Compartmentalization of synthetic cells

Promising compositions of synthetic membranes to design the 'alive' synthetic cell

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Abstract

Synthetic cells are simple, cell-like structures that can be engineered for a specific application or used to study for example dynamics, metabolic pathways or the individual role of proteins. Synthetic cells that resemble natural cells need a semipermeable membrane. The cell membrane is one of the main components that cells have to perform the five characteristics of life: compartmentalization, growth and division, information processing, energy transduction and adaptability. The cell membrane as we know it is a fluid mosaic model with phospholipids, proteins and additional compounds, e.g. cholesterol. The composition of membranes in one cell vary as well as the membranes of different species. However, they have some aspects in common. All membranes need to be semipermeable, stable, fluid and they have to be able to grow and divide.

Synthetic membranes can be designed and constructed in different ways. The two main approaches are named bottom-up and top-down. With the top-down method the genome of a living cell has been stripped down to the minimum number of enzymes and nucleic acids a cell still requires to perform the most basic functions in live. Unfortunately, this way of constructing a synthetic cell may bring up some unpredictable and undesirable results. Changing the genome of a modern cell can cause metabolic pathways to change, resulting in lower production rates, high morality and perhaps unwanted by products. To overcome this problem the bottom-up method is preferred in most studies. With this bottom-up method synthetic membranes can be built by the assembly of non-living components and so increasing the complexity of the cell. Promising components for synthetic membranes are fatty acids, single-chained amphiphilic molecules build of carbon tails and phospholipids, which contain two carbon chains and a polar headgroup of phosphate and glycerol. Fatty acids are found in leftovers of carbon-rich meteorites that were part of a very early bombardment on the prebiotic Earth. Whereby fatty acids are very important for studies searching for the origin of life. Phospholipids are the compounds we know as the molecules that build up cell membranes as we know them right now.

This review focuses on the recent advances in the design of a proper semipermeable membranes for synthetic cells by the bottom up approach. We describe the advantages of fatty acid and phospholipid synthetic membranes and believe the blended membrane with both phospholipids and fatty acids is most promising. Here the "best of both worlds" are coming together. Unfortunately, researchers are still very far away from making this complete "alive" synthetic cell. However, the combination of the exciting challenge to build a 'alive' synthetic cell and all the potential applications in the future, will for sure drive progress in this field for all those years to come.

Abbreviations: C, Carbon atoms; Cmc, Critical micellar concentration; GUV, Giant unilamellar vesicle; LUCA, Last universal common ancestor; LUV, Large unilamellar vesicle; MLV, Multilamellar vesicle; PC, Phosphatidylcholine; pKa, Acid dissociation constant; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; P_s, Permeability coefficient; SUV, Small unilamellar vesicle.

Content

Introduction

400 years ago, Robert Hooke observed a cellular structure from cork materials and coined the word "cell". This observation took place in 1665 and two centuries later, the cell was described by Schleiden and Schwann as the basic unit of life.[1] Schleiden was a botanist and suggested that all structural elements of plants are composed of cells and cell products. A year after, Schwann, who was a zoologist, came with a similar conclusion for animals. He stated that "there is one universal principle of development for the elementary parts of an organisms (…) and this principle is in the formation of cells".[2] Natural cells described by these researchers are complex and compartmentalized and despite the progress the last decades, they are still not fully understood. This complexity of the cell makes cell biology research more intricate. To overcome this complexity barrier, synthetic cells can be used as cell mimics. The big advantage of synthetic cells is simplicity. The cells are simple enough to get understanding of certain mechanism applied to this cell and still the mechanism is performed in a biological system.[3]

Synthetic cells are simple, cell-like structures that can be engineered for a specific application or to study e.g. dynamics, metabolic pathways or the individual role of proteins.[4] It is important to know that terms like artificial cell, minimal cell and synthetic cell are used in literature interchangeably, but they do not necessarily mean the same thing. A minimal cell has minimal properties and can be still called living, while an artificial or synthetic cell is made of artificial components and is not necessarily "alive" or minimal.[5] Notwithstanding the huge diversity between all forms of life, there are specific characteristics that enable these forms of life, and synthetic cells, to exist. These five characteristics are designated as: compartmentalization, growth and division, information processing, energy transduction, and adaptability (Figure 1).[6]

The ultimate goal is to build and design a synthetic cell that can be considered as 'alive' and thus it needs to have all these five characteristics. However, as said, even the simplest form of life on earth is rather complex. This makes it very difficult and a big challenge for researchers to synthesize 'alive' artificial cells.[7] A full molecular remake of the cell needs different cell-metabolites, DNA, RNA, proteins and lipids. Each of these cellular macromolecules does not exist in isolation, but they interact with each other. It builds a complex web of interaction pathways, which makes cells hard to study and hard to use in specific biotechnological purposes. A synthetic cell with, for example, one pathway is very interesting for biotechnological purposes and will give more understanding of this specific pathway. A second advantage of synthetic cells is that the cellular

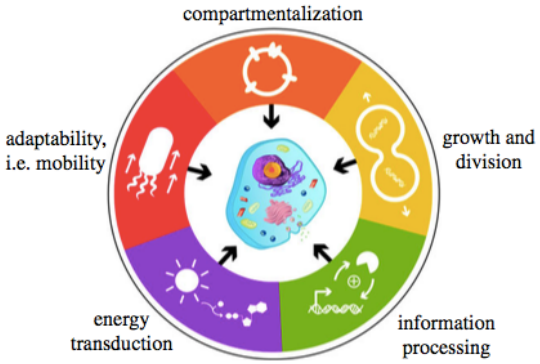

Figure 1: The five characteristics of life [6]
The five different colours and parts of the diagram show five different characteristics of life. These are compartmentalization, growth and division, information processing, energy transduction, and adaptability.

