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Computational studies on membrane proteins and membrane-drug interactions

by

Michal Stepniewski

ACADEMIC DISSERTATION

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ABSTRACT

The cell membrane is a gateway to the cell and immersion point for membrane proteins and thus is of interest for pharmacology and structural biology. This thesis aims to study its interaction with water, small molecules, polymers and proteins through molecular dynamics simulation and statistical analysis.

In the first part of the thesis, I have performed a statistical analysis of membrane proteins present in the PDB databank and enumerated in a structure database of known membrane proteins. Based on a statistical analysis of 127 proteins it was shown that extracellular cysteines are not solvent accessible. This rule has not previously been stated and was poorly followed by the participants of the GPCR DOCK competitions in 2008 and 2010. Thus it can provide qualitative guidelines to improve structural modeling. In a second study, based on a statistical analysis of 39 membrane proteins of three or more transmembrane helices, all of different fold, we have shown and clustered different spatial arrangements that sets of three interacting or consecutive helices can take, in addition to visualizing their abundance.

In the second part of the thesis, I performed 200 ns simulations of both membranes in the gel (DSPC) and liquid-crystalline (DLPC) states with solvent and ions; These simulations were repeated with functionalized PEG polymers included (PEGylation). We also performed 200ns lipid membrane simulations in the liquid-crystalline (POPC) state with hematoporphyrin. Our studies provide a new, more accurate description of interactions between lipid membrane ions and featuring PEG polymers rather as dynamic molecules looping around Na+ ions and penetrating to liquid crystalline membrane rather than just a steric barrier outside of membrane. This sheds new light on the mechanism of liposome protection by PEG as well as triggering the release of liposome content through a heat induced lipid phase transition. Hematoporphyrin was shown to reside in the lipid headgroup carbonyl region. Ionized hematoporphyrin has lower affinity to the membrane as

well as forming stable dimers in the aqueous phase. The research was in agreement with experimental data and has provided a molecular level view of the interactions between photosensitizers and the membrane.

YHTEENVETO

Solukalvo toimii sekä porttina soluun että kalvoproteiinien alustana, joten se on kiinnostava tutkimuskohde farmakologian ja rakenteellisen biologian kannalta. Väitöskirjan tarkoituksena on tutkia solukalvon vuorovaikutuksia veden, pienmolekyylien, polymeerien ja proteiinien kanssa molekyylidynaamisen simulaation ja tilastollisen analyysin keinoin.

Teimme tilastollisen analyysin PDB-tietokannan kalvoproteiineista professori Stephen Whiten ylläpitämään tunnettujen kalvoproteiinien tietokantaan perustuen. 127 proteiinia kattava analyysi osoitti, että solunulkoiset kysteiinit eivät ole alttiina liuottimelle. Tällaista havaintoa ei ole aiemmin esitetty, eivätkä esimerkiksi GPCR DOCK kilpailuun vuosina 2008 ja 2010 osallistuneet ryhmät hyödyntäneet tällaista tietoa. Havainto voikin tarjota kvalitatiivisia suuntaviivoja rakennemallinnuksen kehittämiseen. Analysoimme 39 kalvoproteiinia, joissa on eri tavoin laskostuneena kolme tai useampia kalvon läpäiseviä heliksejä. Analyysin pohjalta osoitimme ja ryhmittelimme kolmella peräkkäisellä tai vuorovaikuttavalla heliksillä tavattavat erilaiset avaruudelliset järjestykset ja havainnollistimme niiden määrät.

Simuloimme solukalvoa 200 ns ajan liuottimen ja ionien kanssa sekä geeli- että nestekidemuodossa (DSPC- ja DLPC-kalvolipidit). Simulaatiot toistettiin PEG-polymeereillä funtionalisoiduilla kalvolipideillä (PEGylaatio). Lisäksi simuloimme 200 ns ajan nestekidemuotoista POPC-lipidikalvoa hematoporfyriinin kanssa. Havaitsimme, että lipidikalvon olomuoto vaikuttaa kalvon vuorovaikutuksiin ionien ja polymeerien kanssa, etenkin ionien ja polymeerien kykyyn tunkeutua ja sitoutua solukalvon karbonyyli- ja ydinalueelle. Tutkimuksemme tarjoaa aiempaa tarkemman kuvauksen lipidikalvon vuorovaikutuksista ionien kanssa ja kuvaa PEG-polymeerit pelkän kalvonulkoisen steerisen esteen sijaan dynaamisina molekyyleinä, jotka kietoutuvat Na^+-ionien ympärille ja tunkeutuvat nestekidekalvoon. Tämä valaisee liposomien PEG-suojauksen ja lämpöindusoidun lipidifaasimuutoksen laukaiseman liposomin sisällön vapautumisen mekanismeja.

Hematoporfyriinin havaitsimme asettuvan lipidien hydrofiilisten päiden

karbonyylialueelle. Ionisoitu hematoporfyriini sitoutuu kalvoon heikommin, minkä lisäksi se ei myöskään muodosta vakaita dimeerejä vesiliuoksessa. Tulokset ovat yhdenmukaisia kokeellisten tulosten kanssa ja tarjoavat molekyylitasoisen kuvan valoherkistäjien ja kalvon välisistä vuorovaikutuksista.

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Michał Stępniewski

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, which will be hereafter referred with Roman numbers. Publications are reprinted with the permission from the publishers.

I Stepniewski M, Xhaard H. Cysteine amino-acids and their significance for modeling membrane proteins. *Submitted*

II Stepniewski M, Xhaard H. 3D helical packing in membrane protein structures. *Manuscript*

III Stepniewski M, Bunker A, Pasenkiewicz-Gierula M, Karttunen M, Róg T. Effects of the lipid bilayer phase state on the water membrane interface. J Phys Chem B. 2010; 114:11784-92. doi: 10.1021/jp104739a

IV Stepniewski M, Pasenkiewicz-Gierula M, Róg T, Danne R, Orlowski A, Karttunen M, Urtti A, Yliperttula M, Vuorimaa E, Bunker A. Study of PEGylated lipid layers as a model for PEGylated liposome surfaces: molecular dynamics simulation and Langmuir monolayer studies. Langmuir. 2011; 27:7788-98. doi: 10.1021/la200003n

V Stępniewski M, Kepczynski M, Jamróz D, Nowakowska M, Rissanen S, Vattulainen I, Róg T. Interaction of hematoporphyrin with lipid membranes. J Phys Chem B. 2012; 116:4889-97. doi: 10.1021/jp300899b

AUTHOR'S CONTRIBUTION

The author was involved in all stages of research from design of the studies, conducting the research, and participating in writing the manuscripts. The author is main contributor of Publications I and II and conducted and analyzed molecular dynamics simulations in Publications III-V.

OTHER PUBLICATIONS NOT INCLUDED IN THESIS

VI Li YC, Rissanen S, Stepniewski M, Cramariuc O, Róg T, Mirza S, Xhaard H, Wytrwal M, Kepczynski M, Bunker A. Study of interaction between PEG carrier and three relevant drug molecules: piroxicam, paclitaxel, and hematoporphyrin. J Phys Chem B. 2012;116:7334-41. doi: 10.1021/jp300301z

VII Magarkar A, Karakas E, Stepniewski M, Róg T, Bunker A. Molecular dynamics simulation of PEGylated bilayer interacting with salt ions: a model of the liposome surface in the bloodstream. J Phys Chem B. 2012;116(14):4212-9. doi: 10.1021/jp300184z

VIII Lehtinen J, Magarkar A, Stepniewski M, Hakola S, Bergman M, Róg T, Yliperttula M, Urtti A, Bunker A. Analysis of cause of failure of new targeting peptide in PEGylated liposome: molecular modeling as rational design tool for nanomedicine. Eur J Pharm Sci. 2012;46(3):121-30. doi: 10.1016/j.ejps.2012.02.009

IX Kepczynski M, Kumorek M, Stepniewski M, Róg T, Kozik B, Jamróz D, Bednar J, Nowakowska M. Behavior of 2,6-bis(decyloxy)naphthalene inside lipid bilayer. J Phys Chem B. 2010;114(47):15483-94. doi: 10.1021/jp103753f

ABBREVIATIONS

AFM	Atomic Force Microscopy
Å	Ångström, 10^{-10} m
DLPC	1,2-Dilinoleoyl-sn-glycero-3-phosphocholine
DSPC	1,2-Disteroyl-sn-glycero-3-phosphocholine
FQA	Fluorescence Quenching Analysis
GSH	Glutathione
GS	Glutathione anion
GS/GSH system	glutathione redox system
Hp	hematoporphyrin
MC	Mass Centre
PCA	Principal Component Analysis
PDB	Protein Data Bank
PDT	Photodynamic Therapy
PEG	Polyethylene glycol
POPC	1-Palmitoyl-2-oleoylphosphatidylcholine
ROC	Receiver Operator Curve
TM	transmembrane helix
XL	extracellular loop

1 INTRODUCTION

Scientists speculate that 3.5 billion years ago a set of amphipathic lipid molecules spontaneously assembled enclosing a set of replicating molecules creating a selectively permeable barrier to diffusion (Schopf et al., 2007; Schopf, 2006; Hamilton and Johnson, 2002; Zhu et al., 2012; Adamala et al., 2016). This event marked the beginning of cellular life as we know it today. Limited diffusion allowed for cell homeostasis and formed proton, ion and other gradients that could be utilized in energy transduction and active transport (Reyes-Prieto et al., 2014) (Strbak et al., 2016). These tasks were and are still performed by proteins folded and located in the membrane. Membranes are natural sites for receptor proteins gathering information about the environment and transducing it into the cell (Pfeil et al., 2014). In the eukaryotic cells that appeared 1.6–2.1 billion years ago (Knoll et al., 2006), biological membranes enclose not only the cell but also its organelles such as the nucleus, lysosome, and the chloroplasts. This allows separation of chemical processes requiring different physico-chemical conditions (Zhu et al., 2012; Adamala et al., 2016). In multicellular organisms that appeared 600 million years ago (Chen et al., 2014), the membrane became the site of cell to cell adhesion, interaction, communication and recognition (White, 2009). Today we find a plethora of different biomembranes (for review, see Chap, 2016). They are composed of a lipid bilayer with proteins embedded or attached. Biological membranes help to maintain stable and often dynamically regulated conditions in cells. The conditions on both sides of membranes can be drastically different e.g. cytoplasm maintains reducing conditions owing to its glutathione (GS/GSH) redox system, while the extracellular milieu is slightly oxidative with pH=7.4 (Couto et al., 2016; Rani et al., 2016; Jeon and Joo 2016). Cellular compartments, for example lysosomes, have very acidic, oxidative conditions (Cao et al., 2015).

