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Review

Physics of the Chemical Asymmetry of the Cell Membrane: Implications in Gene Regulation and Pharmacology

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Abstract: Signalling proteins are key regulators of basic cell physiology and tissues morphogenesis. Whilst signalling proteins are paramount for the cell to function optimally, their down regulation or inhibition is also central to tune the cell and its environment. One process involved in this tuning mechanism is membrane budding, otherwise known as endocytosis. The origin of the physical force driving the budding process and endocytosis has been the subject of much controversy. After two decades the budding process is now well described and it is acknowledged that fundamental principles from soft matter physics are at play. This opens a new window for understanding gene regulations, pharmacokinetic and multi drug resistance in cancer. This review recalls the first steps that have led to a better understanding of cell biology through the use of physics and; how the use of physics has shed light in areas of cell biology, cancer and pharmacology. It is, therefore, not a review of the many enzymes involved in membrane vesiculation and membrane curvature; it is more of an historical account.

Keywords: cell membrane; endocytosis; gene regulation

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1. Vesiculation as a Way of Exchanging Signals

Each cell from every living multi-cellular organism exchanges signals with its close neighbours, which allows it to structure and to control its own physiology, organisation and shape. Such signals are tightly regulated by the organism's genome. Whilst signalling proteins require cognate receptors to activate intracellular pathways, the down regulation of protein-receptor binding involves membrane endocytosis. Endocytosis is linked to the process of membrane budding and vesicle creation. A vesicle is mainly composed of cell membrane and has a diameter of about 50–100 nm. There are different sorts of vesicles, those coated by specific proteins (e.g., clathrin or caveloae) or those not (e.g., pinocytosis or fluid phase vesicle), that are usually created rapidly within two minutes and are transported towards internal compartments where further to fusion with lysosomes, the protein-receptor interaction is inhibited due to the low pH (~5.5) of the lumen. Whilst it can be argued that different types of membrane endocytosis down regulate different families of receptors, it was also demonstrated that a distinction between the various types of membrane endocytosis is not required as they all rely on the fundamental ability of the cell membrane to bend.

2. Vesiculation Mediated by the Coupling between Membrane Elastic Properties and the Transport of Lipids across the Cell Membrane

Membrane budding plays an essential role in the regulation of intra-cellular pathways and about twenty years ago the main question was: "what could force the membrane to bend?"

At the time this apparently naive question was essential as an over expression of clathrin was not able to promote membrane budding beyond control values [1].

In the 1970s, the uniqueness and importance of the bilayer structure of the membrane was underlined in biophysics [2]. A bilayer membrane composed of two lipid leaflets has remarkable properties mostly linked to the mechanical nature of lipids. Lipids are amphipathic molecules that once in water will self-aggregate. In general, the structure of any lipid aggregate is related to the ability of the aggregate to release the stress imposed on lipids, and many lipid phases exist as a result. For example, let us consider a bilayer, the compression of one leaflet of lipids will have a tendency to curve the membrane. In fact, this observation was initially made using red blood cells treated with lipophilic drugs. It was therefore presumed that modulating the phospholipid number asymmetry between the two elastic leaflets of biological membranes would result in the occurrence of a mechanical stress due to the difference in surface area between the two leaflets [3]. This asymmetry could, in theory, lead to membrane budding.

While the physics or biophysics behind such an idea was elegant, the unanswered question was what cellular biochemical process could generate such asymmetry?

In the 1980s, research emphasis focused on explaining the reason why cell membranes are not randomly composed. This work led to the discovery of the existence of an active phospholipid pump, also named "flippase", that actively transports specific phospholipids from the outer into the inner leaflet of the cell membrane in all living cells [4,5]. Although this allowed a better understanding of why cell membrane leaflets are so polarized when their lipid composition is considered, it was difficult to test whether the lipid asymmetry across the membrane generated by the flippase could trigger enough stress to initiate membrane budding and vesiculation.