compounds and pathways can be adjusted making them less dependent on specific nutrients, culture conditions and stress.[3] Finally, the costs to alter the genome of a cell are high and synthetic cells designed for a specific applications have relatively low costs and are still capable of self-organization, self-adaptability and have nanoscale efficienty.[4]

To perform the five essential characteristics of life, biological cells have three main components. The first components are biomacromolecules, such as DNA, RNA and proteins. These molecules carry the genetic material, control the dynamics of the cell and gives the cell the possibility to evolve. Besides the biomacromolecules, biological cells need a series of metabolic pathways used to provide energy, to make them self-renew, self-maintain, and additionally self-process information. Finally, all cells need a stable and semipermeable membrane. This membrane encloses the cell and protects the inside from the damaging external environment. A semipermeable membrane still allows selective material to go inside to exchange energy (Figure 2).[7]

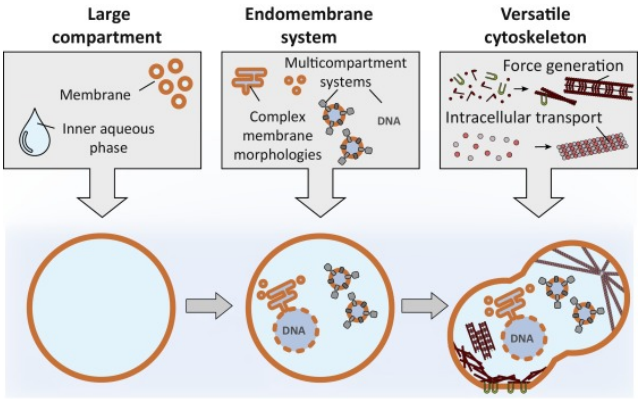

Figure 2: A schematic overview of the components required for a synthetic cell [37]
The required components needed for the 'alive' synthetic cell are divided into three subgroups: large compartment, endomembrane system, and versatile cytoskeleton.

This review focuses on the recent advances in the design of a proper semipermeable membranes for synthetic cells by the bottom up approach. The semipermeable membrane is one of the main components a synthetic cell needs. This review will highlight different approaches, applications, advantages as well as the disadvantages of different synthetic membranes and the future perspective of using synthetic cells.

Membrane compartments in natural cells

Cellular membranes in and around the cell protect and organize the internal environment of the cells. A cell as we know it consists of a bilayer of lipid molecules, the phospholipid bilayer.[8] The bilayer was first described by two Dutch researchers, E. Gorter and F. Grendel. Gorter and Grendel measured the cell lipid surface of an erythrocyte by lipid extraction. They spread the lipids out at an interface of air and saline and measured the respective monolayer surface area. After this they calculated total cell surface microscopically. After comparing these two measurements they found a ratio of approximately 2:1 indicating that the cell membrane is two molecules thick, a bilayer.[9] All cells have an outer plasma membrane and only eukaryotes possess internal membranes. These internal membranes define organelles, including the nucleus and other cytoplasmic organelles, and all have a special composition and specialized membrane proteins to succeed their specific tasks.[10] The outer membrane differs in lipid composition, permeability and the amount of different membrane proteins between different species and environmental conditions (Table 1).[11]

The main building blocks of cellular membranes are phospholipids. These amphipathic molecules consist of two hydrophobic chains and a hydrophilic head group which is composed of a glycerol that links the two chains and the phosphate group. The headgroup of the phospholipid can change as well as the composition of the hydrophobic carbon chains which makes that there are various kinds of phospholipids in one membrane.[10] Besides phospholipids, sphingolipids, and sterols are found in biological membranes. Sterols, such as cholesterol,

Table 1: Lipid composition of different cellular membranes [11]
The lipid concentrations of different lipids is giving in mol%. The different membranes are given at the top of the table in bold. The different lipid types are given in the left column in italic.

Lipid	**Plasma membrane *E.coli***	**Plasma membrane Erthrocyte**	**Rough Endoplasmatic Reticulum**	**Outer Mitochondrial membrane**
Phosphatidylcholine	0	17	55	50
Phosphatidylserine	0	6	3	2
Phosphatidylethanolamine	80	16	16	23
Sphingomyelin	0	17	3	5
Glycolipids	0	2	0	0
Cholesterol	0	45	6	<5

are very important in eukaryotic membranes.[8] Haponoids are the prokaryotic surrogates of sterols. The presence of sterols can influence the fluidity of the membrane. D. Poger and A.E. Mark performed research on the effect of sterols on lipid membranes and found that both sterols and haponoids enhance tighter packing of membrane lipids and have a condensing effect.[12] The model that describes the free movement of individual lipids and proteins through the two-dimensional membrane is called the fluid mosaic model and presented by Singer and Nicholson in 1972.[13]

Membranes are generally characterized as phospholipid bilayers. However, if we look at archaeal membranes a different membrane composition is revealed. Archaeal membranes can consist of a monolayer of tetraether lipids, lipids with a ether linkage instead of an ester linkage. In this membrane a single continuous chain connects two hydrophilic head groups on the inside and outside of the membrane. Another difference in archaeal membranes is the use of enzymes. The lipids in archaeal membranes are synthesized by different enzymes than bacterial lipids. Lastly, the archaeal lipids can have phytanyl side chains connected to the carbons in the chains of the lipids (Figure 3).[14]

