Biology 5357: Chemistry & Physics of Biomolecules - Membranes & Membrane Proteins

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Lecture 1: The discovery of the lipid bilayer cell membrane and the proteins embedded within.

Required reading:

Robertson, J. (2018). **The lipid bilayer membrane and its protein constituents.** The Journal of general physiology 150(11), 1472-1483. https://dx.doi.org/10.1085/jgp.201812153

Singer, S., Nicolson, G. (1972). **The Fluid Mosaic Model of the Structure of Cell Membranes** Science 175(4023), 720-731. https://dx.doi.org/10.1126/science.175.4023.720

Other useful references:

Tanford, C. (1989). **Ben Franklin Stilled the Waves: An Informal History of Pouring Oil on Water with Reflections on the Ups and Downs of Scientific Life in General.** Duke University Press.

Stillwell, W. (2016). **An introduction to Biological Membranes. Composition, Structure and Function.** Elsevier Science.

Part 1. The world before the membrane

The cellular boundary. We can see that throughout biology, cells and their internal organelles are all bounded by the same lipid bilayer membrane structure. Yet, the journey to understand this was long and not always clear. In this lecture, I'll discuss the key discoveries that led to our current understanding of the structure of cellular membranes and the proteins the lie within.

The discovery of biological boundaries in plants. A cell is the smallest structural and functional unit that supports biological life. In 1665, using a simple light microscope, Robert Hooke examined tissue from a cork tree and observed a collection of compartments he termed "cells". We know now that he was visualizing the cell wall, a rigid layer that is dense in cellulose and polysaccharides. However, this would be the first observation in understanding that compartmentalization is a necessary requirement of biology.

Why does biology need boundaries? Let's take a moment to consider the implication of this discovery. The realization that living beings are broken up into simpler functional units is a fundamental discovery in biology. The implicit requirement of Cell Theory is that the cell is distinctly separated from the external world. It must be contained in some manner. The important biological molecules, like DNA, RNA and proteins must be kept in place, and salt and pH must be defined within optimal working ranges for proper biochemistry. A barrier

allows for the storage of information and potential energy, converting cells into batteries that fuel biological work. A barrier allows organisms to live amongst diverse environmental conditions, so that the internal conditions can be precisely controlled. The question is, is the barrier defined by a unique structure particular to each cell or is it something that is robust and general across biology?

Animal plants have boundaries too, but they are invisible. Shortly after, in 1677, Antonie van Leeuwenhoek reported his observations of bacteria and microbes. Following up on work of others, he provided detailed reports about imaging of erythrocytes, showing that the compartmentalization occurs in animal cells as well. Yet, an important observation was noted that animal cells and microbes did not have the distinct cell wall type of structure that was observed in plants. It was clear that they were separated from the external solution, but the structure that was providing the barrier was not observable.

Imaging of other animal cells, and particularly mobile organisms like amoebas, showed that while these cells remained bounded, the encapsulating boundary was flexible. Whatever the bounding mechanism may be, it must be fluid-like, flexible and ideally self-healing in order to sustain the complex requirements of biology.

The first utterance of a cell "membrane". In 1836, C.H. Schultz used iodine to stain the erythrocyte plasma membrane, and with this estimated that the boundary thickness is 22 nm (not far off). In 1839, Theodor Schwann, in collaboration with M.J. Schleiden, proposed "cell theory", tracing the development of animal tissues from embryonic to adult stages. In observing the division of the intracellular compartments, they deduced that there must exist a cellular "membrane" that limits the cellular volume in some way although the structure itself was invisible.

In cell theory, there are three generalizations regarding cells: 1. All living organisms are composed of one or more cells, 2. The cell is the basic unit of structure and organization in organisms. Later, 1855, Rudolf Virchow added a third tenet - 3. All cells arise only from pre-existing cells.

Other theories of cellular boundaries. After Schwann proposed the existence of a cell "membrane", there came out several theories that argued against the idea that there was any sort of encapsulating structure at all. In 1857, Franz Leydig postulated that in most cells the cellular contents, or protoplasm, are colloidal in nature, acting more like a gel than a fluid. He proposed that the protoplasm self-assembles into a sphere-like shape that contains a central body called a kernel, i.e. the nucleus. In this model, the enclosing structure arises from a hardening of the protoplasm at the liquid interfaces. In hindsight, this may sound complex, but there is chemical precedence for such structures. For example, consider the process of "spherificiation" that is being used in molecular gastronomy today (https://en.wikipedia.org/wiki/Spherification), where a flexible shell comprised of a calcium alginate aggregate forms when a sodium alginate solution is introduced to calcium chloride.