To test this assumption biomimetic model systems were used which modelled the asymmetric repartitioning of phospholipids [6]. In the beginning of the 1990s, it was possible to promote vesiculation in giant liposomes. Giant liposomes are simply composed of two leaflets of phospholipids with a size equivalent to cells. To create the transport of lipids between leaflets negatively charged phospholipids (phosphatidyl-glycerol) together with a pH gradient were used. In effect, when a pH gradient is not present, the charged lipids cannot spontaneously cross the plasma membrane due to the strong membrane dielectric capacity. On the contrary, lipids in the inner leaflet and in contact with the low internal pH of the liposome can absorb a proton therefore becoming a lipid of neutral charge. Neutral charge lipids can rapidly (within seconds) jump from the internal leaflet into the external leaflet. Once in the external leaflet the proton belonging to the lipid is lost due to the basic pH of the external medium. As a consequence the re-charged lipid cannot jump back into the inner layer of the liposome and is therefore trapped in the outer leaflet of the liposome. In this scenario, the pH gradient can be considered as a motor force for charged phospholipids between leaflets of the liposome. Such a pH gradient can be generated by making liposomes in an acidic milieu which are used in a basic milieu thus stimulating the formation of vesicles.

Although this model highlighted the importance of lipid number asymmetry in the creation of membrane curvature (an assumption that was initially put forward in the 1970s) [7], the vesicles created were not the size of biological vesicles (~50 nm) as observed in living cells. In addition, in the case of the pH gradient, the shape and number of vesicles is imposed through the surface tension resulting from the limited size of the giant liposome. However, using this simple bio-mimetic system, these experiments demonstrated that translocation of phospholipids from one leaflet to the other has the potential to create membrane bending, without involving specific membrane coats like those made of clathrin proteins for example.

3. Active Trans-Membrane Phospholipid Pumping, a Force for Vesiculation in Living Cells

Subsequent research effort concentrated on living cells focussing on the previously discovered flippase activity [8,9].

These experiments made use of flippase activity to increase the lipid number asymmetry between the two leaflets of the membrane. Some phospholipids such as phosphatidylserine are transported from the outer to the inner leaflet of the bilayer membrane via flippase activity (about 90% of exogenous phosphatidylserine added to the outer leaflet are relocated into the inner leaflet within 30 min). Thus, by adding specific lipids into the outer leaflet it is possible to increase phospholipid number asymmetry of the plasma membrane. As a result, it is expected that an increase in mechanical stress across the membrane, potentially involved in generating the local bending of the plasma membrane, would result in a measurable change in the number of biological vesicles within the cells, observed by an increase in the kinetics of endocytosis. It was indeed measured that a 2% increase in lipid asymmetry results in an increase of the kinetics of the endocytosis process ~300% [10]. These experiments were later modelled using first physical principles [9] (see Figure 1).