In pharmaceutical sciences biological membranes and their components are of interest in a variety of ways.

(1) Membrane proteins are direct targets for more than 50% of drugs on the market (Terstappen and Reggiani, 2001).

(2) Furthermore, the membrane itself is a target for anaesthetics through changing lateral pressure to induce conformational change in ion channels (Cantor, 1997). Photosensitizers like hematoporphyrin aim to disrupt the membrane structure of cancer cells through photo induced creation of free radicals. Additionally, some antibiotics act through creating pores in bacterial membranes (Yang et al., 2013).

(3) In drug delivery an important concern is whether the drug will be able to permeate through the membrane and/or blood brain barrier (Trippier, 2016). This is estimated by measuring drug partition coefficient (log P) or in experimental assays such as Caco2 or PAMPA (see e.g. Hiremath et al., 2009).

(4) Vesicles consisting of synthetic lipids are produced as means of drug delivery through membrane fusion (Cevc and Richardsen, 1993).

This thesis, which is based on computational work, aims partly to discover new rules that govern membrane protein structure and can be later used for *ab initio* modelling. The rate of discovery for membrane protein structures is slower than for soluble proteins due to difficulties in expression, isolation and crystallization (Moraes et al., 2014; Yonath and Ada, 2011). This creates demand for computational studies that aims to build computational models. Computational models are mostly derived through homology modelling, a technique that relies on a known structure of a homologous protein to use as a template (Sali and Blundell, 1993). Nonetheless, it is estimated that only 26% of membrane proteins are accessible to homology modelling (Pieper et al., 2013). Lacking a template, the structure must be predicted from sequence alone through *ab initio* methods (Bonneau and Baker, 2001). *Ab initio* methods utilize rules governing and describing the structure of membrane proteins, for example either the presence of

hydrophobic amino acids at the lipid-protein interface or the excess of positively charged amino acids oriented towards the protein interior (Von Heijne 1986; Von Heijne and Gavel 1998; Rapp et al., 2006). Existence of these rules is connected with embedding of protein into lipid environment or the requirement for packing of transmembrane helices relative to one another.

In publication I, we demonstrated that there are very few single cysteine (i.e. not involved in disulphide bridges or other posttranslational modification) residues accessible to the extracellular milieu. This fact has been disregarded in modelling as represented in previous editions of GPCR DOCK. Publication II considers the packing of helices. Parameters of single helices properties, for example, tilt and swivel have been studied and described previously (Bowie, 1997), but description of overhang has been omitted. We devised a coarse-grained model that includes overhang in the description of one helix. The packing of two helices has been studied with helices represented as single vectors (Bowie, 1997). We have investigated the packing in both membrane leaflets individually. Finally, we investigate the packing of helix triplets, cluster and visualize the ways that triplets can pack and study their abundance. Further work studies the different phases that can be adopted by the membrane (publication III). Interactions between water, ions and lipids in the membrane interphase can be very different depending on whether the membrane is in the gel or liquid crystalline state.

The interaction of smaller compounds with membranes has also been studied. This includes polyethylene glycol with liposomal membranes (publication IV) and hematoporphyrins with biological membranes (publication V). Molecular dynamics simulation indeed proves to be a suitable method to find insight not attainable experimentally (MacDermaid et al., 2015; Kopeć et al., 2013).

Liposomes are vesicles consisting of synthetic lipids produced as means of drug delivery through membrane fusion (Cevc and Richardsen, 1993). Opsonins,

proteins present in the blood plasma (Yan et al., 2005) adhere to liposomes. This leads to their interception by macrophages and removal from the bloodstream within (~1 h) (Moghimi and Szebeni, 2003; Romberg et al., 2007). PEGylation increases blood circulation time by an order of magnitude (Klibanov et al., 1990; Allen, 1994). This happens on the microscopic level and the mechanism cannot be elucidated by experiment alone and is studied by molecular dynamic simulations in Publication IV.

In the case of some classes of drugs like anaesthetics or photo dynamic therapy agents (eq. porphyrins) the lipid membrane is also the target of the drug. Porphyrins are used against bacteria where they are delivered by the heme uptake system (Stojiljkovic et al., 2001), or against cancer tumours (Kuzelova and Brault, 1995) where they are accumulated more than in surrounding healthy tissue. Upon irradiation porphyrins emit toxic free radicals (like singlet oxygen) that damage lipids while diffusing through the membrane. The extent of photodamage depends on the depth of the chromophore component of the porphyrin (Bronshtein et al., 2004). Extensive research has been performed in order to improve delivery of porphyrins and their targeting to tumours and bacteria as well as to increase the photodamage. Understanding the mechanism of these interactions with nanoscale resolution can infer more precise structure-activity relationships. Molecular dynamics simulation is currently the only method that allows us to get insights into these phenomena. In publication V, we employ molecular dynamics simulation to study the interactions of hematoporphyrin with the membrane. We compare protonated and unprotonated molecules in addition to studying aggregation in the aqueous phase.

2 REVIEW OF LITERATURE

2.1 Cellular Membranes

The one characteristic of life on Earth as we know it is its cellular organization (Schrum et al., 2010; Adamala et al., 2016; Zhu et al., 2012). Except for viruses every living being on Earth is composed of one or more cells – spaces enclosed by a semipermeable membrane (Figure 1). Homeostasis of the internal environment is defined within the context of the existence of internal and external milieus separated by a barrier (Adamala et al., 2016). The barrier, i.e. cell membranes, are semi-permeable so that the gradients of molecules can be sustained, created, and or used for production of energy (Pfeil, 201, Strbak, 2016); and allow the cell to react to the information from the outside. Sometimes cells find additional protection in the cell wall.

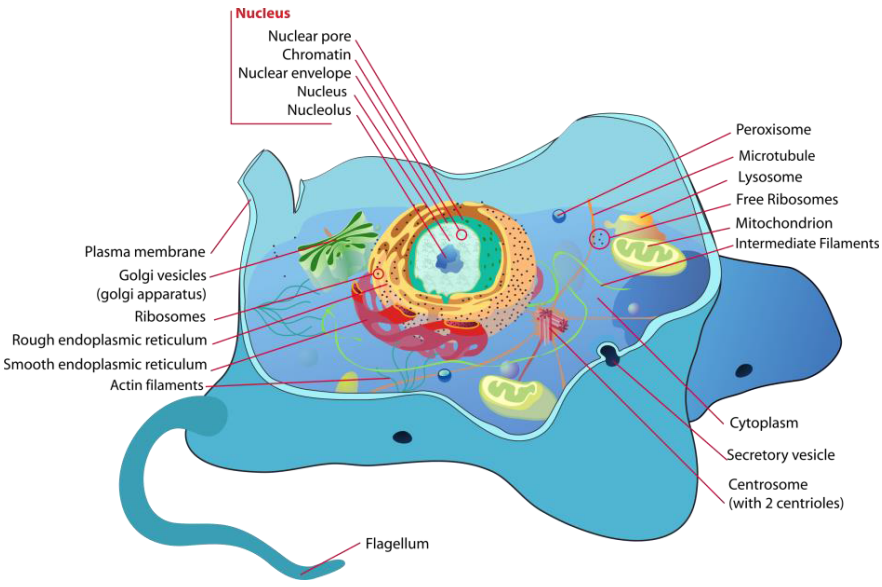

Figure 1. *The cell and its compartments. LadyofHats (Mariana Ruiz). 2006.* File:Animal cell structure en.svg. *[ONLINE] Available at: 'https://commons.wikimedia.org/wiki/File:Animal_cell_structure_en.svg'. [Accessed 06 January 16].*

In his *Micrographia* (1665) Robert Hooke observed that tissues are divided into cells. Moritz Traube (Traube, 1879) noticed that ions could permeate the barrier separating the cells. Meyer and Overton (Hintzensterna, 2002) suggested membranes were composed of phosphatidylcholine and cholesterol. In the next major step Gorter and Grendel combined microscopic surface measurements and monolayer experiments to establish that plasma membranes of erythrocytes are lipid bilayers (Gorter and Grender, 1925). In 1972, a model was proposed in which integral membrane proteins were immersed in the lipid bilayer forming a "fluid mosaic" where all molecules can diffuse freely (Singer and Nicolson, 1972).

In 1988 Simons hypothesized the existence of "lipid rafts" where the diffusion of lipids was limited (Simons and van Meer, 1988; London, 2002). These lipid rafts would have a major relevance on the function of associated integral membrane proteins, but their presence is still debated today (Carquin et al, 2015). Despite years of study using methods like fluorescent probes (Zhao et al., 2015; Kreder et al., 2015), super resolution microscopy (Eggeling, 2015) and cell models (Deleu et al., 2014), definitive unquestioned proof of the existence of lipid rafts in vivo is still not available. Limitations of chosen method are the base of doubts. Some researchers propose other explanations for phenomena attributed to rafts (Sevcsik and Schütz, 2016). Biogenesis of lipid rafts is also a field of discussion (Carquin et al, 2015). Theories range from lipid based with prominent role of cholesterol (Goni and Alonso, 2009; Westerlund and Slotte, 2009; London, 2002) to interactions between proteins and lipids (Lee, 2011; Lee et al., 1995; Shinzawa-Itoh et al., 2007).