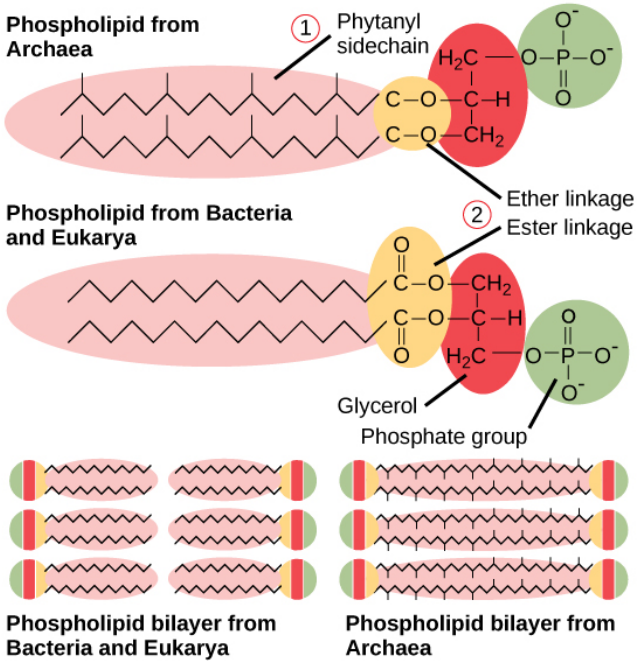

Figure 3: Different phospholipids in eukaryotic, bacterial and archaeal bilayers [15]

The three main structural differences between bacterial and eukaryotic, and archaeal lipids are given: phytanyl sidechains, ester linkage and the connected lipid chains from both layers of the bilayer.

Cell membranes have different tasks to perform. The first one is already mentioned before, protection of the inside of the cell and organizing the cell internal environment. Harmful molecules are not able to go into the cell because of a selective barrier. The selective, semipermeable membrane allows the cell to keep the harmful molecules out and the useful molecules in. Factors that influence this permeability include the chemical nature of the building blocks, the membrane thickness, temperature, the presence of pores and channels, and domain formation in heterogenous memebranes.[16] Uncharged molecules, like nonpolar O_2 and CO_2 and polar H_2O, can all diffuse though the phospholipid bilayer. Large uncharged and charged molecules have problems to diffuse through. The membrane requires transmembrane proteins to transport these larger molecules, another task for the plasma membrane to perform. There are different groups of proteins that can help the diffusion of larger molecules. Channel proteins allow ions to pass the cell membrane. Additionally, carrier proteins bind selective molecules and undergo conformational changes to transport molecules like glucose. Both they act like passive transport. Active transport, were the molecules are transported into an energetically unfavourable direction, is often coupled to the hydrolysis of ATP.[11] Finally, the cell membrane houses receptors and is capable of producing signalling molecules that transmit information to other bacterial cells or other cells in a multicellular organism.

In summary, the cell membrane needs to be able to assemble, grow and divide. It has to be selective, capable of self-reproduction and protein synthesis. All these properties and tasks to perform shows the complexity mentioned before. Showing why it is still a big challenge to make synthetic membranes that represent a real living cell.

Approaches for designing and constructing synthetic cells

There are two main approaches for designing and constructing synthetic cells. These are the bottom-up and the top-down approach (Figure 4). The bottom-up approach starts from scratch.[7] Here a minimal living cell is built of non-living components. In this set up no enzymes and nucleic acids been used, since these macromolecules were not present at the start of evolution. In the top-down approach the genome of a living cell has been stripped down to the minimum amount of enzymes and nucleic acids a cell

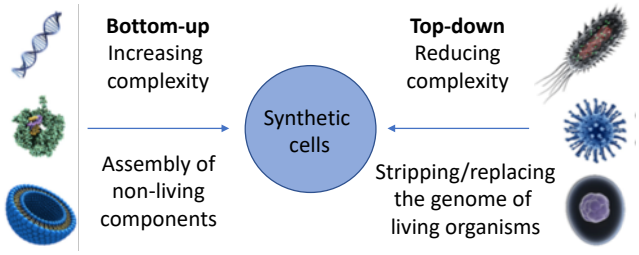

Figure 4: Approaches for designing synthetic cells
The bottom-up approach and the top-down approach are visualized by this picture. In the bottom-up approach, synthetic cells are created by the assembly of non-living components, which increases the complexity, to form a cell that can replicate essential properties of natural cells. In the top-down approach, synthetic cells are constructed by reducing the genome of living organisms, reducing the complexity, and only the minimum substances to maintain the essential of life are still left. This picture is modified from reference 7.

still requires to perform the most basic functions in live.[17] A big difference is that with this approach living organisms are used instead of non-living components.

Top-down

As said before a minimal cell has the minimum number of genes needed to perform the most essential parts of life. The top-down approach can reduce the genome of living organisms to this minimum that still maintains the essentials of life. The inspiration for scientists to develop minimal genomes via this approach, was the parasitic bacteria *Mycoplasma genitalium*. This bacterium was already reported in 1995. At that time *M. genitalium* was, with only 517 genes, among the simplest living organisms known.[7] Over many years later smaller genomes were reported, like the genome of *Carsonella ruddii*, with only 198 protein-coding genes.[18,19] Right now, *Nasuia deltocephalinicola*, discovered in 2013 appeared to have the smallest genome of all bacteria, with only 137 protein coding genes.[14]

Unfortunately, this way of constructing a minimal cell may bring up some unpredictable and undesirable results. Changing the genome of a modern cell can cause metabolic pathways to change, resulting in lower production rates, high mortality and perhaps unwanted by products. To overcome this problem the bottom-up approach can be used. In this approach you only add the minimal components required to perform the desired function, which makes the whole cell system more controllable.[7]