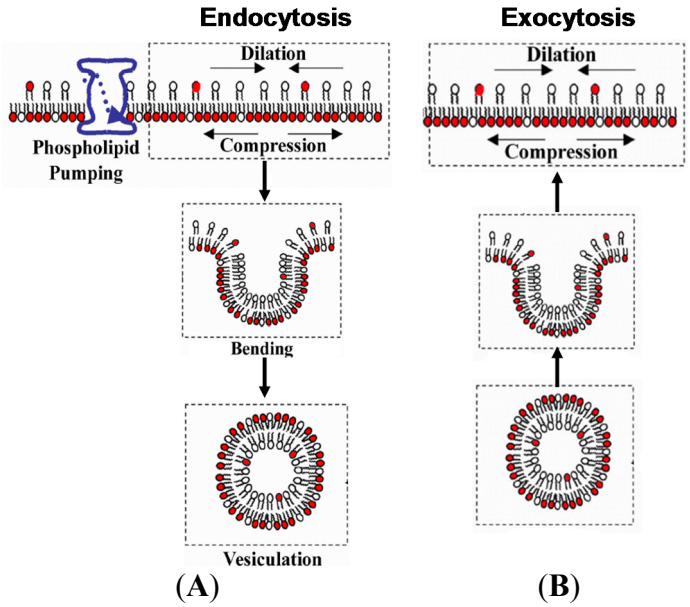

Figure 1. Sketch representing the current model linking fluid phase endocytosis to membrane phospholipid number asymmetry. In (**A**), the translocation of dark-headed lipids into the inner leaflet induces a differential packing of lipids between leaflets leading to membrane bending and vesiculation. Note that the membrane recycling that occurs in cells (**B**), *i.e.*, the exocytosis of vesicles with a size similar to endocytic vesicles, allows the maintenance of lipid asymmetry and thus the maintenance of the differential packing of leaflets at the level of the plasmalemma. The relationship existing between lipid number asymmetry and the vesicle radius is given by $R = 8k_c/hK(\Delta N/N_0)$ (see appendix). Accordingly, lipid number asymmetry has been experimentally deduced from studies on drug sensitive cells (K562) with a value $\Delta N/N_0 = 4\%$ providing a ~35 nm vesicle radius [11].

Finally it was also demonstrated that reversing lipid asymmetry by adding lipids to the outer leaflet that could not be transported to the inner leaflet opposed fluid phase and clathrin endocytosis triggering the exocytosis of caveolae vesicles from the cytoplasm [12]. This highlighted how the physical properties of the cell membrane are able to oppose specific and active biological processes. However, the energy required to impact on specific processes—e.g., pinocytosis *versus* clathrin-coated pit formation—may differ as it was also demonstrated that the energy required to inhibit clathrin-mediated endocytosis is about 100 times higher than that required to stop pinocytosis [12]. So the same conceptual physics is used but the energy differs.

One question remained however, if membrane budding followed by vesiculation is mediated by lipid asymmetry what controls/limits the kinetics of this process? This question was answered much later when the initial data were re-evaluated to discover that the viscous drag applied against membrane budding controls and limits the mechanism of vesiculation [13]. This same work also demonstrated that in addition to the down regulation of membrane receptor expression due to membrane internalisation, pinocytosis was also very efficient at delaying any increase in cell volume due to Laplace's Law. This can be understood simply as follows. When a vesicle is created it takes energy from the membrane stored in the form of lipid number asymmetry. When a vesicle returns to the membrane, the energy contained in the vesicle is put back into the membrane. In general as the levels of endocytosis match those of

exocytosis the energy differences (*i.e.*, what leaves or is brought back into the membrane) cancel each other out. However, when an excess of membrane exocytosis is triggered the membrane will have to absorb this energy which is only possible if this is forced from outside. When cells are incubated in hypotonic media, water should flow inside cells causing them to swell. But the swelling is only possible if exocytosis is triggered, meaning that the hypotonic stress will have to at least match the energy penalty to bring back one vesicle to the membrane. This is why cells do not swell up straight away when incubated in hypotonic conditions but that critical hypotonicity has to be reached before Laplace's Law/cell swelling occurs [13].

4. When Soft Matter Physics and Genetics Converge

Suggesting that the first principles of physics are involved in biology needs to be fully demonstrated. The first experiment performed in this direction used myoblastic cells and Bone Morphogenetic Protein 2 (BMP2), known to generate myoblast-osteoblast transdifferentiation.

When a mechanical stress was applied at the plasma membrane opposing the vesiculation force either by incubating lipids into the outer leaflet or in hypotonic media, inhibition of BMP2/receptor complex endocytosis was observed therefore amplifying several specific post-transcriptional steps. In these conditions, the earliest gene transcript related to BMP2 incubation, named JunB, was amplified from a factor of 3 to 4 when compared to the incubation of BMP2 without stress application taken as a control. As JunB protein is well known to be the transcriptional target of BMP2 repressing the expression of genes involved in muscle differentiation, specific expression of proteins involved in the osteoblastic activity (also controlled by BMP2 incubation) were amplified, only when inhibition of BMP2/receptor complex endocytosis was performed [14].

This work demonstrated that inhibiting the stress asymmetry across the membrane not only alters endocytosis but increases the efficiency of BMP2 involved in the transdifferentiation process. That was the very first experiment signalling the connection between genetics and lipid physics.