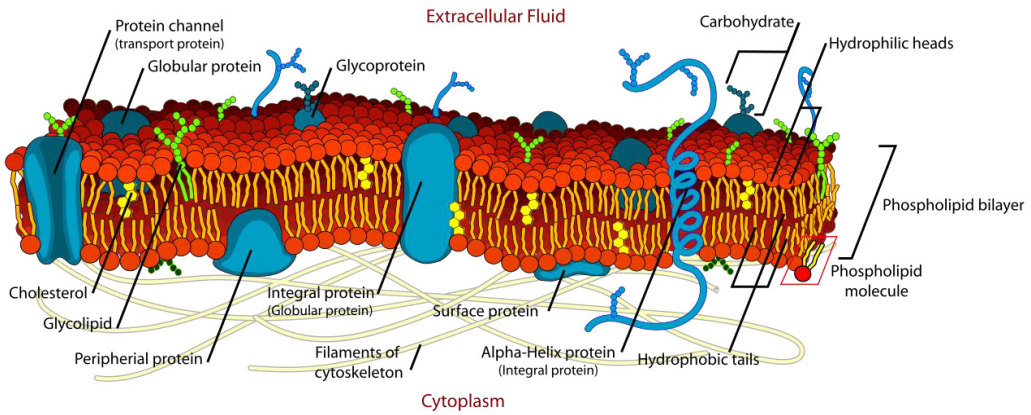

Figure 2. *The cell membrane and its elements. LadyofHats Mariana Ruiz. 2007. File:Cell membrane detailed diagram en.svg. [ONLINE] Available at: https://commons.wikimedia.org/wiki/File:Cell_membrane_detailed_diagram_en.svg. [Accessed 06 January 16].*

2.2 **Membrane composition and properties**

Both the plasma membrane and compartment membranes are bilayers of lipids that contain other components such as embedded proteins (Figure 2). The exact protein and lipid composition varies depending on cell type and/or organelle enclosed (Lauwers et al., 2016). For instance, the myelin sheath is 80% lipid and 20% protein; plasma membrane 50% lipid and 50% protein; mitochondrial inner membrane: 25% lipid and 75% protein (Stillwell 2013). The lipid composition includes three classes of amphipathic lipids: phospholipids, glycolipids and sterols. Llorente et al. (Llorente et al., 2013) found cell membranes to be composed mostly of phospholipids (72.2%), cholesterol (19.2%), sphingomyelin (6.9%). Amongst phospholipids phosphatidylcholine (68.1%), phosphatidylserine (7.7%), phosphatidyletanoloamine (14.5%) were most prevalent. The fatty acid composition of tissue lipid varies across species and has been suggested to be an important characteristic of the metabolic rate associated with each specie (Hulpert et al., 2007). The lipidome is not only cell-specific but also dynamic through time, as reviewed for brain cells (Lauwers et al., 2016). Lipids have the ability to regulate membrane trafficking and control protein activity (Lauwers et al., 2016).

Phospholipids are amphiphilic and assemble into bilayers spontaneously (in water) (King and Marsh, 1987). Each phospholipid molecule is composed of two hydrophobic lipid tails (buried inside the bilayer) bound to glycerol bound with polar head facing the water solution. The nonpolar lipid tails interact with each other with van der Waals forces. Hydrophobic effect shielding them from water is the main force forming the lipid bilayer. In the polar layer phospholipid headgroups form –P-N+ dipoles. Their interaction with each other and through water molecules stabilize the bilayer structure and limit water penetration into the bilayer (Bechinger and Seelig, 1991).

The central region of the membrane, composed of phospholipid tails, is hydrophobic and its width depends on the membrane type and ranges from 30 to 40 Å (Lewis and Engelman, 1983). A partially hydrated region extends a further 3 Å (Marsh, 2001; Marsh, 2002) and the polar head groups extend an additional 8-9 Å (Nagle and Tristram-Nagle, 2000) in both directions. The content of cholesterol can increase bilayer thickness through an ordering effect on the lipid tails (Kucerka et al., 2009). The relation between the membrane thickness and $CaCl_2$ concentration is more complex with the thickness increasing, then decreasing and increasing again with growing concentration (Pabst et al., 2007).

Fluidity is an important property for regulation, uninterrupted function and cooperation of embedded proteins, lipids, signalling processes etc. while maintaining integrity of the cell and compartment (Helmreich, 2003). It is also crucial for proper cell division (Mercier et al., 2012). The lipid type (in particular balance between saturated and unsaturated lipids) together with temperature and pressure decides the fluidity of the bilayer (Gennis, 1989; Heimburg, 2007). The presence of cholesterol for example has a critical effect on membrane fluidity (Marquardt et al., 2016). Increasing concentration of NaCl decreases self-diffusion of POPC lipids and orders the lipid chains (Bockmann et al., 2003).

Biological membranes usually maintain a positive surface charge, eq. electrostatic potential difference across an unexcited axon is ~-70 mV (Pekker and Shneider, 2014). The surface charge depends on lipid composition, which can be incidentally altered by lipid metabolism (Goldenberg and Steinberg, 2010). Permeability of the membrane is altered by the proteins (channels) and environment of membrane is used by enzyme proteins (Kraml and Lojda, 1977).

Lipid membranes can be studied experimentally. Liposomes are vesicles that form naturally upon fragmentation of lipid bilayers (Lasic, 1998). Initially used as lipid bilayer models from 1970s (Allen and Cullis, 2013) they are applied in drug delivery. Their advantage is the fact that they tend to target to tumours and sites of infection (Krasnici et al., 2003). Nevertheless the surface of the liposomes adheres blood serum proteins (Yan et al., 2005). This process known as opsonization marks the liposome for removal from the bloodstream by the macrophages within 1 h from injection (Yan et al., 2005). Opsonization can be inhibited by altering the liposome charge (Chonn et al., 1991) or grafting hydrophilic polymers (like polyethylene glycol) on the liposome surface (Moghimi and Szebeni, 2003).

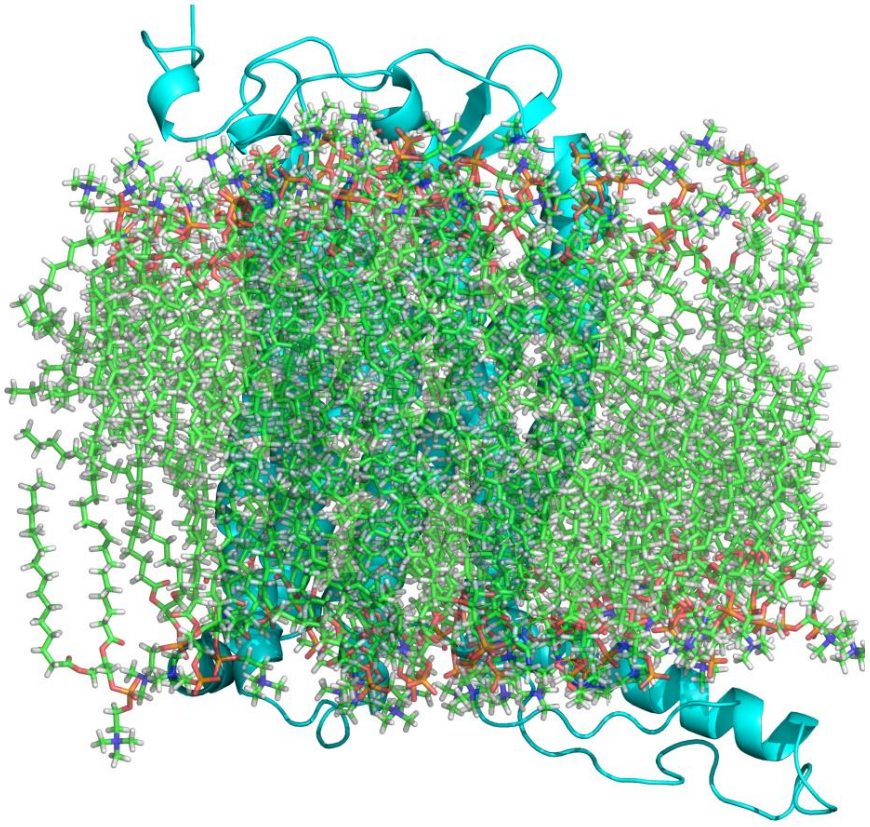

Figure 3. *Membrane protein. Human CCR5 chemokine receptor X-ray structure (PDB: 4MBS)(blue; cartoon representation) shown in POPC membrane model(carbon: green, hydrogen: white, oxygen: red, phosphorus: orange) after 20ns of molecular dynamics simulation performed using the GROMACS package.*

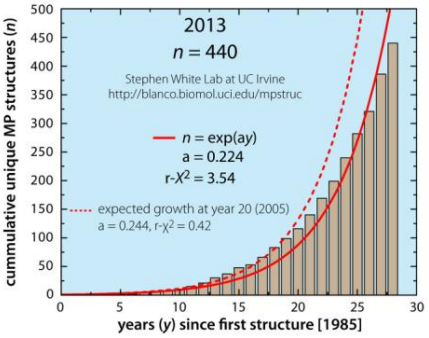

Figure 4. *The growth in the number of membrane protein structures available in the RCSB PDB database. Used with permission from Prof. Stephen White. Stephen White laboratory at UC Irvine. 2014. MEMBRANE PROTEINS OF KNOWN 3D STRUCTURE. [ONLINE] Available at: http://blanco.biomol.uci.edu/img/MP_structures_2014.jpg. [Accessed 06 January 16].*

2.3 **Membrane proteins**

Proteins are a major constituent of biological membranes (Figure 2; Figure 3). Some proteins are associated with the membrane, others are just attached to the membrane, while others are embedded into the whole width of the membrane (Karp, 2009). These later example are named integral membrane proteins. 20-30% of all genes encode membrane proteins. Some proteins serve to reinforce membrane shape and structure (McKinley et al., 2015). They can also allow for cell-cell interaction and identification (cell adhesion molecules) (White, 2009). Some proteins use proton gradient for energy production, other use energy (from ATP) for transport of molecules through the bilayer opposite to their gradient (Physiology; Cooper and Geoffrey, 2009). Other proteins transmit, transform and amplify information signals across the lipid membrane (Cuatrecasas, 1974; Dautzenberg and Hauger, 2002; Rivière et al., 2009). They are key gateways for the cell or cell compartments for information, ions, molecules and targets for over 50% of all drugs (Hessa et al., 2005).

The structural study of membrane proteins is usually done using X-ray and to a lesser extent using NMR (Moraes et al., 2014; Bill et al., 2011). Determination of membrane protein structure involves protein expression, isolation and crystallization (Pandey et al., 2016). Protein isolation without destroying the structure is difficult and requires use of carefully selected detergents, crystals are more difficult to obtain than for soluble proteins (Moraes et al., 2014). That is why while membrane proteins constitute up to 30% of the human genome (Wallin and von Heijne, 1998), less than 1% of protein structures deposited in the Protein Data Bank belong to membrane proteins (Berman et al., 2000). However, the number of experimental membrane protein structures solved at atomic resolution is growing exponentially and follows the growth in number of soluble protein structure (White, 2009) (Figure 4).

