Lipids and Their Functions

12-07-2022
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Bachelor's Thesis Life Sciences (WBBY901-05)

Table of Contents

Summary
Cellular membranes contain thousands of different lipids. Of many of these lipids the function is still unknown. The objective of this thesis is to provide an explanation for the enormous diversity of lipids observed in membranes. This thesis presents a broad overview of different functions of membranes and lipids, the effect of membrane composition on these functions and how they are maintained. New lipid functions are still being discovered, indicating that the complexity of membranes and their composition reflects their many tasks in the cell. Lipid metabolism also plays a crucial role in tackling the global obesity epidemic.

Introduction
Cell membranes are crucial for life of cells, as they mediate the compartmentalisation of cells that makes life possible in the first place. All living organisms, whether they are eukaryotes or prokaryotes, have plasma membrane around their cells. It is estimated that 15 to 39% of the human proteome is dedicated to membrane proteins (Almén et al., 2009), with similar numbers for *Escherichia coli* (21%) and *Drosophila melanogaster* (20%) (Krogh et al., 2001). Despite their ubiquity, there is a large degree of variation in membrane composition: eukaryotic cells contain thousands of different lipid species (Harayama and Riezman, 2018, Agmon and Stockwell, 2017). These differences can be very subtle, such as the position of a double bond in the hydrophobic tail, but the backbone and headgroup can also be completely different. This thesis will give a broad overview of different lipids and their functions, the role they play in different membranes and how they are maintained in the cell, giving an explanation for their enormous variation.

What makes a membrane?
Around 50% of the mass of animal cell membranes consists of lipids, with the rest being membrane proteins (Alberts, 2015), there are around 10^9 lipid molecules in the membrane of an animal cell. Lipids are amphiphilic molecules, meaning that they have a hydrophilic end and a hydrophobic one. This allows the lipids to spontaneously assemble into lipid bilayers, with the hydrophobic ends next to each other and the hydrophilic ends facing outwards. These lipid bilayers form the basis for cellular membranes. Proteins and lipids can move freely laterally throughout the membrane, which is a critical property for membrane functions such as signalling and transport (Cooper, 2000). Movement from the inner leaflet to the outer leaflet and vice versa are energetically unfavourable and therefor occur rarely. Cells can mediate this movement by using enzymes called lipid flippases and scramblases. The hydrophobic part or tail of lipids contain long acyl chains (usually between 14 and 24 carbon atoms). The tails are connected to a backbone, to which headgroups can attach. A *cis*-double bond in the tail leads to a kink in the chain. Usually, one of the tails is unsaturated and the other is saturated. This variety in components gives rise to an enormously diverse group of molecules. The most abundant membrane lipids are phospholipids, which have a polar head group containing a phosphate group and two tails which are usually fatty acids. The most common phospholipids in animal cells are glycerophospholipids (GPLs), which have a three-carbon glycerol backbone. Sphingolipids are also an important class of phospholipids, which have a sphingosine backbone instead of a glycerol one.

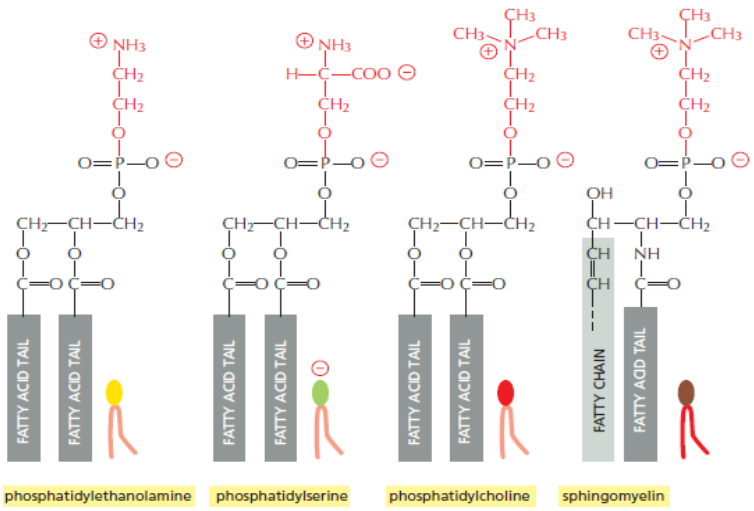

Figure 1: The chemical structure of the most common phospholipids in mammalian cells. The various head groups are coloured in red. (Alberts, 2015)