5. When Soft Matter Physics and Pharmacology Converge

The many lipid bilayers composing our body are well known to affect pharmacokinetics. There is a set of rules well known by the pharma industry named after the scientists who discovered them: Lipinski's rules. These rules try to address, before very costly clinical trials are performed, what would be the best physic-chemical properties of a drug. One rule stands out by its apparent simplicity stating that a drug must have a molecular weight (MW) below 500 [15]. Whilst it is normal to expect a limit as far as drug size and delivery are involved, this limit has remained obscure for years. Note here that the drug size refers to its volume as there exists a linear relationship between the MW and the volume (or size) of a drug chemical.

Lipid asymmetry was again required to solve this problem. If a difference in mechanical stress exists across the cell membrane this stress should select compounds that cross the membrane passively. We can see this process as one that would mechanically squeeze drugs into the membrane. If a drug cannot cross the membrane quickly due to its size it will stay trapped in the membrane for a long period of time. This should result in drugs diffusing into the membrane and being actively transported out by membrane transporters [16] thereby dropping their pharmacodynamic properties. This theory was also successfully applied to multi drug resistance in cancer [16–19] (Figure 2).

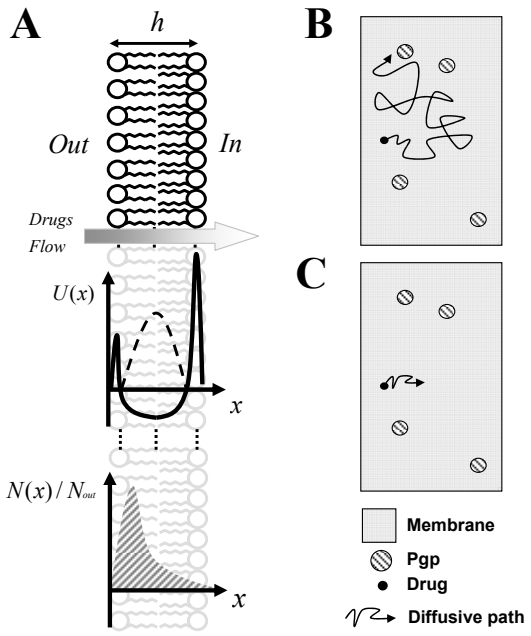

Figure 2. (**A**) Representation of the different energy barriers involved when a drug traverses the bilayer cellular membrane. Two leaflets have been represented with an inner leaflet containing more phospholipids related to the increase in the difference of surface tension (upper graph). Energy profiles of both surface tension in leaflets (plain curve-middle graph) [20] and hydrophobic core of membrane (dashed curve-middle graph) [21] are both involved in providing penalty energy with regard to the drug transbilayer movement. For the sake of simplicity only averaged values of these profiles have been taken into consideration. The dashed area (lower graph) represents the probabable density of drugs in the membrane. In effect, due to the membrane barrier energy, more drugs are found in the outer leaflet than the inner leaflet. (**B**) Effect of the residency time on drug/P-glycoprotein interaction. During its residency time in the membrane, we assume that the drug diffuses laterally over a length that is related to the membrane barrier energy to bypass. As a result a drug may encounter a Pgp. (**C**) Effect expected on drug/Pgp interaction when the drug lateral path is shortened. A decrease of the lateral path length is likely to decrease the probability of a drug meeting and being extruded by Pgp, which in turn may result in a higher intracellular drugs accumulation.

This simple process allowed a better understanding of basic pharmacokinetics and also of drug resistance that heavily relies on the transport of drugs from the cell membrane.

6. Conclusion

First principles of physics are no stranger to biology, and it is by understanding the physics and the biology of living systems that unifying views will emerge. It is often stated that living systems are complex because of the numerous elements involved in them. However, whatever the order of complexity, it cannot override evolution which requires a certain amount of flexibility. It is very probably physics that confers this flexibility allowing systems to be highly sensitive to external elements. In this context it is essential to review the way we educate students, as creating an excessive distance between physics and biology will always play against science. Finally we think the next challenge

regarding the membrane lipid asymmetry will probably be related to a better understanding of the interaction that exist between membrane mechanics and the optimal functioning of membrane proteins.

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Author Contributions

Cyril Rauch, Paula Williams and Ziad Omran conceived and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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