The membrane imposes a set of specific physicochemical constraints on the 3D structure of membrane proteins, which can be enunciated as "rules" (Von Heijne 1986; Von Heijne and Gavel 1998; Rapp et al., 2006; Hönigschmid and Frishman 2016). These rules govern both the 3D structure and membrane insertion and trafficking and can be perceived at the level of amino acid sequence. It has long been thought that these rules could help in achieving the holy grail of protein structure determination, i.e. the calculation of protein structure from sequence alone, much more easily than that for soluble proteins (Bogdanov et al., 2014). They are used to provide guidance and constraints for the 3D reconstruction of membrane proteins from sequence alone by computational methods (Attwood et al., 2016; Li et al., 2016; Feng and Barth 2016).

Several sequence analysis bioinformatics applications have been devised to take advantage of these rules (Attwood et al., 2016; Li et al., 2016; Feng and Barth 2016). Common rules about the structural organization of membrane proteins are presented below.

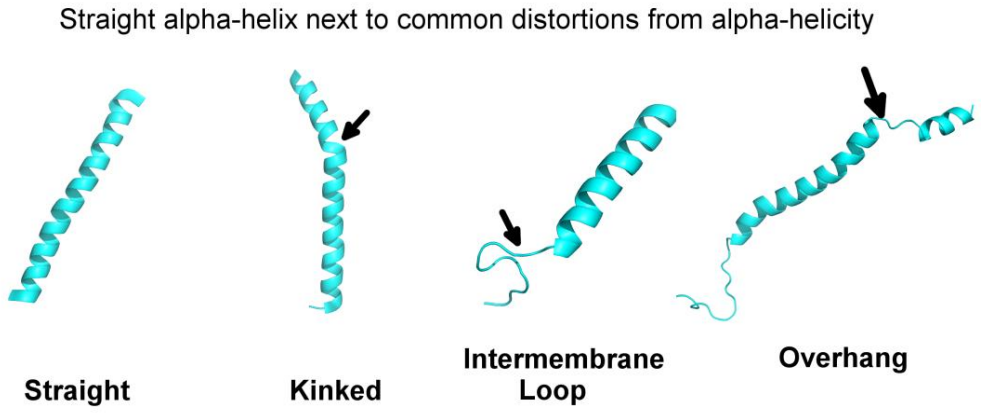

Figure 5. *Common distortion with respect to a-helicity observed in transmembrane segments. Phase shifts following Overhangs and Kinks are not represented.*

2.3.1 Membrane proteins are alpha helical

The membrane part of the integral membrane protein is usually folded into one or more alpha-helices. This means that they form a right handed helix, where the N-H group of the amino acid is connected through a hydrogen bond with the C=0 group of the amino acid four residues earlier in the sequence. The number of amino acids per on helix turn is 3.6 and the pitch of the helix (vertical distance between consecutive helix turns) is 5.4 Å.
A notable exception is bacterial membrane proteins which are anchored into the membrane with beta-barrel (von Heijne, 2006).

Some transmembrane segments are separated in the middle with a non-helical unstructured fragment known as an overhang. Sometimes overhangs are combined with kinks, a bend inside the structure (Figure 5). Kinks are much more prevalent in membrane proteins than in soluble proteins (Chamberlain et al., 2003). Average kink angles are 21(+/-11) degrees when a centrally positioned proline residue is present and 16(+/-11) degrees when central proline residue is absent (Chamberlain et al., 2003). The membrane environment acts as a stabilising factor, increasing the tolerance for such irregularities (Chamberlain et al., 2003).

2.3.2 Preferred tilt and packing angles

When considering a transmembrane helix in isolation, the membrane limits and influences the helical tilt with respect to the surrounding lipids (Bowie J. Mol. Biol., 1997). The preferred value (peak middle) is 21 degrees while smaller values (<21 degrees) are more common than larger values (>21 degrees). The mismatch of transmembrane helix length and bilayer width tilt (so called ‘hydrophobic mismatch’) can help to predict helical tilt.

When considering pairs of transmembrane helices, certain packing angles are preferred by pairs of interacting helices (Bowie, 1997). Arrangement of a pair of helices can be described by their distance, crossing angle, both tilt angles, and their rotations around the inner axis, which decides the contact interface. Packing angles around +20 degrees (while it is -35 degrees for soluble proteins) are preferred (Bowie, 1997). The sign of the angle is positive for anti-clockwise rotation of the near helix with respect to far helix and negative for clockwise rotation (Chothia et al., 1981).

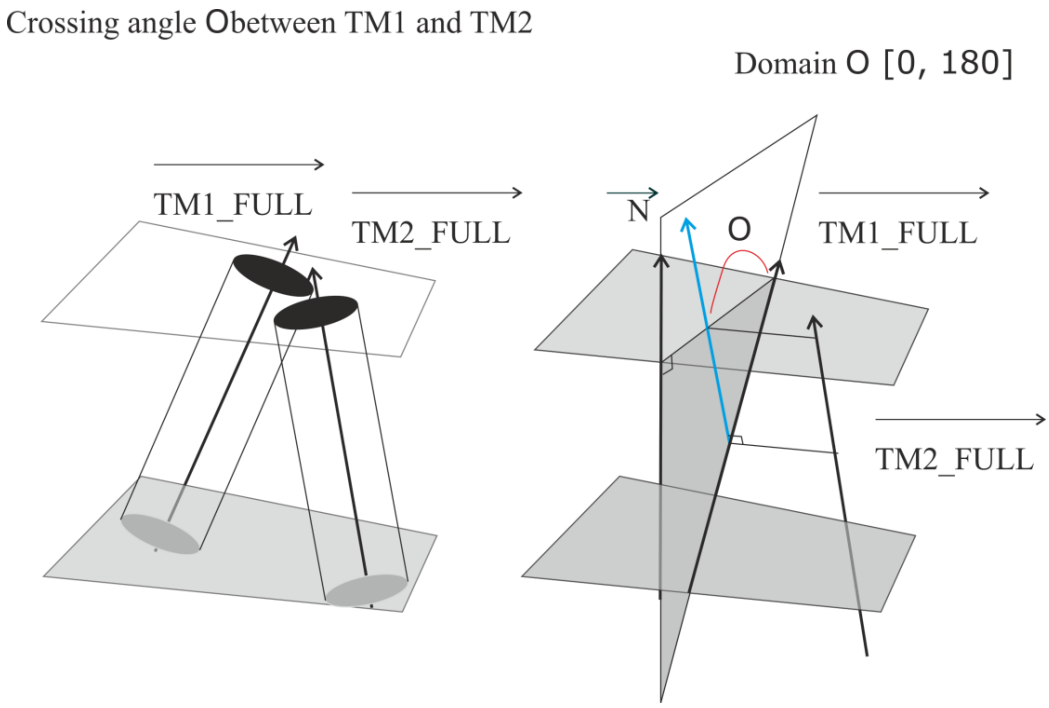

Figure 6. *Packing angle between two helices. Taken from Publication II.*

Certain amino-acid motifs like GxxG are preferred at the contact interface (Senes et al., 2000).

Walters and DeGrado (2006) have shown that around 75% of transmembrane helix pairs belong to one of five tight (RMSD<1.5 A) clusters (Walters and

DeGrado, 2006). The first cluster is around the crossing angle of -156.5 (+/- 10.1) degrees and distance of 8.61 (+/-0.8) Å. The second cluster is at146.4 (+/-13.6) degrees and 8.57 (+/-0.99) Å. The third cluster is at-37.9 (+/-7.5) degrees and 7.93 (+/-0.88) Å. The fourth cluster is at 13.8 (+/-16.6) degrees and 9.77 (+/-1.18) Å. The fifth cluster is around 178.0 (+/-20.8) degrees and 9.14 (+/-1.47) Å.

Feng and Barth have suggested that triplets of helices adopt six preferred clusters (Feng and Barth, 2016). In both these cases the RMSD after structural superimposition was used as a measure of structure comparison.

2.3.3 Hydrophobic amino acids are facing the membrane lipid tail

The membrane spanning fragments inserted amongst the hydrophobic lipid tails are hydrophobic. They can be identified from the amino acid sequence using a hydropathy plot (Kyte and Doolittle, 1982). Modern protocols use machine learning methods such as support vector machines or neural networks, sometimes as an ensemble prediction (Wang et al., 2007).

Some transmembrane helices are amphipathic i.e. they have both a hydrophilic and hydrophobic interface through their whole length (Sharadadevi et al., 2005). In this case the hydrophobic surface faces the lipids while the hydrophilic face interacts with the protein or lines the inner channel of the protein (Sharadadevi et al., 2005). The method of the hydrophobic moment plot is used to speculate the location of the membrane (Eisenberg et al., 1984).

The notable exceptions, i.e. non hydrophobic transmembrane segments, are reentrant loops (Yan and Luo, 2010); fragments located in the lipid bilayer but not crossing through its full-width and, on the other hand marginally hydrophobic

helices immersed in the membrane core (Hedin et al., 2010) and not interacting directly with lipids. In addition, some helices are located in the middle of the transmembrane bundle (Hedin et al., 2010). They form interactions only with other transmembrane helices and their composition is only marginally hydrophobic.

2.3.4 Conservation in sequence alignments provides clues about the three-dimensional fold

The protein interior is more conserved than the exterior, which is also used in sequence analysis to locate the protein core/membrane (Illergård et al., 2011). Furthermore correlated mutation patterns can elucidate which amino acids are connected in tertiary structure, since co-evolutionary information seen in sequence alignments has been suggested to be an indicator of proximity in the 3D structure (Park and Kim, 2012; Gulyás-Kovács, 2012; Halabi et al., 2009).

2.3.5 Amino acid propensities

Amino acids have different propensities for structural features connected to their properties (Ulmschneider and Sansom, 2001; Huang and Chen, 2012; Senes et al., 2000). Proline can sterically disrupt the helical structure with its side chain (Huang and Chen, 2012); in membrane proteins it is well accommodated and forms kinks in alpha-helices or helix caps. Glycine also has the propensity for breaking helical structure while it also forms motifs connected with interhelical interaction spots (Senes et al., 2000). An additional stabilising factor are the interactions of ring amino-acids with the interphase of lipids with soluble compartments (Ulmschneider and Sansom, 2001).

The intracellular surface of the protein has excess of positively charged amino acids compared to the extracellular side ('positive-inside rule', von Heijne and Gavel, 1988).

