Protein-Protein Interactions in Membranes

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Abstract: In this article we review the current status of our understanding of membrane mediated interactions from theory and experiment. Phenomenological mean field and molecular models will be discussed and compared to recent experimental results from dynamical neutron scattering and atomic force microscopy.

Keywords: Protein-protein interactions, membrane inclusions, lipid mediated interactions, membrane theory, membrane dynamics, neutron scattering.

1. INTRODUCTION

Understanding how proteins interact with each other is a major goal of biological science and one of its greatest challenges. Whether it is for molecular trafficking, signal transduction, structural support or storage, proteins play a vital role in the function of a cellular membrane. Protein interactions have been studied for many years, both experimentally and theoretically. Here we provide a review of theoretical models of protein-protein and protein-ligand interactions within a membrane, supplemented with advanced experimental methods. It is our goal to inspire further research into protein interactions; a field that may greatly contribute to our current understanding of cellular functions.

Protein communication may be important for understanding macromolecular function in a cellular context. It is common sense that biological systems act as coherent entities, but the degree of cooperation and, more importantly, the level of biological organization where this cooperation occurs are mostly unknown. Recent experimental results suggest that the basic building blocks, such as lipids and proteins, interact and already show concerted dynamics [1-3]. Besides the interest in fundamental science, the study of biological membranes is motivated by applications in nanotechnology, bio-inspired materials science, as well as the biomedical field. Trans-membrane and membrane-associated proteins are, for instance, the first to be attacked in many infectious diseases. Protein-protein interactions are also speculated to play an essential role in Alzheimer's disease and apoptosis, two pressing fields of research.

One of the main building blocks of cellular membranes are lipids, which form a two-dimensional fluid matrix in which membrane associated proteins are able to carry out their various functions. In 1972, Singer and Nicolson [4] proposed the "fluid mosaic" model to describe the structural features of biological membranes. In this model integral proteins are considered to diffuse more or less freely in a two-dimensional, viscous phospholipid bilayer solvent. Since then, the plasma membrane has been shown to be considerably more complex. An example are lipid rafts, regions of membranes enriched in certain types of lipids and cholesterol, which appear to act as platforms for the co-localization of proteins involved in intracellular signalling pathways. Attractive protein-protein interactions may lead to protein aggregation. It is speculated that protein aggregation plays an important role in neurodegenerative diseases, such as Alzheimer's disease. While protein aggregation can be considered as the final state, dispersed proteins allow the investigation of protein-protein interactions in experiments. The dispersed state may thereby be metastable, i.e., separated by an energy barrier from the aggregated state, or energetically stable, i.e., a global energy minimum, as pictured in Fig. (**1**). The purple membrane, which will be discussed in the experimental section, is an example of a system where the proteins arrange on a 2D lattice and form an almost crystal like structure. This well ordered state enables the comparison of theoretical and experimental results and to investigate fundamental aspects of a possible membrane-mediated interaction between membrane proteins. Modern computer simulations give insight into molecular level details of lipid-protein interactions. It was for instance found from molecular dynamics simulations [5, 6] that such interactions play important roles for stability and function of membrane proteins. Interactions between membrane embedded proteins will most likely depend on membrane composition and physical properties.

2. THEORETICAL APPROACHES

2.1. ANALYTICAL THEORIES

Inclusions in lipid membranes, such as membrane proteins, cause deformations in the bilayer. In order to minimize the exposure of nonpolar parts of the inclusion to the aqueous environment, the bilayer thickness adjusts to match the thickness of the hydrophobic region of the inclusion ("hydrophobic matching"). Interactions between inclusions may arise from direct interactions between the proteins, such as electrostatic, steric, and van der Waals interactions. Inclusions may also communicate by means of membrane-