Of course, this does not explain the movements and flexibility that were observed in organisms like amoebas, where appendages could be observed to extend and contract. In 1863, Max Schultze proposed a model to consolidate this discrepancy. He suggested that cells contain lumps of contractile protoplasm that are held together because of their inability to mix with the

surrounding aqueous solution. This offered some rationalization for the flexibility that was observed in mobile organisms with the colloidal protoplasm model.

As we go on, we will find out that the hardened sphere theories for cellular boundaries is not the solution that biology employs. However, it is interesting to consider that we are now observing this type of phase separation phenomenon exhibited in membrane-less organelles, such as P-bodies, stress granules and the nucleolus and the ideas of phase separation within cells. Therefore, this is a phenomenon that may play an important role in biology, it just does not explain the behavior of the encapsulating structure of most cells and organelles, as we will see once the field turns to quantitative biophysical studies.

Introducing physics to cellular studies - plasmolysis under osmotic stress. In 1748 Jean-Antoine Nollet first documented the observation of osmosis. He took a vial of alcohol that he carefully purged of air, sealed it tightly with a pig's bladder and submerged the vial into water. After 6 hours, he noticed that the pig's bladder was bulging. He then pricked the bladder and liquid from inside the vial shot up through the air, about 1 foot in height. He had discovered that the water entered the vial, through the pig's bladder, by osmosis.

In 1773, William Hewson observed osmotic swelling and shrinking in erythrocytes and also deduced the existence of a cell membrane as a structure surrounding the protoplasm. He did not use the term membrane, and this was a conclusion was largely ignored in the scientific community.

These studies would be revisited ~80 years later in plants. In 1855, Karl von Nageli began the osmotic studies on plants. This was easily observable using a microscope, as the inner boundary could be observed as pulling away from the cell wall. While still qualitative, it approached a quantitative level due to the outer boundary of the cell wall acting as a reference. Sugar and salt were used to control the osmotic conditions and von Nageli found that the process was reversible, as long as the cell did not become ruptured. Also, it was observed that isolated vacuoles from plant cells exhibited the same reversible behavior. One important thing to note, is that in all of the plasmolysis studies conducted during this time, is that the permeability characteristics varied quite a bit. Cells were generally permeable to water, but the rates varied amongst cell types and different organisms.

In 1886, Jacobus Henricus van't Hoff noticed that osmotically driven processes in dilute solutions follow the ideal gas laws:

$$\pi = icRT$$

Where π, is the osmotic pressure defined as the minimum pressure which needs to be applied to a solution to prevent the inward flow of its pure solvent across a semi-permeable membrane, i is the dimensionless van't Hoff index, c is the solution molarity, R is the ideal gas constant and T is temperature in Kelvin.

It is this discovery that would provide the foundation for careful physical and quantitative studies of behavior of cells that would reveal that cells were enclosed by membranes and put the hardened shell theory to some rest.

Overton provides a masterclass in the benefits of systematic quantitative science. Over the course of seven years, from 1895-1902, Charles E. Overton returned to plasmolysis studies with the new physical information provided by van't Hoff. He quantitatively compared over 500 substances used to control the osmotic pressure. Essentially, any compound that was available at the time in his laboratory, was tested. He quantified the results by visual measurements under a microscope, or by measuring changes in cell weight. He found that polar substances induced plasmolysis, however, aliphatic alcohols and non-polar substances has little effect. Relating his results back to van't Hoff's osmotic pressure law, he concluded that these there must be no barrier for non-polar species. Therefore, whatever is enclosing the cell must act as a semi-permeable membrane. It provides no barrier to non-polar species, is somewhat permeable to water, and is generally impermeable to salts and polar species.

The cellular membrane is made up of lipids. At approximately the same time, Hans Horst Meyer in 1899 and Overton in 1901, also tested the effect of non-polar substances that were known to act as general anesthetics in a clinical setting. They both found that the clinical efficacy of these molecules showed a strong correlation to their partitioning into olive oil. Putting these results together, Overton concluded that the cell barrier was lipoidal, and likely to be made of known biological lipoidal molecules like phospholipids and cholesterol.

An interesting property of olive oil. In 1623, Pliny the Elder was observed spilling oil on the sea, claiming that it had the ability to "still the waves". In 1774, Benjamin Franklin decided to repeat this experiment, dropping a teaspoon of olive oil onto Clapham pond and observing it to spread out to the full size of the pond, about a half of an acre. He saw that the oil spread out to a very thin layer and demonstrated prismatic colors. We know that the oil is forming a lipid monolayer at the water air surface, and is one molecule thick. Back when Franklin conducted this experiment, he had all of the information he needed to calculate the molecule thickness of this lipid layer, but did not go as far as to quantify it.