The energetics of the insertion of membrane proteins has been well studied but are not yet fully understood (Cristian et al., 2003; Bond et al., 2008). The folding of alpha-helical integral membrane proteins has been suggested to occur in two stages (Popot and Engelman, 1990). First, the sequence folds into secondary structure elements like transmembrane-helices. These elements are transported by translocon machinery into the membrane, where they arrange against the membrane and each other. The "translocon code" has been deciphered and is thought to contain structural information at the sequence level (Hessa et al., 2005).

2.4. **Computational studies on membrane proteins**

Computational studies aims to provide insights to elucidate those aspects of membrane and membrane proteins that are difficult or even impossible to study by available experimental methods alone (Punta et al., 2007). In the case of alpha-helical integral membrane proteins there is hope that their common plan of structure plus the structural constraints provided by lipid membrane and helical packing mentioned in previous paragraphs can guide structure prediction (Gao and Li, 2009).

Membrane protein structure prediction involves prediction of membrane protein topology (in particular prediction of transmembrane helices) and membrane protein 3D structure (Punta et al., 2007).

2.3.6 Predictions of topology from sequence

The first methods of transmembrane segments prediction that were developed, for example SOSUI (Hirokawa et al., 1998; Mitaku and Hirokawa, 1999; Mitaku et al., 2002) used hydropathy and amphihilicity index, charges of amino acid and length of sequence

Other methods utilize Hidden Markov Models (HMMs), e. g. HMMTOP (Tusnady and Simon, 2001; Tusnady and Simon, 1998). HMMTOP predicts the membrane protein topology (including localization of transmembrane helical segments in sequence) by choosing the topology with maximum likelihood from the set of all possible topologies.

Other servers, like PredictProtein (Yachdav et al., 2014) return comprehensive information including multiple sequence alignments, predicted secondary structure, solvent accessibility, transmembrane helices and strands, coiled-coil regions, disulphide bonds and disordered regions. Other methods aim to identify functional regions (ConSurf (Glaser et al., 2003)), predict subcellular localization (LocTree3 (Goldberg et al., 2014)), protein-protein binding sites (ISIS2), protein-polynucleotide binding sites (SomeNA (Yachdav et al., 2014)) and predict the effect of point mutations (non-synonymous SNPs) on protein function (SNAP2 (Yachdav et al., 2014)).

2.3.7 Predictions using three-dimensional structure

Some predictions can be run based on the 3D structure of the proteins. For example, the contact maps between transmembrane segments (Fuchs et al., 2009) or the relative solvent accessible surface (Xiao and Chen 2015). The insertion of the protein in the membrane can be predicted using the TMDET methods, which

computes the optimal location of the plane corresponding to the hydrophobic region of the lipid bilayer with respect to the protein surface (Tusnády et al., 2005).

2.3.8 Predictions of three-dimensional structure directly from sequence

Known protein structures can be used as templates to predicting structures of their homologues (>20% sequence identity) in a method called homology modelling (Sali and Blundell, 1993; Marti-Renom et al., 2000). In this process we align the sequence of the query on the known structure of the template(s) (Olivella.et al., 2013). In membrane proteins, the transmembrane bundle usually shows higher conservation than soluble loops (with the exception of some features present in loops like disulphide bonds or ionic locks) (Gao and Li, 2009). This means that while the transmembrane bundle can be reliably modelled from homology, in soluble loops alignment is not reliable and we have to model the structure practically from sequence alone (Kmiecik et al., 2015; Yu et al., 2015). One of the most used software tools for homology modelling is Modeller (Sali and Blundell, 1993).

Also the structure of other proteins, without a close homologue of known structure has to be predicted from sequence alone. Fragment-based *ab initio* methods like Rosetta utilize a library of short (usually less than 20 residues long) fragments extracted from known protein structures (Chen et al., 2014; Alford et al., 2015; Bonneau and Baker, 2001).

2.5. **Molecular dynamics simulation of membrane and interacting molecules**

Molecular dynamics simulations can be used to study the interactions between single atoms that occur in atomic resolution and timescales less than 1 microsecond

(MacDermaid et al., 2015; Kopeć et al., 2013). The interactions that we are interested in are the interactions that occur in the lipid-water interphase between the lipid polar headgroups, ions, waters and polymers grafted to the lipid membrane surface and small molecules. These interactions: maintain the structure of the lipid bilayer, define the area per lipid value and limits penetration of water and ions into the bilayer by interaction with ions influence the net charge of the membrane surface and thus its propensity for opsonisation (Berkowitz et al., 2006). Interactions between lipid atoms and small molecules define the permeability, position and mobility of small molecules in the membrane (Bemporad et al., 2004).

The first simulation of lipids was performed by Kox et al. (1980) consisting of 32 lipids simulated for 80 ps. Since then studies have developed in size, timescale, complexity of composition and complexity of model used. Mukhopadhyay et al. (2004) studied interactions of ions with hydrated membrane bilayers. In 2011 Jurkiewicz et al. has combined fluorescence solvent relaxation experiments combined with MD simulation to study lipid hydration, mobility and the effect of ions (Jurkiewicz et al., 2011). Even though lipid membrane simulations can extend over 1000 lipids and 1 microsecond (Dror et al., 2012; Grouleff et al., 2015; Kirsch and Böckmann, 2015; Venable et al., 2015; Pluhackova and Böckmann, 2015) the main challenge of MD is to extend timescale and size scale in order to study nanoparticles like liposomes or long timescale phenomena like membrane transport in full to compare with biophysical measurement results (Bond and Khalid, 2010). This can be achieved by coarse-graining where one bead represents several atoms (Marrink et al., 2004, 2007; Marrink and Tieleman, 2013). Molecular dynamics is extensively applied in studying small molecule interactions with the lipid membrane (Kopeć et al., 2013) in drug delivery and action.

3 AIMS

The main aim of the thesis work is to discover new rules that governs membrane protein structure and can be later used for *ab initio* modelling.

Aim 1. To test the hypothesis that single cysteine residues are not accessible to the extracellular milieu and how this 'new rule' is applied in computational modelling.

Aim 2. To study the orientation of single helices and the packing of 2 or 3 transmembrane helical segments.

The other aims are to conduct molecular dynamics simulations to gain understanding into the interaction of small molecules with membranes.

Aim 3. To study the effect of the lipid phase (gel or liquid-crystalline) on lipid membrane interactions with water and ions on microscopic level.

Aim 4. To elucidate how PEG polymers compete with lipid headgroups in binding ions and water in gel and liquid-crystalline bilayer.

Aim 5. To describe the behaviour of ionized and unionized hematoporphyrin in the lipid bilayer.

4 MATERIAL and METHODS

4.1 **Dataset(s) collection and preparation.**

We collected three datasets: (A) for studying cysteine solvent accessibility; (B) for studying helix packing; (C) models submitted to GPCR DOCK competitions. Dataset (A) contained 127 integral membrane proteins with less than 75% percent sequence identity representing all alpha-helical IMPs of known structure solved before 2011.03.08. (B) contains 39 proteins, all of different fold found in all alpha-helical IMPs of known structure solved before 2011.03.08. (A) and (B) were collected using Stephen White's membrane protein known structures database. (Stephen White laboratory at UC Irvine. 1998. MEMBRANE PROTEINS OF KNOWN 3D STRUCTURE. [ONLINE] Available at: http://blanco.biomol.uci.edu/mpstruc/. [Accessed 08 March 11]; White, 2009)

GPCR Dock is a community-wide competition meant to assess the state of human G protein-coupled receptors modelling and docking. Up to date three GPCR DOCK competitions (in year 2008, 2010 and 2013) have been conducted. Each competition precedes the release of experimental structure(s) and invites the community to submit their own structural models obtained theoretically. The competition results can guide method development and assessment. In our work models submitted to GPCR DOCK serve as the dataset to test the presence of solvent accessible extracellular cysteines in community submitted models and its effect on model correctness. Dataset (C) was collected from GPCR DOCK website (JOINT CENTER FOR INNOVATIVE MEMBRANE PROTEIN TECHNOLOGIES. 2008. *Critical Assessment of GPCR Structure Modeling and Docking 2008*. [ONLINE] Available at: http://jit.usc.edu/gpcr_dock.html.

[Accessed 08 March 11]; Abagyan lab. 2010. *GPCRDock2010*. [ONLINE] Available at: http://ablab.ucsd.edu/GPCRDock2010/. [Accessed 08 March 11]).

The structure preparation and analysis was conducted using python using scipy module (for code see https://github.com/michalstepniewski/Science.git). The proteins were oriented so that the system z axis would coincide with the membrane bilayer normal and centre of the membrane core would be located at the z=0 Å plane. In dataset (A) and (C) proteins were immersed in a model POPC membrane. Rotations and translations were calculated by TMDET software and found in PDBTM database (Gábor E. Tusnády, 2004). *Detection of transmembrane regions by using 3D structure of proteins*. [ONLINE] Available at: http://tmdet.enzim.hu/. [Accessed 08 March 11]; Tusnady et al, 2004).