Bottom-up

The bottom-up approach is more challenging than the top-down approach. However, this is an approach multiple studies rely on. The goal of this approach is to create a cell by assembling a stack of non-living components. Considering that most life on earth is supposed to have initiated from the non-living components, this method is very important in designing a synthetic cell that can be called alive. Working with this approach will definitely give more information about the origin of life and the relation between non-living and the living world.[7]

The bottom-up approach can construct the cell membranes for the synthetic cells. Although they are very complex, membranes need to contain different critical features of the natural membranes to be part of the synthetic cell as well. Such as fluidity and the hydrophobicity, which were already mentioned before. One of the most important components in natural cell membranes are lipids and proteins.[7]

Composition of synthetic membranes

The construction of typical synthetic cells is considered as one of the most important pillars of the synthetic biology. Typical synthetic cells preferably have the same characteristics as alive cells, in contrast to the untypical synthetic cells.[7] Membranes as we know, contain phospholipids and the main component of these molecules are most likely hydrophobically fatty acid chains. Besides this, the membrane of the last universal common ancestor (LUCA) is most probably build of lipids.[20] This makes the use of lipids and fatty acids to compartmentalize cells most interesting.

Fatty acid membranes

Fatty acids are single-chained amphiphilic molecules. They are built of a saturated or unsaturated carbon tail and a hydrophilic carboxylic and acid headgroup. The tails are mostly between the four and 28 carbon atoms (C) long. The fatty acid synthesis is highly conserved within all kingdoms of life. Here acetyl-CoA molecules are connected to each other by an enzyme complex that uses ATP and NADPH. This kind of synthesis causes the fatty acids to grow two carbon molecules at one time.[21] The C6 till C18 fatty acids can also be synthesized by Fischer-Tropsch-type reactions from CO and H_2. This reaction uses a nickel-iron

catalyst under conditions that have occurred in primitive Earth period. The period where life started.[7]

The origin of fatty acids is very important and interesting. The molecules are found in leftovers of carbon-rich meteorites that were part of a very early bombardment on the prebiotic Earth. They are the first molecules that could form vesicles and so the first protocell membranes.[22] Protocells are simple cell-like entities that are capable of self-replication, metabolism and Darwinian evolution. Fatty acid vesicles are still a very important model for protocells and studies searching for the origin of life. However, the cell we know is made of phospholipids and it is very unlikely that phospholipids could be synthesized in this primitive Earth conditions. Phospholipid synthesis needs fatty acids, glycerol and phosphate molecules in a mixture and there is a very small chance all these compounds were present at one time. This makes it very unlikely that the first lifeforms had phospholipid membranes.[7]

The ability of fatty acids to form vesicles was first reported by Gebicki and Hicks in 1973.[7] The fatty acids can form bilayers, vesicles and micelles due to their amphiphilic character.[23] Micelles of fatty acids start to form when the concentration of free fatty acids in a solution is constantly higher than the concentration where it starts to transit to micelles. This point of micelle transition is the critical micellar concentration (cmc).[24] Besides the cmc, the pH and the acid dissociation constant (pKa) of the carboxylate headgroups in the membranes play a big role in aggregation of the fatty acids. At a pH lower than the pKa the fatty acids are precipitates in water or oil, with a pH higher than the pKa they start to form micelles. This formation is due to the repulsion of the charged carboxylate groups. However, when the pH is close to the pKa, dimers are formed. These dimers are more stable and start the formation of a bilayer, presumably due to the hydrogen bonds between ionized (COO) and the neutral acid (HOOCR) in the headgroup. Finally, this bilayer can curve onto themselves and an enclosed vesicle can be formed (Figure 5).[23] The pH controls the headgroup ionization and the concentration of the soluble fatty acid the size of aggregates formed.[25]

Fatty acid vesicles are capable of division, growth and self-reproduction, which is triggered by the environment and the presence of other vesicles in the solution. Fatty acids can grow because of the rapid exchange between the different stages. The external addition of fatty acids and absorbing other

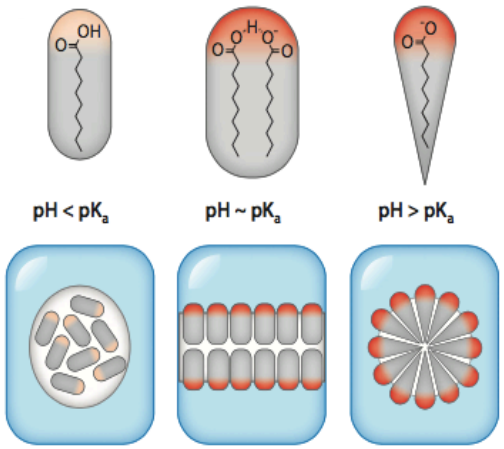

Figure 5: The aggregation of fatty acids in different pH[23]
Fatty acids spontaneously aggregate above a certain concentration. It depends on the pH of the environment and the pKa of ~7 to 9 if the fatty acids form vesicles.

fatty acid vesicles can cause the vesicles to expand in size. They have a special kind of growth, first described by the group of J.W. Szostak, where vesicles grow through the formation of filamentous membrane tubes instead of growing spherically.[23] This is a consequence of the multilamellarity of the initial vesicles. It begins with formation of a thin unilamellar filamentous hump from the outermost bilayer membrane. This is presumably due to the increased lateral pressure of absorbing more fatty acids in the membrane. Thereby, there is a faster increase of surface area relative to the volume which forms the vesicle to a filamentous shape.[26] When the size reaches a critical value, the filament will split up and divide while retaining their contents (Figure 6). This is also a consequence of the high shear stress and the thermodynamically instability. This filamentous growth is osmotically constrained and T.F. Zhu et al. showed that removing this constrain resulted into no filamentous growth. [27] The filamentous growth shows us that fatty acid vesicles have remarkable self-