The four major phospholipids in mammalian membranes are phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer) and phosphatidylcholine (PtdCho), which are phosphoglycerides, and sphingomyelin (Alberts, 2015; van Meer et al., 2008). Over 50% of the phospholipids in most eukaryotic membranes are PtdCho. These lipids and the structure of their head groups is shown in Figure 1. Charges are also shown in these structures, these are charges at physiological pH. Except for PtdSer, they are zwitterionic, meaning they contain an equal number of positive and negative charges. The zwitterionic nature of the headgroups helps stabilize the membrane and pack the phospholipids tightly. The headgroups are oriented perpendicularly with respect to the membrane, with the positively charged end close to the negatively charged phosphate group of its neighbour (Chen et al., 2018). Membrane composition can change dynamically, such as during cell division (Atilla-Gokcumen et al., 2014) but is also under homeostatic control. When the balance of lipids in the membrane is disturbed, cells have mechanisms that can restore it.

Lipid analysis

Lipidomics is a relatively new scientific discipline that focusses on studying cellular lipids on a large and analytical scale (Yang and Han, 2016). Developments in Mass Spectrometry (MS) have greatly influenced the emergence of the field. The workflow for a typical lipidomics analysis consists of sample preparation, spectrometry analysis, data acquisition and data processing. The most commonly used method is the so-called Modified Bligh and Dyer method, which is suitable for small biological samples. It uses chloroform to separate the total lipid fraction from the rest of the cell. Once a lipid fraction has been isolated, the most common techniques used are shotgun lipidomics and liquid chromatography based lipidomics. In LC based lipidomics, the sample is separated via liquid chromatography before mass spectrometry analysis. The advantages of this technique are that it can be used for highly sensitive analysis but it has a low throughput. Shotgun mass spectrometry is a faster and simpler technique, requiring no adjustments to a lipid sample before injecting it for mass spectrometry.

Composition of Organelles

During the introduction, the four major phospholipids in mammalian cells were mentioned. Mammalian cells however, like all eukaryotes, have multiple different membranes in one cell, forming not only the outer barrier of the cell but also enclosing different organelles inside of it. In human cells, PtdCho makes up around 50% of all phospholipids in membranes (van Meer et al., 2008). Each organelle has a different function and the membrane composition reflects this. The membrane of the endoplasmic reticulum for example, needs to be more fluid to facilitate membrane transport, while the plasma membrane needs to be more rigid to perform its barrier function. Mono- and poly-unsaturated fatty acids are harder to pack together because of the kinks in their chains. The endoplasmic reticulum has a higher degree of unsaturated fatty acids, creating a thinner and more fluid membrane (Holthuis and Menon, 2014). The plasma membrane, in contrast, contains more saturated fatty acids which produces opposite effects. Protein liquidity can also help promote protein-protein interactions. Some lipids are also used to covalently modify certain proteins. These modifications can increase membrane affinity and can also help to promote protein-protein interactions.

Mitochondria

Another interesting membrane composition can be found in the inner mitochondrial membrane (IMM), which consists for about 20% of cardiolipin. Cardiolipin is both localised and synthesised in the IMM (Paradies et al., 2019), while other phospholipids are mainly synthesised in the endoplasmic reticulum. It plays a vital role in the functioning of the mitochondria, not only by maintaining the shape of the IMM but also by interacting with IMM membranes including enzymes in the electron transport train. Cardiolipin has a unique dimeric structure which allows it to grant the curvature needed to create the cristae of the IMM that protrude into the mitochondrial matrix (Ren et al., 2014). After synthesis, the acyl chains of cardiolipin are heavily remodelled so that it contains mostly unsaturated fatty acids. This so called mature form of cardiolipin help in providing stability to the IMM shape. Cardiolipin also

occurs in the outer membrane of prokaryotes, where it has been shown to create membrane domains that appear to be involved in functions such as binding, cell division and energy metabolism (Mileykovskaya and Dowhan, 2009).

Lipid Rafts

Next to the phospholipids and sphingolipids mentioned in the introduction, another important building block of membranes are sterols. In mammalian cells, the main sterol found in membranes is cholesterol. In Cholesterol inserts itself in the membrane close to the polar head of the phospholipids, reducing the mobility of that region and thus decreasing the permeability of the membrane. Cholesterol tightens the bilayer and decreases the fluidity. The cholesterol content of membranes in a human cell also varies widely. The outer membrane has a cholesterol:phospholipid molar ratio of 1:1, while organelles all have ratios below 1:2 (van Meer et al., 2008).