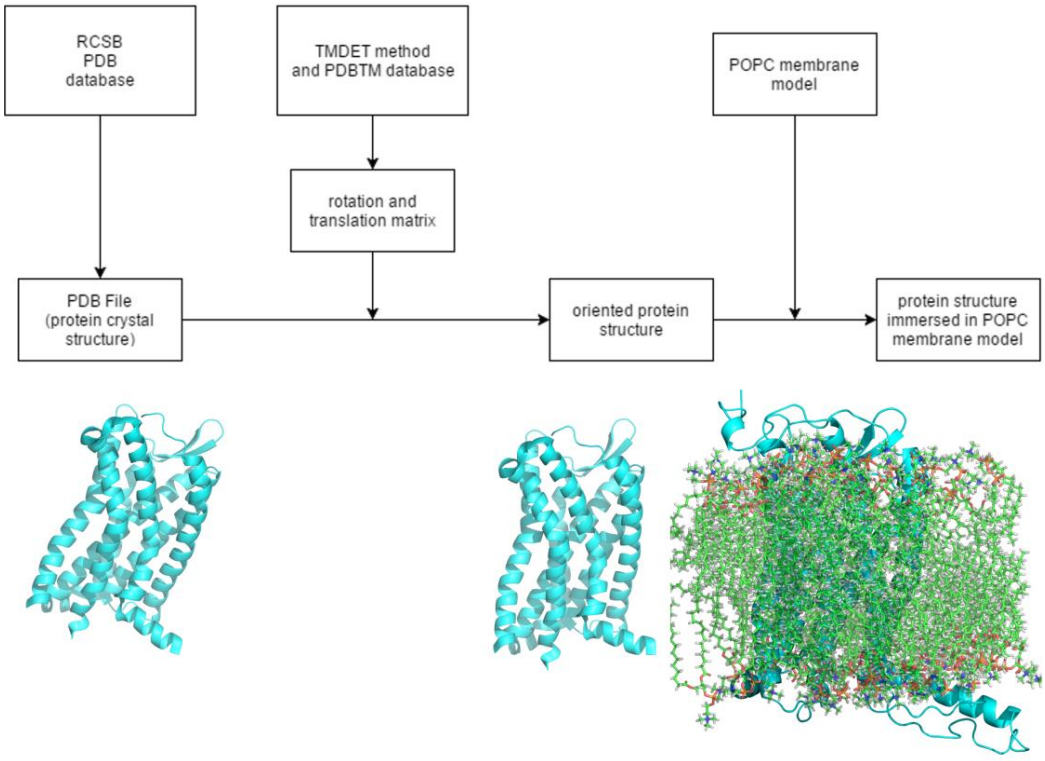

Figure 7. *Structure preparation.*

The TMDET algorithm tests different positions and directions of the molecules within the membrane with respect to the protein and calculates the objective function which has a structural and surface component (Tusnady et al., 2005). The surface component is the percentage of the membrane exposed surface that is hydrophobic and the structural component depends on the number of loops, chain endings within a membrane and linearity of consecutive amino acids. This assignment leaves some uncertainty and is difficult to verify as many proteins are crystallized without the membrane. This uncertainty is difficult to assess. Nonetheless, good agreement is usually found with the presence of co-crystallized lipids.

Solvent accessibility was measured using NACCESS software (datasets (A) and (C)) S. Hubbard and J. Thornton. 1992. *Naccess V2.1.1 - Atomic Solvent Accessible Area Calculations*. [ONLINE] Available at: http://www.bioinf.manchester.ac.uk/naccess/. [Accessed 08 March 11].). We considered sidechains as solvent-accessible if the side chain surface area relative to Ala-Ala-Ala tripeptide was above 30%. Solvent accessibility criterion was chosen to exclude cysteines buried inside protein core.

NACCESS is an implementation of the rolling ball algorithm (Lee and Richards, 1971), which rolls a ball shaped probe of a given radius around a van der Waals surface of the protein structure model. Here we used default probe radius of 1.4 Å.

In Publication II helicity was measured using DSSP. DSSP assigns secondary structure of protein segments using hydrogen bond pattern. The algorithm (Kabsch and Sander, 1983) removes hydrogen atoms from the structure, adds new hydrogen atoms to structure, finds hydrogen bonds and calculates their energy and then uses two best hydrogen bonds for secondary structure assignment. The helicity criteria

was chosen at 50% in order to exclude transmembrane loops but not helices with short loop fragments like overhangs or kinks.

Interfaces and domains were assigned using the manually curated Orientations of Protein in Membrane database (Lomize et al., 2006).

4.2 **Membrane protein structure analysis.**

The work was conducted using scripts written by Michał Stępniewski in python programming language (version: 2.7; https://github.com/michalstepniewski/Science). Analysis and Visualisation of the internal spherical coordinates was performed using a combination of the R language in the R studio package in addition to the KS library.

4.2.1 Automated definition of transmembrane segments.

Transmembrane segments were defined based on the z coordinate value between -15 Å and 15 Å after the protein is placed into the membrane by applying the coordinate transformation suggested by TMDET. Further checks were conducted to demonstrate that amino acids are found both below and above the z=0 Å plane.

For each transmembrane helix we controlled the number of amino acids contained between the 3 pairs of section planes (z= -12 Å, z= -6 Å; z= -3 Å, z= 3 Å; z= 6 Å, z= 12 Å) i.e. for further analysis we selected only transmembrane helices, where the number of amino acids contained between each of the 3 section planes was between 4 and 12. In addition, we checked the helicity (as estimated by DSSP).

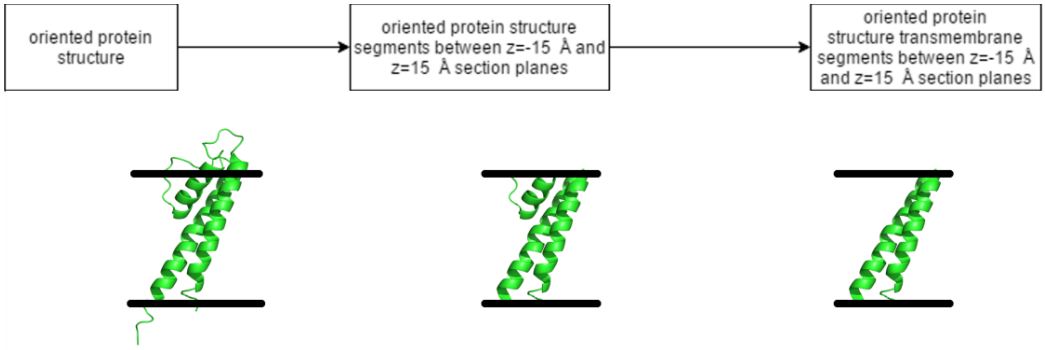

Figure 8. *Automated definition of transmembrane segments.*

Simplified representations of membrane proteins were developed. First, point representation uses 3 points to represent one helix. Points used are Mass Centers (MCs) of three 6 Å wide slices parallel to z axis and centered on z= -9; 0 and 9 Å section planes. The parameters (width and location of slices) were chosen empirically to include at least 4 alpha carbons in each slice.

The second, vectorial representation was developed consisting of one or two vectors representing axes of complete (-12 Å<z<12 Å) or half-segments considered helical, spanning between (z=-2 Å and z=12 Å) and (z=-12 Å, z=2 Å). The crossing points of vectors with z=0 Å plane were part of the representation. Principal component analysis is used to approximate the axis since the PC_1 corresponds to the direction of the largest variability in the data (the atomic coordinates).

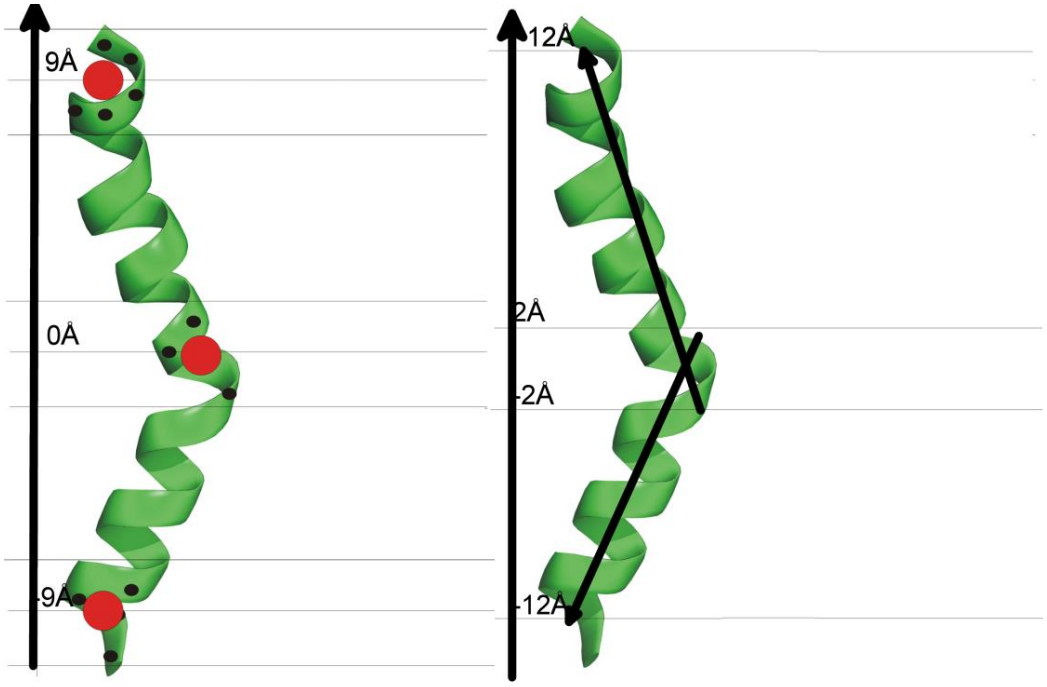

Figure 9. *Transmembrane helix representation.*

We represent each segment as either one vector spanning the entire membrane width or two vectors one for positive and one for negative values of z coordinate, i.e. one for each membrane leaflet. For the second representation we used 2 vectors representing the axes of half-segments (hypothetically half helices) spanning between z=-2 Å and z=12 Å, and z = -12 Å, z = 2 Å. The crossing points of vectors with z=0 Å planes were part of the representation.

Helical contact was defined as two atoms contained in two different transmembrane segments with separating distances of less than the sum of their van der Waals radii plus 0.6 Å.

4.2.2 RMSD calculation and definition

The Mass Center representations of TM triplets were aligned using the Kabsch

algorithm (Kabsch, 1976) implemented in python yielding values of RMSD between each of two representations. All 6 possible pairings between helices in the triplet pair were exhaustively tested and the lowest possible RMSD retained.

4.2.3 Clustering using k-medoids algorithm and hierarchical clustering

In order to separate the dataset into groups of triplets of similar packing we have clustered the resulting RMSD matrix using the k-medoids algorithm implemented in the scipy python library. We tested different numbers of clusters and chose those that yielded the clusters of about 10 Å of mean variance. As the number of resulting clusters was larger than 10 we decided to hierarchically organize the obtained clusters. With this aim we have performed hierarchical complete linkage clustering implemented in R on the set of resulting centroids – triplets representing each packing.

4.3. Molecular dynamics simulations

By dividing time into discrete units we can numerically simulate the evolution of our system in time. Through MD simulation microscopic interactions can be elucidated. The potential energy of a molecular model is determined by the interactions between its atoms. The same interactions create forces that guide the evolution of the system in time.

In order to computationally calculate potential energy or simulate the evolution of the system in time we need to calculate strength of those interactions and their contributions to the forces acting on each atom as well as their contribution to potential energy. A set of mathematical functions and parameters used to describe atomic interactions is known as a force field. The most common way to categorize atomic interactions is into bonded and nonbonded. Bonded interactions result from the energy needed to deform molecular orbitals forming bonds between atoms in

the same molecule. Nonbonded interactions result from attraction or repulsion between atomic charges (electrostatic interactions) or deforming atomic orbitals (vdW).