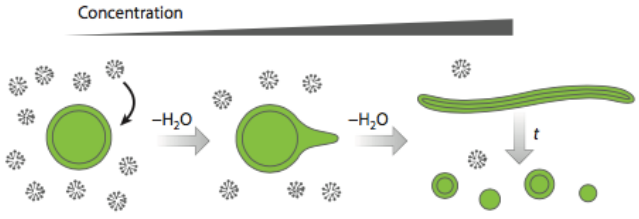

Figure 6: Filamentous growth of fatty acid vesicles [25]
The filamentous growth of fatty acid vesicles is visualized in this figure. A higher concentration of fatty acid micelles causes the bilayer vesicle to grow and this spherical form changes to a filamentous form as the concentration increases. Increasing the concentration is given as removing H_2O ($-H_2O$). At a certain timepoint, t, the filament will split up in vesicles.

organizing behaviour of the small amphiphilic molecules and besides this, they predispose the vesicle for division by mechanical or photochemical processes.[26]

Fatty acid membranes are more permeable to small charged molecules than the phospholipid membranes. A study of M.G. Sacerdote and J.W. Szostak showed that the permeability coefficient (P_s) of molecules differs for different fatty acid and phospholipid membranes.[28] The vesicles not have protein channels or transporters. This means that they need other properties, such a simple diffusion, to transport macromolecules to survive. The group of J.W. Szostak showed that adding Mg^{2+}, a cation ribozymes need, to fatty acid membranes can significantly increase the permeability of macromolecules. In this research they tested this with nucleotides that normally do not transfer across the fatty acid membrane, however with Mg^{2+} they do. Besides the nucleotides, sugars, such as ribose, are capable to diffuse over the membrane.[7] The P_s for ribose is going up till 31,0 cm/s in contrast to 5,7 cm/s for lyxose, another pentose.[28] The selective permeability for ribose over other pentoses could explain why it is used as the nucleic acid backbone.[7]

The connection of self-replication and self-reproduction in fatty acid vesicles is shown by K. Kurihare et al. Both are studied extensively, but most of the times carried out independently. Kurihare showed that self-replication of DNA is linked to the self-reproduction of the membrane. They observed that after the amplification of DNA the division of the vesicle was accelerated, so the replication of genetic information and the membrane can be done at the same time. This was a big step forward in the construction of a model for a synthetic cell.[29]

Vesicles made of fatty acids are highly dynamic. They perform more flipflops than phospholipid membranes. This dynamic aspect can be a big disadvantage if you want to do research on these vesicles. C. Hentrich and J.W. Szostak developed a method were the fatty acid vesicles were immobilized on glass slides and the rapid Brownian motion was disregarded. This makes long time observation of growth of the dynamic vesicles possible.[26] However, they are less robust and can form bilayers in a smaller range of conditions than the phospholipid membranes.[23]

Phospholipid membranes

Phospholipids are the compounds we know as the molecules that build up cell membranes as we know them. Two hydrophobic carbon chains connected by glycerol and a phosphate, the structure that was mentioned before in this review (Figure 7a). Different molecules can be fused to the phosphate molecule in the polar head, causing the headgroup to change. The same can be done with the hydrophobic tails. These can be varied in length, but because of the synthesis there always an even number of carbons. The tails can be saturated or non-saturated. These characteristics makes that there are different phospholipids and a membrane is composed of different kind of phospholipids. In water, phospholipids can spontaneously self-assemble into a spherical membrane with a bilayer.[7] The diversity of phospholipids makes the membrane more fluid, but can influence the phase behaviour, elasticity and the fundamental bilayer properties. An example of a membrane phospholipid is phosphatidylcholine (PC), which is phospholipid with a choline molecule connected to the headgroup (Figure 7b)[30].

Phospholipid vesicles are used for compartmentalization of more complex artificial cells. Their structure makes that they are stable in a wide range of conditions in comparison with the fatty acids.[23] The lipids used in lipid vesicles are purified and derived from e.g. plants and animal cells. Next to these natural phospholipids, synthetic lipids are being

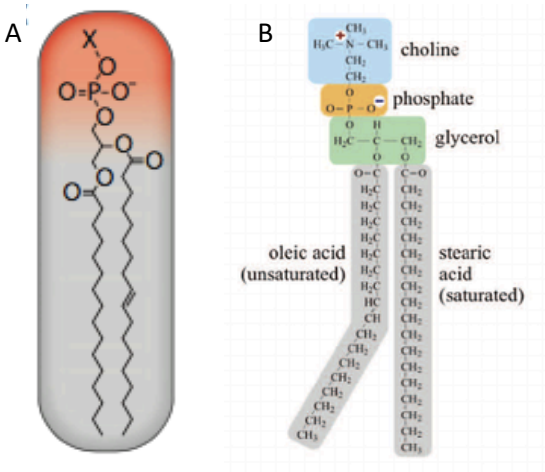

Figure 7: Phospholipid molecule [38,23]
a) Shows a simplified picture of a phospholipid molecule, The headgroup is coloured orange and the unsaturated and saturated chains grey.
b) shows a more detailed version of the phospholipid molecule. The choline molecule if coloured blue, the phosphate yellow, the glycerol is green, and de grey part shows the two fatty acid chains.

used for designing membranes. The headgroups of these lipids are modified for, for example, better coupling reactions and chelating metal ions. Synthetic lipids are, for example, used in the pharmaceutical industry for drug delivery.[31] Despite the high diversity in lipids, the phospholipid vesicles are classified by their size and the number of bilayers (Figure 8). The unilamellar vesicles are in the artificial cell research of biggest interest and are divided into three different types: small, large and giant. The multilamellar vesicles (MLVs) are more used in drug delivery studies and in research in nanotube-vesicle networks.[30]