Lipid rafts are specialized domains in the membrane that compartementalize specific cellular processes by keeping related signalling molecules near eachother in the membrane. Lipids in rafts are more tightly packed than the rest of the membrane but rafts are able to float freely through it (Simons and Ehehalt, 2002). Lipids in the lipid bilayer exist in a liquid-disordered state, in which the lipids can move relatively uninhibited. Lipid rafts are comprised of cholesterol and sphingolipids in the outer leaflet which are connected to cholesterol and phospholipids in the inner leaflet. Cholesterol acts as a spacer between the sphingolipids and as glue to keep the raft together because it has a higher affinity for the sphingolipids in the raft than for unsaturated phospholipids. If cholesterol is removed, the proteins in rafts dissociate. Lipid rafts are assembled in the Golgi complex. Both cholesterol and ceramide, the precursor of sphingolipids, are produced in the endoplasmic reticulum but most sphingolipid head groups are attached in the Golgi complex. Lipid rafts appear to be blocked from retrograde movement from the Golgi complex to the endoplasmic reticulum, keeping them moving towards the plasma membrane. The concentrations of cholesterol and sphingolipids are tightly regulated so the supply is limited.

Vesicular Transport

Transport of substances between organelles and intake/excretion are mediated by vesicular transport. Many organelles also form membrane contact sites (MCSs) with other organelles if they are in close proximity, the endoplasmic reticulum in particular, which forms MCSs with nearly all other organelles. Phosphoinositides are derived from phosphatidylinositol (PtdIns), which is found in the cytoplasmic leaflet of eukaryotic cells (Posor et al., 2022). They are signalling lipids that are involved in many cellular processes and play a critical role in intracellular transport. Phosphoinositides can be phosphorylated at multiple positions of their inositol headgroup. PtdIns 3-phosphate recruits proteins to early endosomes and helps define their identity. Different organelles have different PtdIns variants and in different ratios as well. For example, the Golgi Apparatus is characterised by PtdIns 4 phosphate and the plasma membrane is enriched in PtdIns 4,5-biphosphate. Vesicles used in exocytosis also contain a small amount of PtdIns 4,5-biphosphate, which helps guide it towards the membrane and helps with vesicle docking. A key feature of MCSs is the recruiting of a phosphoinoisitide binding protein by one of the organelles. For instance, the ER is connected to the plasma membrane via synaptotagmins. A major function of MCSs is lipid transfer between the linked organelles. Lipids can either be transported along their gradient or be linked to the transport of another. An example of this is the PtdIns4P gradient between the ER and the Golgi apparatus. PtdIns4P is synthesised in the Golgi apparatus but its hydrolysis is mostly done in the ER, resulting in a gradient between the two. The energy from this gradient is used to power the export of lipids like cholesterol from the ER.

Cell Division

During cell division, cells undergo a change of shape, forming a small structure called the midbody. At the midbody, cleavage between the daughter cells occurs. Lipidomics research has shown that cells actively regulate the production and localization of lipids during cell division (Atilla-Gokcumen et al., 2014). For instance, the concentration of triacylglycerol increased 54 fold at the midbody, while

remaining unchanged for the cell as a whole, indicating that it is recruited to the midbody during cell division. Unfortunately, the study does not provide any answers as to what the function of these lipids is or what drives their transport or synthesis during cell division. It does indicate that lipids play an important role in this biological process as well. Possible functions of these lipids could for instance be stabilising the membrane while it undergoes the stress of fission and ensuring a tight seal after membrane fission, to make sure that the cells are not open to the environment at any point.

Protein Interactions

There are many lipids that recruit proteins to specific membrane domains via interactions with a lipid-binding domain. Phosphoinositides are the best understood lipids that engage in protein recruitment. Phosphoinositides can be regulated dynamically via phosphorylation and dephosphorylation. They are involved in multiple cellullar functions including vesicular budding and membrane fusion (De Craene et al., 2017). Although they are present in small quantities, their role in the cell is important. Imbalanced phosphoinositide concentrations are linked to various cancers and other diseases. PtdSer is also an important protein-interacting lipid. It is involved in intracellular trafficking and also is an important signal for phagocytes during apoptosis.

Other lipid-protein interactions are based on membrane propterties. An example are the BAR-domain containgin proteins. The BAR-domain is a banana-shaped domain that favorably binds to curved membranes, allowing the proteins to detect membrane curvature. As discussed before, the lipid composition greatly affects the curvature of the membrane. When cholesterol is depleted, the increased density of PtdSer causes the membrane to become more curved, because of the repulsion between the charged head groups. It has been shown that this increase in curvature results in the recruitment of endophilin, a BAR-domain containing protein, resulting in endocytosis (Hirama et al., 2017).