Bonded interactions:

The most common way to systematize bonded interactions is to divide them into interactions preserving bond length, angle value and torsion value.

(1) Bond length

$$V_{bond} = \frac{1}{2} k_{ij}(r_{ij} - r_0)^2$$

k_{ij} – force constant; r_{ij} – interatomic distance between atom i and j; r_θ – equilibrium bond length

(2) Angle value

$$V_{angle} = k_\theta(\theta_{ijk} - \theta_0)^2$$

k_θ – force constant; θ_{ijk} – angle between atoms i, j, k; θ_0 – equilibrium angle between atoms i, j, k

(3) Torsion value

$$V_{dihedral} = \Sigma\, k_\phi(1 + \cos(\mathrm{n}\phi - \phi_0))$$

C_n – dihedral constants; φ – dihedral angle;

Non-bonded interactions:

(4) Electrostatic interactions are long-range interactions resulting from the

attraction or repulsion between electronic charges. They can be represented by:

$$V_c(r_{ij}) = \frac{q_i q_j}{4\pi\varepsilon_0 r_{ij}}$$

V_c – Coulomb interaction energy; ε_0 – dielectric constant; q_i, q_j – atom charges for atoms i and j

Particle Mesh Ewald (PME) method was used for treatment of electrostatics throughout the simulations used in this study.

(5) VdW interactions. VdW interactions are short-range interactions resulting from deforming electronic orbitals.

$$V_{LJ}(r_{ij}) = 4\varepsilon_{ij}((\frac{\sigma_{ij}}{r_{ij}})^{12} - (\frac{\sigma_{ij}}{r_{ij}})^{6})$$

V_{LJ} – Lennard-Jones interaction energy; r_{ij} – interatomic distance between atom i and j; ε_{ij} – potential depth; σ_{ij} – potential zero point

In our studies we have simulated the following systems.

Time:	System:
200ns	288 DSPC with 125 nM NaCl
200ns	512 DLPC with 125 nM NaCl
200ns	256 DSPC, 32 DSPE-PEG with 125 nM NaCl
200ns	464 DLPC, 48 DLPE-PEG with 125 nM NaCl
200ns	128 POPC, $4Hp^0$
200ns	128 POPC, $4Hp^0$
200ns	128 POPC, $4Hp^{2-}$
200ns	128 POPC, $4Hp^{2-}$
200ns	128 POPC, $4Hp^{2-}$

Table 1. *Simulated systems*

5 SUMMARY OF RESULTS

5.1 **Publication I**

We tested the hypothesis that single cysteine residues are not accessible to the extracellular milieu and checked how this 'new rule' is applied in computational modelling. In order to do so, we investigated cysteine amino acids in experimentally determined structures of membrane proteins present in the PDB database: their presence, location, bonded state and environment (solvent accessibility). We compared the findings to computational structural models submitted to the GPCR DOCK competition. We found that in general the 'new rule' was not followed by the participants of the GPCR DOCK competitions. Although following the rule did not fully correspond to model quality in terms of RMSD, it guarantees compliance with general chemical knowledge and should drive efforts for better prediction of the extracellular domain.

5.2 **Publication II**

We studied the orientation of single helices and the packing of 2 or 3 transmembrane helical segments. For one helix we introduced a novel representation as two vectors, one for each membrane leaflet and measure the overhang value. For two helices we studied the packing of two helices in each membrane leaflet separately. For sets of consecutive and interacting three helices we used an internal spherical coordinates representation and clustering (k-means and hierarchical) using RMSD as distance measure. We found that certain packing arrangements are preferred (i.e. more common), which corresponds to the existence of N k-means clusters.

5.3 **Publication III**

We studied the effect of the lipid phase (gel or liquid-crystalline) on lipid membrane interactions with water and ions on a microscopic level by performing 200 ns simulations of molecular dynamics simulations of gel and liquid-crystalline membrane models with water and ions. We found that the interactions of PC head groups with water and ions with partial dehydration and deionization in the gel phase that can be explained by a more tight structure of the bilayer in this phase.

5.4 **Publication IV**

To elucidate how PEG polymers compete with lipid headgroups in binding ions and water in the gel and liquid-crystalline bilayers. We aim to provide an accurate view of the interactions of ions, PEG and lipids on the liposome surface through experimental and molecular dynamics simulation study.

Our study combining Langmuir monolayer film experiments with all-atom molecular dynamics simulation provide a new model for grafted PEG polymer interactions with lipid bilayer, water and ions. Instead of forming a neutral hydrophilic layer outside the membrane interacting only through steric forces as previously accepted PEG chains interact and loop around Na+ ions and (for the case of the tighter gel membrane) exclude Cl- ions from the membrane vicinity in addition to penetrating into the lipid core of the looser structure of the liquid crystalline membrane. Observed interactions help to elucidate how PEGylation influences the phase transition of membrane as well as how it prevents liposome opsonisation through affecting the interaction between the liposome and bloodstream proteins.

5.5 **Publication V**

We aim to describe the behaviour of the ionized and unionized forms of the photodynamic therapy agent hematoporphyrin in the POPC lipid bilayer by means of molecular dynamics simulation. We study depth, localization and orientation of porphyrin with respect to lipid molecules. We investigate the dynamics including rotation and diffusion within a membrane. We study interactions between porphyrin, lipid and water molecules (charge pairs and hydrogen bonds). Formation and alignment of dimers observed in the solvent phase is also studied. We find that the hematoporphyrin molecule is located in the phospholipid head group close to the lipid carbonyl groups, parallel to the membrane lipids. The dianion form has lower affinity to enter the membrane and the ionized groups tend to face the solvent influencing the molecular orientation. The dianionic form is also able to form stable dimers in the aqueous phase, with molecules arranged in parallel.

6 DISCUSSION

6.1 Membrane lipids and membrane proteins

As previous studies indicate (Marino and Gladyshev, 2010) cysteine residues are not abundant on protein surfaces owing to their function. Our results agree with that finding, adding that they are particularly rare on extracellular surface of proteins. Our findings could be utilized to devise new model scoring functions and knowledge-based *ab initio* methods for membrane protein structure prediction.

6.2 Could bridging cysteines located nearby improve the models? Receiver Operator Curve analysis of model distance based disulphide connectivity classifiers.

Even though pairs of cysteines bonded in respective target structures are often within a 5 Å or 10 Å vicinity in models submitted to GPCR Dock 2008 and 2010 editions, it is not clear how this could be utilized to improve model quality and disulphide bridge prediction rate. Histograms showing the distance of disulphide bonded cysteines to disulphide bond partner and other cysteines are shown in Figure 6. For cysteines forming XL3 bridge in more than 20% of cases disulphide bonded partner is found within 5 Å vicinity and in 65% of cases it is 10 Å vicinity. In less than 4% of cases we find an additional cysteine is found within 10 Å and in only 2% of cases within 5 Å.

For cysteines forming the XL2-TM3 bridge in more than 58% of cases a disulphide bonded partner is found within a 5 Å vicinity and in more than 80% of cases – within a 10 Å vicinity. In less than 2% of cases another cysteine is found within 5 Å, and in less than 2.2% of cases it is found within 10 Å. For XL1-XL2 bridges in hAA2AR and N-XL3 bridge in hCXCR4 in more than 5% of cases the disulphide bonded partner is found within 5 Å and for more than 14% of cases it is

found within 10 Å, while in less than 3.8% of cases other cysteine is found within 5 Å while in less than 20% of cases it is found within 10 Å. Therefore a distance based classifier would be reliable for the XL2-TM3 bridge to be conserved, intra-loop XL3 bridge that is connecting cysteines only 3 positions away but not for the XL1-XL2 and N-XL3 bridges that are connecting cysteines many positions away and is thus consequently less conserved and less expected.

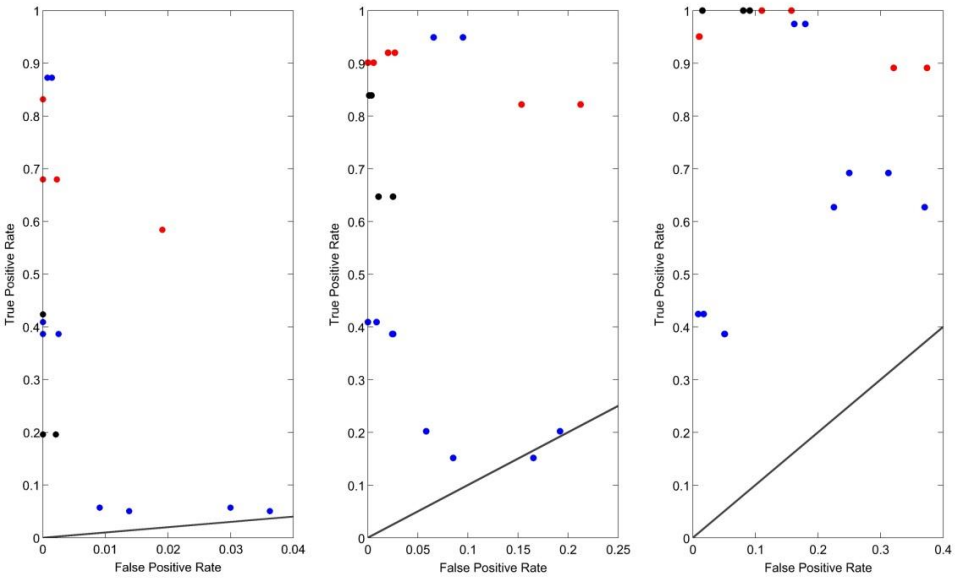

Figure 10. *ROC curves for function classifying cysteine as disulphide bond partner of cysteine studied based on distance for A) 5 Å; B) 10 Å; C) 20 Å distance thresholds. Function tested on models submitted to GPCR Dock competition editions in 2008 and 2010. True Positive Rate is plotted on the Y axis versus False Positive Rate plotted on the X axis with Y=X function plotted as solid line for reference. Results for different cysteine residues studied are shown in different colours based on the type of bridge they are involved in native structure with: XL2-TM3 bridge red; XL3 bridge – black; XL1-XL2 or N-XL3 bridge – blue.*

6.3 Effect of parameters and representation on describing transmembrane helices.

Visual analysis suggests that for short helices the MC representation is often more accurate in representing the helix main axis and its tilt from the membrane normal than principle component analysis. Partially overlapping helices atom sets are often optimal for extracting the set direction through Principal Component Analysis. The correct adjustment of parameters is difficult due to factors like differences in width across membranes (Figure 11). The study on helix packing is also limited by insufficient information regarding the definition of which helices are actually packed and/or interacting (Figure 12). There are also uncertainties related to the determination of membrane localization with respect to protein.