Phospholipid vesicles of all different sizes are prepared in different ways. The formation of MLVs first reported in the 1960s by the group of Bangham. However, this multilamellar character is often a limitation and it was found that small unilamellar vesicles (SUVs) could be made out of MLVs by sonication. The SUVs were a bit too small to really function as an artificial cell membrane, since most of the cellular life is bigger in diameter than 20 nm.[4] A solution for this is the use of stable large unilamellar vesicles (LUVs). LUVs can be made by repeated extrusion under pressure of the MLVs through a polycarbonate filter. This is done in combination with freeze and thaw protocols and has the advantage that it can be done very fast and no organic solvents are used.[32] A third class of unilamellar vesicles are giant unilamellar vesicles (GUVs). This type of vesicles can be made by electroformation, a technique that uses ionic strength. GUVs are mostly used when imaging or visualization with fluorescent labelling techniques is preferred to be performed.[33] GUVs attracted much attention as biochemical reactors for macromolecules and another advantage is the possibility of puncturing these vesicles with a micropipette.[7] Besides these methods a multitude of other methods exists to prepare different unilamellar vesicles (Figure 9).[30]

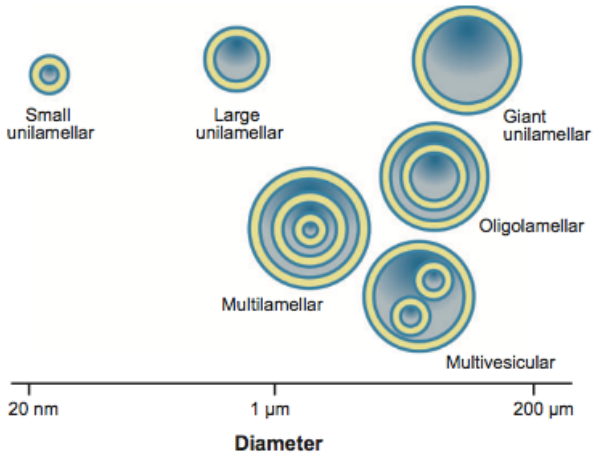

Figure 8: Schematic representation of the most common liposomes[30]
The scale shows the diameter size of the different liposomes in µm. On top the SUV, LUV and GUV are presented.

The exchange of phospholipid molecules between different membranes is not possible because the molecules are essentially permanently anchored to the membrane itself. The growth of the phospholipid vesicles is possible by fusion with other vesicles. The big downside of this way of growth is the potential leakage of contents of the vesicles. The growth of phospholipid vesicles needs constant supply of appropriate substrates for lipid synthesis. The division of the vesicles can be induced by phase separation or by an enzymatic reaction after the vesicle reach a certain size.[23] This is in contrast to the filamentous growth of the fatty acid membranes.

The permeability of phospholipid membranes can be altered by changing the phospholipid

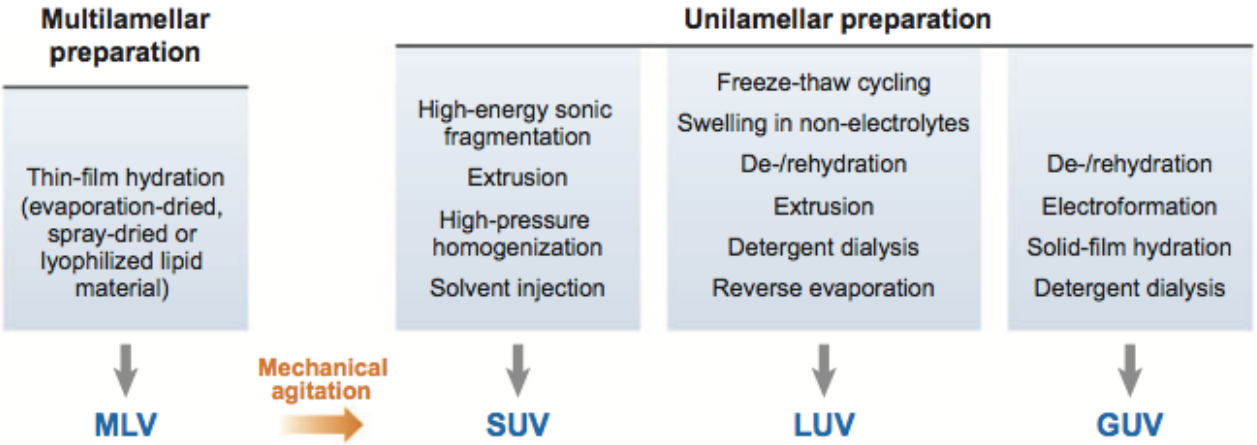

Figure 9:Different methods to prepare different unilamellar vesicles [30]
The different methods are categorized by lamellarity and size range. MLVs can be transformed into unilamellar vesicles by various means of mechanical agitations (shown in orange).

composition of the membrane itself. Shorter hydrophobic chains of the phospholipids can cause small molecules to pass and keep macromolecules at the outside. Steroids can decrease the permeability as well, for example the diffusion of arabinose over the membrane. The P_s of arabinose decreases from 2,8 cm/s to 1,1 cm/s when cholesterol is added to the membrane. This was shown by M.G. Sacerdote and J.W. Szostak.[28] However, to achieve efficient and specific transport, the use of transmembrane proteins, channels and carrier proteins is needed. Nevertheless, two challenges are coming up in designing stable membrane proteins. The first one is the regulation of the channels and the second one is to generate membrane proteins with a stable and correct orientation and stoichiometry.[30] GUVs are very suitable for imitating the cytological processes of biological cells.[7]

