ends in the opposing membrane. The red and green loops coils when injection occurred and thereby form the hair-pins.

Viral proteins:

A plethora of viruses are known. They are studied widely to prevent disease, for they insert their genetic information into cells to procreate and in so doing harm the host organism. Many virus-types exist, each with their own mode of infection. Somewhere along the way, these minimal organisms need to cross the cell membrane. Hence, they must either inject their genetic material through the membrane, or undergo bilayer fusion. Understanding viral infection and prevention of membrane fusion is therefore of major medical interest[70].

The most common viruses to infect man are enveloped in a protein coated membrane, a phospholipid bilayer. To have its genetic contents enter the cell, fusion between this viral envelope and the host's cell membrane needs to occur. To this end, the virus employs fusion proteins that activate when exposed to the conditions where fusion is most advantageous to the virus. Some viruses employ mechanisms that work under slightly acidic conditions so that fusion occurs after endocytosis occurred. Others have proteins that work at neutral pH and cause membrane fusion before they are taken up by the host for digestion[71].

Viral fusion-proteins generally function in a different way than SNARE proteins. Though there are some specialised viruses that act on natural proteins that hosts express, most viruses. cannot rely on specific markers presented by the host. As such, the fusion proteins for viruses need to be inserted in the host membrane. The structure of Influenza hemagglutinin, a model for type 1 viral proteins, is shown in *figure 10*. Viral fusion proteins of this type are kept in an inactive state until the virus encounters specific conditions, which cause the protein to expose a point that inserts itself into the opposed bilayer. It will then form a hair-pin and draw the bilayers close together. This allows the process of fusion to occur[72].

Type 2 viral fusion proteins employ a slightly different strategy. These viral proteins are folded up so that a hydrophobic

region is under steric pressure. As soon as an acidic environment is detected, the protein responds through a conformation change. In that conformation change, a hydrophobic section is forcefully inserted into the opposing membrane. Again, the formation of a hairpin results in fusion, but the effect of this type of protein doesn't end there. The form of the hair-pin structure is such that it induces membrane bending, and thus stabilizes the formation of a stalk[73]. Whether proteins such as these are capable of inducing enough tension in the bilayers to induce diaphragm rupture is still in question, however.

Conclusion:

The consensus in the field of membrane fusion studies is that biological membranes fuse through bent bilayer intermediates. The process of fusion begins with the approach of the two bilayers. The two bilayers, which are normally covered with a hydration shell will come into contact through a point-like protrusion that causes local dehydration of the bilayers so that a contact-site is formed. This process may occur in systems without protein assistance but in real biological systems it is commonly directed through the presence of proteins that keep the bilayers close together.

Tail splay governs the next step of bilayer fusion. Here, one of the tails of a few lipids cross into the outer bilayer leaflet of the opposing vesicle and cause the two bilayers to curve towards each-other to form a stalk-structure. This stalk structure was confirmed through imaging experiments to be a real fusion intermediate. Tension in the vesicle's outer leaflets, either induced by proteins or by other mechanic causes of stress such as osmotic pressure or lipid composition, causes the stalk to be pulled open. The inner leaflets will now come in contact to form a hemi-fusion diaphragm. The outer leaflets of the vesicles have become one outer leaflet for both vesicles. If the expansion of the stalk into the diaphragm border rim is fast, the diaphragm comes under large tension and will rupture. The contents of the two vesicles can mix and the inner leaflets merge to become one.

An alternative pathway for fusion has been proposed wherein tension does not cause the stalk to expand but where the bilayer of one of the vesicles ruptures close to the stalk. The stalk structure will then expand along the edges of the pore, drawing from the bilayer of the opposing vesicle. That way, a pore is formed between the two vesicles. This alternative pathway allows for a small amount of vesicle content to leak out.

For various reasons, this mechanism for fusion is not commonly accepted as a biologically relevant. Naturally, the pure phospholipid bilayer fusion mechanism through the hemi-fusion diaphragm is not completely realistic either. Organisms require a careful regulation of vesicle fusion. For this reason, there are many mechanisms by which vesicle fusion can be influenced.

A good example of biological control of fusion is that of local ion concentration. The influence of ions on biological systems can vary greatly but the most important ion for the fusion mechanism is Ca^{2+}. It is capable of forming a trans-complex with two lipids from the outer leaflets of opposing bilayers and causes disorganisation in the hydration shell surrounding the bilayers. It thereby facilitates contact-site formation. A low pH and magnesium ions can also give rise to a similar effect but their interactions are less strong.

Another very common molecular machine for membrane fusion in biological systems is the SNARE complex. It forms when two proteins in opposing membranes bind. The molecules coil together and so, the two membranes are brought close together. This enables the first few processes of fusion to occur a lot faster.

The same process can be reproduced by attaching complementary DNA-strands to a lipid anchor in two opposing bilayers. The DNA-mediated mechanism provides easier options for fusion control, however. For this reason, DNA-mediated fusion is of great

importance for the future of chemistry and directed drug delivery.

To the medical world, viral infection is of much greater concern. To that end, the fusion machinery of viruses has been studied extensively. The two most important types of viral fusion proteins function through insertion in the opposing bilayer. They differ in how this insertion is achieved. After successful insertion, the protein goes through another conformational change and now folds into a hair-pin structure. In that way, the bilayers of the virus and host are brought close together, so that the initial steps of fusion can more easily occur.

So-far, it seems that proteins and other molecular fusion inducing machinery are only capable of affecting the initial steps of bilayer fusion. This may, however, simply be a side-effect of the current experimental limitations. Both in simulation and in experimental set-up, it remains difficult to study the later steps in fusion as there are many processes and intermediates preceding them. The limits of our current imaging technologies also still apply and complicate matters even further. As such, it is to be expected that new insights and experimental techniques will continue to be discovered and these will grant further insight into the fundamental processes of phospholipid bilayer fusion.

Though a complete picture of the progression of fusion has been proposed in the form of the modified stalk-pore theory, the truth of this theory in the biological setting of living organisms cannot yet be assumed unconditionally. The credibility of the hemi-fusion diaphragm as intermediate in biology could benefit from more thorough experimentation as experimental evidence is of limited detail, especially when related to the effects of proteins and other biological processes. Nevertheless, the stalk-pore fusion theory is currently the most complete explanation, and I have no doubt it will only become more complete over time.

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