Measurements of the thickness of lipid monolayers. This quantification would have to wait more than a century. In 1890, Lord Rayleigh repeated Benjamin Franklin's experiment, measuring the area to which a given volume of oil would expand. He reduced the experiment to a laboratory scale, 0.8 mg spread out to 5500 cm^2, and measured the thickness of the layer to be approximately 1.6 nm.

Further developments into the accuracy of the measurement of thin oil films would require the work of Agnes Pockels. She was a 19 year old homemaker, taking care of her elderly and sick parents while her brother, Friedrich Pockels, studied Physics at Gottingen University. She did not have a formal education, as was customary for women at that time, but she did have a passionate interest in science and particularly physics. She did however do much of the housework, and part of this included cleaning the greasy kitchen pots and pans. During this, she regularly observed the thin films of oil and their prismatic colors on the surface of water. Curious about this, she developed a device, made out of her kitchen pans, that would measure the surface tension of the thin oil film. She showed her results to the physicists at Gottingen who showed no interest. Then in 1890, she learned of Lord Rayleigh's similar interest in this research and her brother advised her to write a letter to him. Rayleigh took note of her scientific advancements and helped to communicate Pockels' work. In 1891, her first paper was published in Nature, and this led to many more impactful discoveries that founded the field of thin layers. With her device, a prototype for the Langmuir trough that is used today, she measured a thickness of the olive oil monolayer as 1.3 nm. When her brother died in 1913,

she stopped publishing as she no longer had access to the University and scientific community.

In 1917, Irving Langmuir publishes on the molecular orientation of oil molecules on water, building a higher resolution "Langmuir trough" to measure surface tension based on Pockels' design. With this, he established that the oil layer was a single-molecule that was 1.3 nm in thickness. He is often recognized as the pioneer of surface chemistry.

Cell membranes as lipid bilayers. These technical developments for measuring the properties of thin films was essential for the study of cellular encapsulation. In 1925, following these improvements in measuring surface tension, Gorter & Grendel carried out an experiment of extracting the membrane fraction from erythrocytes, and then depositing it into a Langmuir trough to measure the surface area. The oil fraction spread out to a layer with a defined area. When taking into account the number of cells, and the surface area of each cell, then determined that the area on the trough was twice the surface area of the cells. With this, they proposed that the cell membrane was a lipid bilayer.

This was actually a serendipitous conclusion, because their paper contained errors that cancelled out. Furthermore, when others repeated the study under different extraction conditions, they would get different conclusions. It was not a robust study, but it did put forth the idea that the cell membrane was comprised of a lipid bilayer. Another experiment was conducted at the same time, which quite robust but is often ignored. Also in 1925, Hugo Fricke carried out electrical impedance/resistance measurements of erythrocyte suspensions. Assuming that the membrane is made up of oil, as Overton proposed, he used a low dielectric constant of 3, and measured the hydrophobic thickness of the membrane as 3.3 nm thick, a value that stands to this day. However, he did not interpret this as a bilayer in his paper. In hindsight, given Rayleigh's, Pockel's and Langmuir's measurements of 1.3-1.6 nm for the olive oil monolayer, Fricke's data is also supportive a lipid bilayer structure.

The paucimolecular model of Davson and Danielli. In 1935, Davson and Danelli proposed a model that would consolidate the results from Fricke, Gorter & Grendel into a lipid bilayer, but one that could be potentially thicker due to a lipoid core. At the same time, Danielli & Harvey found that proteins are adsorbed onto the outer surface of egg cells, and that these proteins must protect and stabilize the lipoid molecules that are not stable when in contact with the aqueous solution. This begins to introduce the ideas of permeability in membranes. They proposed the formation of stable pores by these protein layers to allow for differential permeation.

Seeing the lipid bilayer cell membrane. In 1957 J.D. Robertson, an electron microscopist used KMnO4 fixation in his preparations of the myelin sheaths of Schwann cells. He observed the consistent appearance of trilaminar units, two dark bands with a light core, at all of the positions where cell membranes and barriers were expected to be. The KMnO4 was expected to have stained the protein layers that bound to the membrane surface, as was described in the Davson & Danielli model, and that these layers were separated by a light lipoid core. Robertson present the 'unit membrane' model that was very similar to the Davson & Danielli model, with the exception that the membrane was formed as a true lipid bilayer.