Since lipid membranes often rely on the use of transporters and channels it is important to know how these proteins can be inserted into the membrane. This is mostly done by the use of detergents. Detergents, such as Triton X-100, can solubilize the membrane protein. Detergents can form micelles and have an own cmc. After adding detergent to lipid vesicles with a membrane protein, the hydrophobic part of the detergent will bind to the hydrophobic part of the lipid molecules, thereby replacing the lipid molecules and introducing the protein in detergent molecules. Later on, the protein solubilized in detergent and detergent-phospholipid combinations are combined and the removal of the detergent will cause the membrane protein to fuse with the available phospholipids (Figure 10).[10]

The solubility of phospholipids is very low in contrast with the fatty acid molecules.[26] A big advantage of phospholipids compared to fatty acids is that phospholipids do not inhibit any polymerases. This means that e.g. PCR in these phospholipid vesicles is possible. This can be important if they are used in clinical applications, yet for research of the origin of life.[16] The bad permeability of phospholipid vesicles can be a disadvantage but an advantage as well, since this feature prevents degradation by proteinases from the outside. Another big advantage of phospholipid vesicles is the possibility of communication with other cells and giving response on changes in the environment.[7]

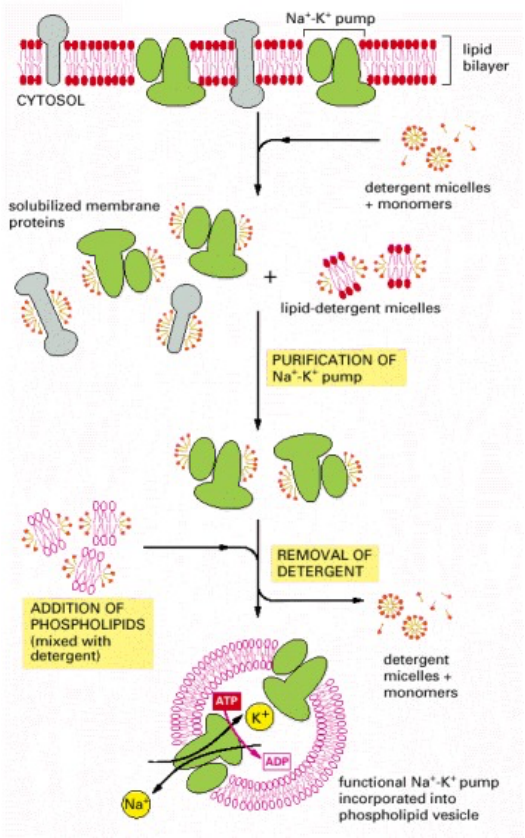

Figure 10: The use of detergents for solubilizing, purifying and reconstituting functional membrane protein system[10]
This overview shows how an Na⁺-K⁺ pump of a lipid bilayer is incorporated into a phospholipid vesicle by using detergent..

Blended membrane

Last years, researchers look at blended membranes as well. With this term, this review indicates membranes with both fatty acids and phospholipid molecules. You could think that this membrane could give “the best of both worlds”. One of the research groups that is performing research on this subject is the group of Szostak. The blended membrane is an intermediate found in the transition of fatty acid membranes to modern phospholipid membranes. It differs from the modern membranes found in nature these days. Szostak et al. showed that phospholipids are needed in a membrane to promote vesicle growth, increase stability and stabilize membrane proteins that could be inhibited by fatty acids. However, what was said before, pure phospholipid membranes are highly impermeable for cations and small polar molecules in the solution.[34]

In a study of Lin Jin et al. of the group of Szostak, the blended membrane that was used was composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), a phospholipid, and oleic

acid as a fatty acid. This research indicates that the stability of the membrane is higher with more phospholipid in the membrane but he permeability gets lower. They tested this in the presence of Mg^{2+}, an important compound needed for RNA catalysis, but also known as a compound that disrupts fatty acid membranes. The permeability was tested with Magnesium green, a dye that gets fluorescent in presence of Mg^{2+}, inside the vesicles. Figure 11a shows that after 2 minutes the Mg^{2+} diffuses into the vesicles with 50% OA and 50% POPC and thereby the inside of the vesicle was stained green. For the POPC membrane no fluorescence was observed. Figure 11b shows quantitative data that after OA addition to the vesicles the fluorescence in the vesicle gets higher. Which means that the permeability gets higher with more fatty acids in the membrane. Blended membranes are still permeable for Mg^{2+} and also nucleotides till a concentration of 75% POPC.[34] This blended membrane made of OA and POPC shows that combining membrane building blocks of two different kind of membranes could also combine properties.

Other membranes

Although fatty acid and phospholipid membranes are the most studies models for synthetic cells, there are multiple different typical synthetic membranes designed and used. Membranes could also be built up from proteins, a membrane we refer to as a proteinosome. Protein molecules are attractive as building blocks because of the biocompatibility, biodegradability, and biofunctionality. The elastic compartments are happening to be semi-permeable, temperature-sensitive and enzymatically active.[7] Kamat et al. left the proteins out and built membranes only composed from polymers. These vesicles are known as polymersomes. Synthetic cells with these polymer membranes are most used for study nongrowing cells because the polymers make the membrane incapable to divide and grow.[23]

In addition, research groups use semi-permeable nylon capsules or inorganic membranes, called colloidosomes. The nylon capsules have multiple medical applications, mainly replacing or supplementing deficient cell functions. Colloidosomes may find applications as delivery for controlled release of drugs and cosmetic or food supplements. [7,35] Recently atypical synthetic membranes are studied. These are synthetic cells based on compartmentalization with non-membrane types of compounds that mimic the compartments. An