Lipid Metabolism

In the last decades, nearly all steps of lipid metabolism have been assigned to corresponding enzymes, giving great insight into the driving forces behind the creating and maintaining of lipid diversity and ratios. The main production of phospholipids and cholesterol takes place in the endoplasmic reticulum. One feature of these lipid-metabolizing enzymes that contributes to the chemical diversity of lipids is their promiscuity, which means that one enzyme uses multiple similar yet different substrates. This means that only a few enzymes are necessary to generate various GPLs with different acyl-chains.

Fatty acids can be de novo synthesised from acetyl-CoA by the enzymes Acetyl-CoA carboxylase 1 & 2. These fatty acids are combined with glyceraldehyde 3-phosphate to synthesise phosphatidic acid (PtdA), which is the predecessor for GPLs and essentially a GPL without a headgroup. Different enzymes can add different head groups to PtdA. PtdA is first converted to diacylglycerol by Phosphatidate phosphatase enzymes, after which choline can be attached by choline phosphotransferase 1 and ethanolamine by ethanolamine phosphotransferase 1 to synthesise PtdCho and PtdEtn respectively. Diacylglycerol kinase catalyses the reverse reaction from glyceraldehyde 3-phosphate to fatty acids.

Lands' cycle

After GPL synthesis, the GPLs enter the remodelling pathway, also known as Lands' cycle (Hishikawa et al., 2014). Lands' cycle creates lipid diversity by removing acyl chains from phospholipids via phospholipase A enzymes (PLA_2) and re-attaching acyl chains using lysophospholipid acyltransferase (LPLAT). This progress is also important for the production of lipids containing poly unsaturated fatty acids (PUFAs). Mammalian cells cannot synthesise PUFAs by themselves (except for mead acid), meaning they have to derived from dietary supply. PUFAs are metabolised from (α-)linoleic acid (also known as omega-3 and omega-6 fatty acids in common terms) from food and incorporated into GPLs using the aforementioned Lands' cycle.

When Less than 1-2% of total calories are provided by these essential fatty acids (EFAs), people enter a state of essential fatty acid deficit (EFAD) (Ichi et al., 2014). EFAD has been linked to serious diseases such as coronary artery disease and diabetes (Simopoulos, 1999). Since PUFAs are important for multiple cellular processes and membrane-associated enzymes, there is a pathway in the cell to synthesise mead acid when it enters a state of EFAD. The cell uses oleic acid as a starting material for this, and uses fatty acid elongases and desaturases that are normally used in the metabolism of EFAs.

Diacylglycerol kinase

Diacylglycerol kinase (DAGK) occupies a pivotal point in the synthesis of new phospholipids, catalysing the conversion of diacylglycerol (DAG) to phosphatic acid (PA). When cells are not stimulated, the activity of DAGK is low, allowing for the normal synthesis of new lipids. Once it gets activated by the phosphoinositide pathway, its activity increases. Both DAG and PA themselves are also linked to the regulation of various cellular functions, giving DAGK the effective function of a switch by terminating one signal while enhancing the other.

Homeostasis

As mentioned earlier, the membrane composition is under homeostatic control and the composition of a also membrane plays an important role in cellular and organelle function. So even though membranes are very complex and dynamic structures, their physical properties need to remain stable. The composition of membranes is constantly remodelled through regulatory metabolic processes (Agmon and Stockwell, 2017).

Membrane composition is regulated by both synthesis of new lipids and lipid trafficking. Lipid synthesis is partially controlled by lipid composition sensors that can up- and downregulate the activity of lipid enzymes. Lipid trafficking is mediated by lipid transfer proteins. The lipids can either be transported from diffusing lipid droplets, which are small storage organelles found throughout the cell (Olzmann and Carvalho, 2019), or through direct contact between organelles. The endoplasmic reticulum is central in the transport of lipids, since it is the primary secretory organelle. Transfer proteins are highly selective and many are unidirectional between organelles, which allows the cell to maintain the differences in membrane composition between different organelles.

Membrane fluidity can also be regulated in response to thermosensors that are embedded into the membrane. This process is called homeoviscous adaptation (Mendoza, 2014). Although it is less important for human cells, since our temperature is regulated and does not fluctuate much, it is a vital process for both prokaryotes and poikilotherm animals, such as fish.