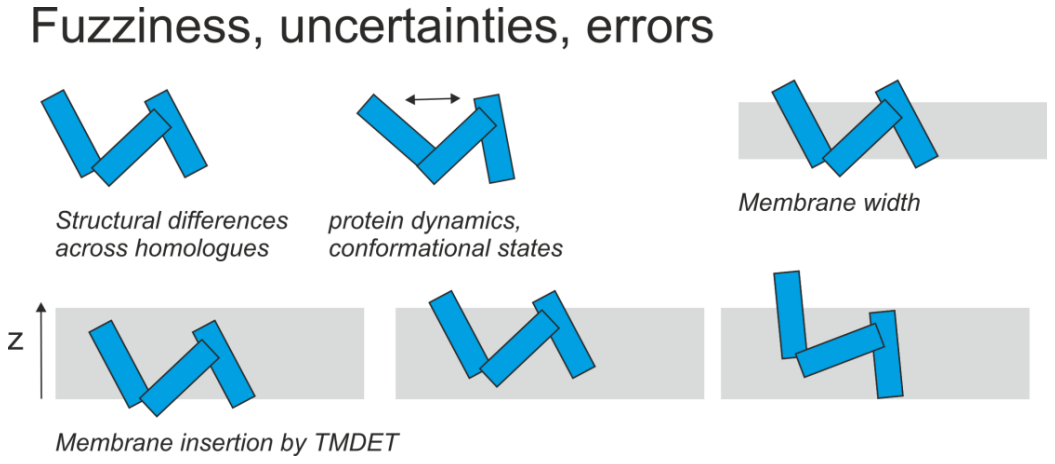

Figure 11. *Discussion about the different interferences in the TM packing study. Biases due to dataset composition are not illustrated.*

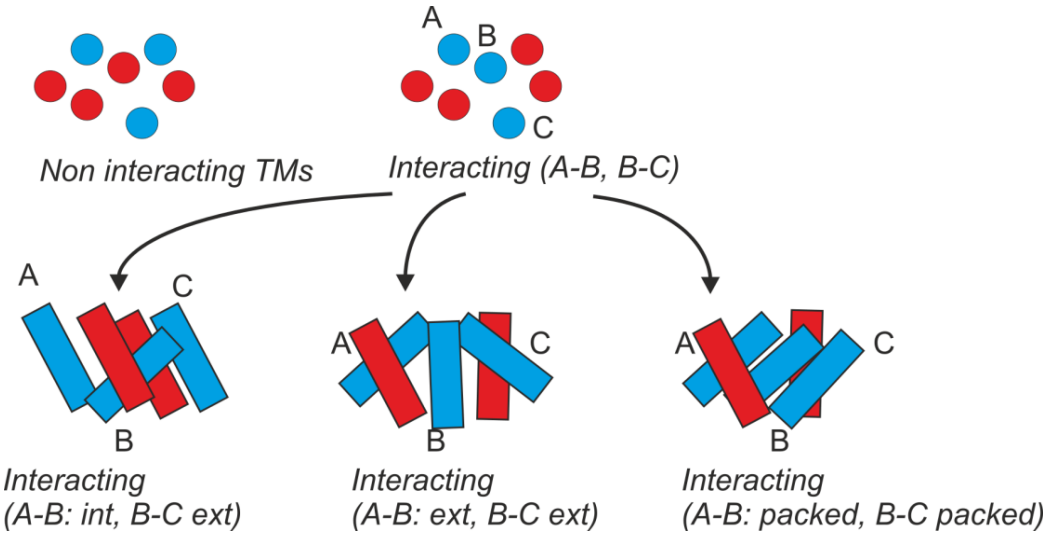

Figure 12. *Types of interactions between TMs.*

Our preferred values for helix tilt at 20 degrees are in accordance with literature (21 degrees in Bowie, 1997). We complement previous studies by representing helix as two cylinders and study tilt separately in each membrane leaflet and studying overhang. Preferred packing angles for pairs of helices with main peak at 27.5 degrees and smaller peak at 60 degrees are similar to those reported by Bowie (Bowie, 1997) at 20 degrees. While Feng and Barth clustered helix triplets into six preferred clusters (Feng and Barth, 2016) we organize the MC representations of triplets into 19 clusters. We also present preferred angles for internal spherical representations.

6.4 Effect of bilayer inner structure and dynamics on its interactants and interactions.

Throughout the studies contained in this thesis one recurring motif is demonstrating how including the inner structure and dynamics of lipid bilayer at atomic resolution leads to painting a more accurate picture of its interactions with water, ions, small molecules, grafted polymers and membrane proteins. The simplified model of membrane as a continuous steric barrier characterized only by permeability is not sufficient to explain experimental results and may lead to

artifacts. The inner structure and dynamics of the lipid membrane needs to be taken into account when describing lipid membrane interactions.

6.5 **Membrane bilayer interface as seen in the atomic resolution**

The looser structure of the liquid-crystalline state allows for water and ion penetration into the carbonyl region. This also sharpens the shape of electrostatic potential and decreases permeability creating a barrier for cation penetration into the membrane. X-ray and neutron scattering techniques can elucidate interactions between biomembranes and the extent of their hydration water (Berkowitz et al., 2006). Infrared spectroscopy can be used to study hydrogen bonding and fluorescence spectroscopy to study the dynamics of water molecules in the hydration shell (Berkowitz et al., 2006). More experiments using these techniques could corroborate our findings.

6.6 **Membrane interface and grafted polymers: atomic interactions**

Instead of being a general hydrophilic polymer layer outside the membrane (Kenworthy et al., 1995; Kuhl et al., 1994; Pantusa et al., 2003; Warriner et al., 1998; Needham and Kim, 2000; Hill, 2004) PEG penetrates the membrane in the liquid-crystalline phase. While the ordered, tight structure of gel phase excludes the PEG to aqueous phase, the looser, disordered structure of membrane in liquid-crystalline phase allows for PEG polymer penetration into the carbonyl region and is itself affected and disrupted by PEG polymer through an increase in the area per lipid, lowering in the acyl chain ordering parameter and penetration of water and ions with the PEG. These findings can help explain how PEGylation can influence liposome permeability and lower the temperature for triggered release of liposome contents.

6.6.1 Gel versus liquid-crystalline structure can mean a difference between a neutral and effectively charged PEG layer.

In our simulations PEG polymer binds Na^+ ions, which confirms results of previous studies(44-59). While the looser structure of membrane in liquid-crystalline phase allows for the presence of hydrated Cl^- ions between the PEG chains, in the membrane in gel phase the Cl^- ions are effectively excluded from the vicinity of the membrane leading to the charged PEG layer. While a neutral PEG layer can shield the liposome from bloodstream opsonins, the charged PEG layer can lead to an increased rate of opsonisation and liposome clearance from bloodstream. It is necessary to consider these facts when designing polymer coatings for liposomes and interpreting experimental results. I suggest further experiments in particular AFM are required to corroborate our findings.

6.7 Membrane inner structure and dynamics effect on its permeability for small molecules

For nonpolar compounds logP (partition coefficient logarithm) is a good predictor of drug permeability into the membrane. However for ionizable compounds, such as hematoporphyrin logD (distribution constant) is the correct descriptor as it accounts for a different ionization state in physiological pH. For the case of PDT the extent of photodamage depends also on the depth of chromophore immersion. The depth of the hematoporphyrin molecule depends on its ionization state with mass centres of Hp^0 and Hp^{2-} located about 1.5 and 1.7 nm from the centre of the POPC membrane. The effect of deprotonation on hematoporphyrin depth is relatively small in comparison to the effect on its orientation. With the porphyrin ring aligned with the acyl chains at ~30°, the deprotonated carboxylic groups preferentially located to the polar headgroup region and out of the membrane into the solvent while protonated carboxylic groups showed no such preference. Furthermore a study by Kepczynski et al. (2002) suggests that the

values of logP or logD do not always correlate with the binding constant to liposomes.

Results of our molecular dynamics simulations agree with experimental results, for example in (Kepczynski et al., 2002; Gramlich et al., 2004) porphyrins protonated and deprotonated at carboxylic moieties can both partition into the membrane.

(45) The dimerization equilibrium constant of porphyrin at neutral pH and 310K was found to be $2.8 * 10^5 M^{-1}$. In our simulations we observed the formation of porphyrin dimers in the aqueous phase. Membrane depth penetration by fluorescent molecules such as hematoporphyrin can be studied using the parallax method utilizing fluorescent quenching by spin-labeled phospholipids (Chattopadhyay and London, 1987; Ladokhin, 2014). Fluorescence Quenching by potassium iodide (KI) has been performed by Bronshtein et al. (Bronshtein et al., 2004) to find that indeed the tetrapyrrole ring is located deep in the membrane with its depth limited by the location of carboxylic groups at lipid-water interface.

7 CONCLUSIONS

The atomic resolution provided by molecular dynamics simulation of solvated and/or grafted lipid bilayer models as well as statistical analysis of crystallographic structures deposited in the PDB database is necessary to provide insight into phenomena such as solvation and ionization of lipid membrane, influence of grafted polymer, local differences in amino-acid abundance in proteins, helix packing and membrane insertions of proteins.

Unlike the case for known membrane protein structures, models submitted to the GPCR DOCK competition contain free cysteine thiols accessible to the extracellular milieu. Burying or bonding free cysteines could improve model quality and correctness.

Characteristic of transmembrane helix like tilt can take fairly independent values in different leaflets of lipid membrane. This applies also to the packing of helix pairs and triplets. There are preferred packings of helix triplets that do not stem from the preferred modes of pair packings.

The looser structure of the liquid-crystalline membrane leads to deeper penetration by water and ions as well as a reduction in rotational dynamics of the DSPC headgroups.

It also allows for PEG penetration into the carbonyl region and the disruption of the membrane structure, thus lowering the temperature for triggered release.

LogP often does not correlate to the actual partition of the molecule into the lipid bilayer and understanding of the cellular uptake of photosensitizers, partition, localization and orientation of molecules. The mass centre of hematoporphyrins is

located in the carbonyl region while the carboxylic group rests in the polar headgroup region or even in the aqueous phase when the groups are deprotonated.

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