The unit membrane hypothesis. Robertson observed the trilaminar structure in all preparations of cells, and for all of the organelles - anywhere where there was expected to be a

cellular boundary. With this, he even went to propose that the membrane was continuous, bringing in ideas of trafficking between the cellular compartments. While this is not the case, it does highlight the fact that it is in principle, the same membrane structure shared across all of these regions, and that they are capable of exchanging under the right conditions.

The lipid bilayers are fluid. In 1970, Frye & Edidin conjugated mouse and human cells with fluorescein or tetramethyl-rhodamine labelled antibodies, then fused the cells together. When they examined the cells 5 minutes after fusion, they observed separated domains within the membranes. However, after the course of 40 minutes, they found that the entire membrane compartment had mixed completely. This indicated that the cell membrane, as formed by the lipid bilayer structure, exhibited fluid properties.

Over the years, we can see that the concept of the cellular boundary changed from a hardened shell of colloid, to a lipoidal membrane to finally a lipid bilayer structure. Now, the missing part of the puzzle was what accounted for the differential permeability characteristics of cell membranes, and where were the proteins that were known to be associated?

The problem of putting proteins into membranes. At this time, it was deemed unlikely for proteins to exist within the membrane itself because of the presumed instability of the polypeptide chain in a non-water environment. Not only would the protein be unstable, but the act of putting a protein in the membrane was expected to dissolve the membrane. This is why the Davson & Danielli model proposed that the membrane was lined by layers of protein, and that the permeability characteristics defined by extensions of these layers across the membrane.

However, in the 1960s, freeze-fracture electron microscopy would show key evidence that proteins could span the full lipid bilayer. In the first experiments by Moor & Mühlethaler, they found that they could fracture cells along the interface of the lipid bilayer, and imaged each separate leaflet. They noticed that the images showed defects in the membrane that provided positive and negative relief as if they fit together like a puzzle. Thus, they could show that a protein in one leaflet left a depression from where it was in the opposing leaflet, showing that it spans the full membrane.

The fluid-mosaic model. In 1972, Singer & Nicholson developed the fluid mosaic model for cell membranes. In this model, they propose that the membrane is a lipid bilayer, a thermodynamically stable structure comprised of phospholipids and membrane proteins. The lipid bilayer is a fluid solvent, constrained in 2-dimensions. Proteins are embedded within the membrane and responsible for selective permeability. The lipid bilayer is the solvent for the membrane protein reactions. The proteins and the membrane are thermodynamically connected, i.e. the reaction is driven by the free energy of the system as a whole. In 1976, Israelachvili and others bring a more complete physical understanding of the membrane in which the lipid bilayer can self-assemble around membrane proteins in a thermodynamic balance.

The first structural studies of membrane proteins. The final piece of evidence to solidify these theories were structural studies of membrane embedded proteins that would show directly that these proteins have the same dimensions as the lipid bilayer. The first of these studies relied on the observation that the halophile H. Halobium that survives in > 4.3 M salt solutions had large purple patches in its cell membranes. In 1967, Stoeckenius & Rowen isolated these

membranes, and in 1970, Osterhelt & Stoekenius identified that these patches were comprised of a 26 kDa molecular weight species that we now know is bacteriorhodopsin, which obtains its purple color due to the binding of retinal. In 1975, Hendersen and Unwin determined the first membrane protein structure of bacteriorhodopsin by EM diffraction at 7 Å resolution, which clearly showed the alpha-helices that correspond to the dimension of thickness of the lipid bilayer. It was this discovery that led to the general acceptance of membrane embedded proteins.

It would take another 10 years before the first high-resolution structure of a membrane protein was determined, this time by x-ray crystallography. In 1985, Deisenhofer, Huber, Michel and others solved the structure of the photosynthetic reaction centre of Rhodopseudomonas viridis at 3 Å resolution. The critical step to the success of this experiment was the ability to solubilize membrane proteins stably with detergent. While this advancement now enabled structural studies of membrane proteins to take place, it must be stressed that this remains a serious challenge in the field, which can be seen by the limited numbers of membrane protein structures in the protein data bank compared to soluble proteins. Still, the experiments are possible and with recent advances in cryo-EM imaging, have become even more accessible within the field.

Summary. Our understanding of cellular boundaries have gone from hardened shells to oily lipid bilayer membranes to membrane with self-assembled structure. We are now at a point where we are starting to understand this substructure and the physics associated with it. The lipid bilayer is a unified mechanism of bounding biological systems. It turns out nature goes for the robust common solution instead of one that is particular to each organism. This seems to be a common theme in biology. Throughout these lectures, we will learn more about the complex environment of the lipid bilayer membrane and the membrane proteins that are embedded within.