Although many lipid-metabolizing enzymes are promiscuous, they also have different substrate preferences, which leads to different product ratios for different enzymes. The combination of promiscuity, substrate preferences and redundant enzymes give a cell great control over membrane composition, resulting in a large amount of membrane diversity between different tissues. Unfortunately, the transcriptional regulation for different tissues has not yet been studied thoroughly but it seems a very promising field for finding explanations for membrane diversity.

Health and Disease

Proper membrane metabolism is vitally important for the functioning of cells. Over 80 diseases have been identified as complex lipid metabolism disorders. In such disorders, normal levels of lipids are disturbed because lipid-metabolizing enzymes do not work properly (Natesan and Kim, 2021). Hyperlipidaemia refers to disorders where there is an increased level of unwanted lipids in the blood. Hyperlipidaemia produces more health disorders than disorders characterised by a lower amount of lipids. Obesity is the most common lipid metabolic disorder, characterised by an increased amount of body fat. If the excessive lipids are not able to be removed from the blood, they damage blood vessels and accumulate inside them, leading to complications such as heart failure and strokes. The storage of excess lipids can also lead to organ damage, which causes diseases such as Gaucher's disease. In general, hyperlipidaemia disorders do not produce any noticeable symptoms until it is too late. A

heightened level of lipids in the blood can be easily monitored however, and lifestyle changes (diet & exercise) are often enough to bring lipid levels down to a desirable level. In the case of high cholesterol levels, lifestyle changes are the least effective. If there is an acute danger for the patient, statins can be administered. They are inhibitors of HMG-CoA reductase, which is an enzyme found in the liver that is responsible for cholesterol synthesis (Taylor et al., 2013). Worldwide, over 200 million people take statin daily (Blaha and Martin, 2013).

Dietary Balance

As humans evolved, our diets have changed quite drastically. Since the 1800s, our total fat intake has nearly doubled, as shown in Figure 2. Also seen in the graph is a doubling of saturated fat intake, a sharp rise in trans fatty acids and a shift in the balance between omega-3 and omega-6 fatty acids. Trans fats occur naturally in the milk and body fat of cows and sheep, so a small amount of natural trans fats is part of a normal human diet and not harmful. Problems started to arise when the processed food industry started mass producing vegetable oils. Trans fats are a by-product of partially hydrogenating these vegetable oils and their use was widespread in products like fast food and baked goods. Trans fatty acids increase membrane cholesterol when they are incorporated and are considered a major health risk factor for coronary heart disease (Niu et al., 2005). In most of the western world, artificial trans fats are now banned but in developing countries they are still widely consumed. It has been estimated that this results in around 500,000 excess deaths per year (de Souza et al., 2015). The shift in balance of the essential fatty acids can also be attributed to the increased intake of vegetable oils. In western diets, the ratio of omega-6:omega-3 fatty acids has shifted from a natural 1-2:1 to approximately 25:1. A high intake of omega-6 fatty acids leads to a shift in physiological state to higher blood viscosity and vasoconstriction (Simopoulos, 1999).

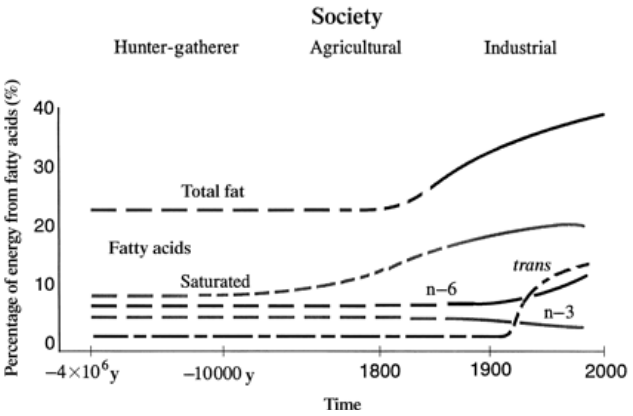

Figure 2: Changes in human intake of fats through their diet (Simopoulos, 1999)

Programmed Cell Death

Programmed cell death is an important part of the life cycle of a cell. An adult human loses between 50 and 70 billion cells per day to apoptosis. It is also extensively linked with lipids and lipid metabolism. An excess of saturated fatty acids causes lipotoxic stress which eventually leads to autophagy and apoptosis (Thürmer et al., 2022). Programmed cell death is characterised by a decrease mono-unsaturated fatty acids (MUFAs) and phosphatidylinositol (PI). Healthy cells can counteract this process by converting the saturated fatty acids into MUFAs using stearoyl-CoA desaturases (SCDs). A newly discovered lipid that is derived from an SCD that appears to promote cell survival is PI(18:1/18:1).