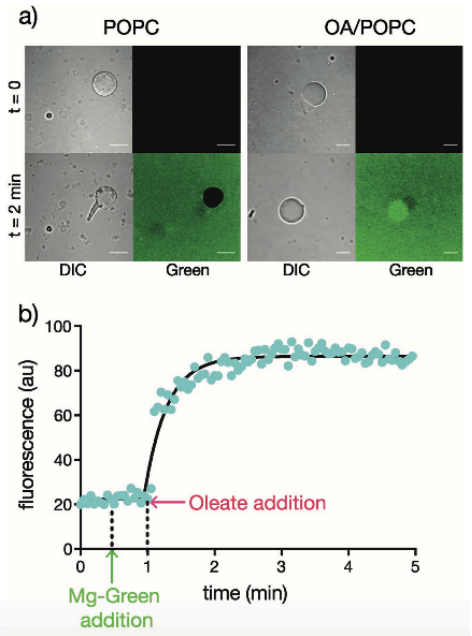

Figure 11: Fatty acids enhance the permeability of phospholipid vesicles[34]
a) Microscopic images of POPC and OA/POPC (50/50) giant vesicles at time point 0 minutes and timepoint 2 minutes. DIC stands for differential interference contrast microscopy image and Green stand for the cells where Magnesium green was added. The presence of Mg^{2+} is indicated by the increased green fluorescence and so Magnesium green. The scale bar of POPC vesicles is 20 µm. The scale bar for OA/POPC vesicles is 10 µm. B) Quantitative data of a fluormeter study. The X-axis shows the time in minutes and the Y-axis the fluorescence in arbitrary units. After addition of oleate to the pure phospholipid vesicles containing Mg^{2+} the Mg^{2+}diffuses out of the vesicle. This was measured by an increase in Magnesium Green fluorescence outside the vesicle.

example of these kind of membranes are water-in-oil emulsions. These droplets can be fed by other vesicles, grow and divide by shear forces or extrusion. The big downside of these atypical membranes is that they are far away from the standard biology. There is a miniscule chance that this kind of compartmentalization can be used in designing an "alive" artificial cell.

Conclusion

Despite the impressive progress in the design of a synthetic cell, there is still a big gap between a real biological system and a synthetic system. Creating a cell that has all hallmarks of life in one is not yet been accomplished. To achieve this goal, we believe researchers need to work multi-disciplinary. Chemists, biologists, physicists and even informaticians need to work together to find a solution for constraints and design problems they are dealing with. Such constraints are, fotr example,

thermodynamically defined. Computer sciences could help in this research. Most of the times computer models are faster prepared than lab models and thereby you could make models that biology would not accept. This could help in understanding cellular life and see which parts, compounds and genes are really important and a necessary for the synthetic cell.

This review focussed on the bottom-up creation of an artificial cell. This bottom-up approach is relatively more challenging, but still most studies apply this approach. Additionally, top-down synthetic biology can provide more insight into the processes that are essential for cells to live.[6] We think different research groups should focus on one approach of synthetic biology and should combine the found results of both.

The advantages of the use of fatty acid and phospholipid membranes are presented. However, we believe the most promising membrane is the blended membrane with both phospholipids and fatty acids. Here the "best of both worlds" is indeed coming together. For the size of the vesicles a LUV would be preferred this vesicle has the correct size to resemble a natural cell. The phospholipids create a stable membrane with the possibility of incorporating membrane proteins. The blended membrane is probably capable of self-replication and assemble as the fatty acid vesicles, it can transport the small molecules though the membrane, and bigger molecules with channels, transporters and carrier proteins. The permeability, stability and dynamics can still be adjusted by varying the chain length of fatty acids and the use of different lipids as done by the non-blended membranes. Since not only fatty acids are used the bilayer can be formed in a wider range of conditions and it is still a robust membrane.

The only big downside is that this kind of membrane does not represent a biological cell as we know it. We believe that this could probably be compensated with the fact that a normal membrane is not only composed of phospholipids on its own. The membrane can be seen as a fluid mosaic model with lipids, but membrane proteins and cholesterol in it as well. The fatty acids cause more fluidity and can possibly be a part of this mosaic model. Besides this, a fatty acid is a "small" phospholipid and a precursor of it. Still the consequences of using this as a synthetic membrane for an alive cell need to be researched further.

Synthetic cells have different advantages that makes them so interesting for applications. It could provide a conceivable theory for the origin of live, it could provide a less interfering way to research cellular life and could connect the non-living world with the living world. Researchers could even add non-biological new functions to the cells. This makes that synthetic cells could have attractive opportunities to fields in biotechnology, where they may replace engineered organisms to produce fuels and pharmaceuticals. Furthermore, synthetic cells could be used in biomedical applications.[7] Such as advanced drug-delivery technology, in which synthetic cells are engineered to control the timing and the site of the release of a certain drug.[23]

Additional to the compartmentalization problem, multiple questions are still unanswered. Like how to provide cells with informative biomacromolecules and designing metabolic pathways to provide energy to the cell. Even more daring is introducing applicable feedback mechanisms in synthetic cells that switch on and off and can respond to the environment.[36] Al these hallmarks are part of the five characteristics of life and very important in making a synthetic cell. When a cell contains all the hallmarks of life, we can satisfy NASA's definition of life: 'a self-sustaining chemical system capable of Darwinian evolution".[6,7] Unfortunately, researchers are still very far away from making this complete "alive" synthetic cell. However, the combination of the exciting challenge and all the potential applications in the future, will for sure drive progress in this field for all those years to come.

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