Lipids are involved in multiple forms of programmed cell death. It makes sense that the membrane is a target for controlled destruction of a cell, since it is such a vital component in keeping the cell alive. During apoptosis, caspases cleave proteins and DNA into smaller pieces. As the cytoskeleton begins to degrade, small cellular bodies called blebs are released from the dying cell, to be recycled by phagocytic cells. The outer membrane of mitochondria is a target for apoptosis pathways. As apoptosis continues lipid flippases and scramblases increase the amount of PtdSer in the outer leaflet, which provides a signal to nearby phagocytes that the cell is dying.

Ferroptosis is a relatively newly discovered form of programmed cell death. It is characterized by a large amount of iron accumulation and lipid reactive oxygen species (Li et al., 2020). During ferroptosis, the mitochondria shrink and the cristae are reduced or disappear. The accumulation of reactive oxygen species occurs because the cysteine uptake of cells is disturbed. The cysteine is

normally used in the production of glutathione, which is used by the cell as an antioxidant. As cysteine uptake is blocked, the antioxidant capabilities of the cell are weakened, leading to the accumulation of lipid reactive oxygen species. This affects the mitochondria the most since that is where most of the reactive oxygen species are formed in the electron transport chain. PUFAs are especially sensitive to lipid peroxidation and are therefore one of the essential elements of ferroptosis. Ferroptosis plays a role in the development of several diseases, including tumours, acute kidney injury and certain neurological diseases. The ferroptosis pathway is a putative target for treatment in these diseases, as activating or blocking it could alleviate their progression.

Membrane Lipid Therapy

Around 60% of currently known drug targets are membrane proteins (Aguayo-Ortiz et al., 2021; Overington et al., 2006). As discussed previously, lipids directly interact with membrane proteins by influencing protein conformation, localization and protein-protein interactions. This means that altering a patients' membrane composition can be used to target a specific protein (Escribá et al., 2015). There are multiple ways to influence membrane composition. The lipid composition can be changed by making dietary changes, by regulating the activity of lipid-metabolising enzymes or by altering the gene expression of these enzymes. Since lipids play such an integral role in programmed cell death, membrane lipid therapy can be used to induce or suppress it. Two ferroptosis inducers have been identified that prevent tumour growth in mice models (Yang et al., 2014).

Lipid-based Nanoparticles

Another promising medical application of lipid membranes are lipid based nanoparticles (LBNPs) for drug delivery. These nanoparticles aim to mimic the membrane properties of liposomes. Once more is known about the intricacies of liposome targeting and transport, this could enable drug carriers to deliver drug directly to a target organelle in a non-toxic manner. An obvious example of this in action are the Pfizer-BioNTech and Moderna vaccines for COVID-19 that were successfully deployed at a global scale in 2021. The advantages of LBNPs include high stability, cheap production costs that can be realised at an industrial scale and low toxicity (García-Pinel et al., 2019). LBNPs are also used in cancer treatment, with the drug doxorubicin commonly being administered via LBNPs. Another technique used is active targeting. Here, an LBNP is modified with antibodies that recognise the target tumor cells

Conclusion & Discussion

The objective of this thesis was to find an explanation for the large variety of lipids in membranes. The conclusion is that the huge variety of lipids corresponds the large amount of complex and varied tasks that membranes perform in the cell. A lot of the functions of lipids are still unknown and as more and more lipid functions are discovered the total picture will also become more clear. As more lipid functions are discovered, the impact of the small structural differences between them may also become better understood. Obesity and its consequences are major challenges on a global scale. Perhaps a better understanding of lipid metabolism can help us combat this issue. While the enzymes responsible for lipid metabolism have nearly all been identified, a more complete understanding of regulation and feedback will be needed to understand how specific lipid compositions are achieved and what the effect of changes in the composition might be. The sheer amount of lipids makes it difficult to analyse the function of every one, compounded by the promiscuity of enzymes involved in enzyme metabolism. There are also many transient lipid species which are poorly understood, as it is difficult to analyse them due to a lack of tools. A tool that could help understand what happens when lipid composition is altered is Molecular Dynamics Simulation, which could also give some insights in lipid functions and behaviour. At this point, scientists have only really uncovered the tip of the iceberg when it comes to lipids.

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