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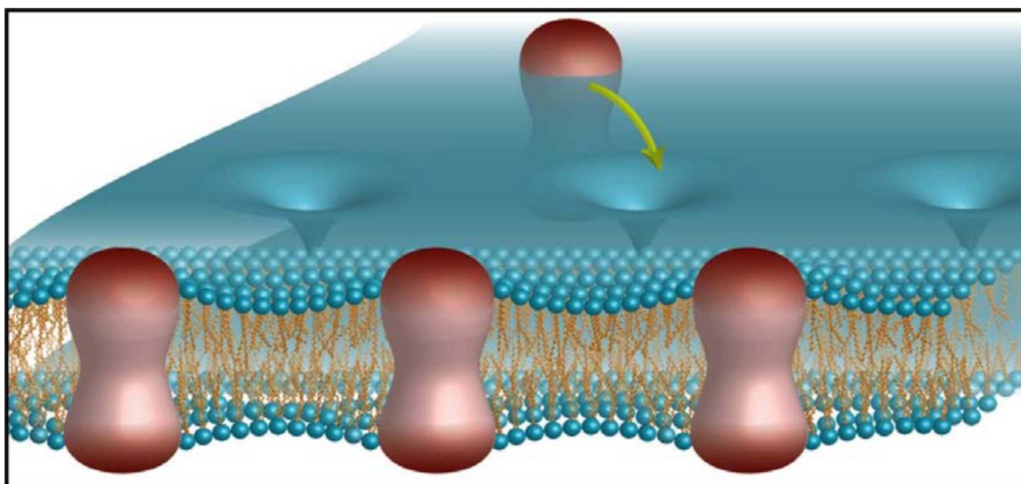

Figure 1. Hydrophobic mismatch and spontaneous membrane curvature may lead to proteins arranging themselves with a finite spacing. The foreground displays the physiological schematic, where the darker ends of the protein represent the hydrophilic region and the lighter central portion is hydrophobic. The background demonstrates the free energy landscape for the placement of proteins in the membrane as a result of the aforementioned factors.

induced interactions, arising from the perturbation of the bilayer structure. While electrostatic interactions are repulsive between like inclusions and decay exponentially with separation, van der Waals interactions are always attractive. However, as typical distances between membrane inclusions are 6 nm and more, the latter interactions are expected to lead to relatively small contributions to a possible interaction, only. The driving force between protein-protein interactions is most likely a membrane mediated interaction. Such an interaction may be repulsive or attractive, as will be discussed below. The lipid-mediated interaction was first investigated theoretically by Marčela using a mean field theory of chain orientational order in lipid membranes [7]. There is a large body of theoretical work today addressing protein interactions in membranes. We will, in the following, discuss selected recent papers that we consider to particularly be enlightening and representative.

One of the first studies addressing the lipid mediated interactions between inclusions took into account two types of membrane distortion, namely variations in membrane thickness and the surface area per lipid molecule [8]. Thickness and area were coupled to keep the membrane volume constant. A monotonically attractive short-ranged interaction was predicted. It was later realized that the hydrophobic membrane core exhibits elastic behaviour when its thickness is varied and that mechanical parameters, such as bending and compressional moduli, must be taken into account. While early studies mainly addressed the question of protein aggregation in membranes, accounting for the bending energy in subsequent studies brought forward a metastable state characterized by a definite spacing and potential two dimensional regular arrangement of inclusions. This ordered state is separated from the aggregated by an energy barrier.

Present theoretical models can be distinguished into 1) phenomenological models, which employ phenomenological parameters, such as interfacial tension, stretching and bending elastic moduli of the membranes, and 2) molecular models, which explicitly account for molecular parameters and interactions of the lipid molecules, such as van der Waals, electrostatic, chain interaction energy and configurational entropy. The basic model is sketched in Fig. (**2a**). Depending on the size of the hydrophobic part of the inclusion, l_0, and the equilibrium thickness of the bilayer, h, there is a hydrophbic mismatch $h_0=(l_0-h)/2$. A positive (or negative) hydrophobic mismatch therefore refers to a larger (or smaller) thickness of the hydrophobic region of the protein compared to that of the host bilayer. Although the bilayer is entirely flat, the upper and lower leaflets have to bend in order to accommodate the hydrophobic portion of the inclusion. $u(r)$ is the thickness of the membrane as a function of distance from the center of the inclusion and reflects the distortion of the membrane and the perturbation profile.

The free energy per amphiphile of the monolayer can then be written as [9, 10]:

$$f(u,a_l) = f_o(u,a_l) + K(a_l)\left(\nabla^2 u - \kappa(a_l)\right)^2. \quad (\mathbf{1})$$

The first term is the free energy of a flat monolayer given by $f_o(u,a_l) = \gamma a_l + G(u)$, where γ is the surface tension between the aqueous media and the hydrophobic amphiphile tails, and $G(u)$ a compression-expansion term of the amphiphiles. The thickness of the membrane, u, and the area per amphiphile molecule, a_l, are functions of the distance with respect to the inclusion, i.e., $u(r)$ and $a_l(r)$, and are related by an incompressibility condition. The other terms stem from bending of the monolayer indicated by the local monolayer curvature $\nabla^2 u(r)$. $K(a_l)$ is the bending stiffness per molecule, so that $K(a_l)(\nabla^2 u)^2$ represents the energy related to bending the leaflet. The last term corresponds to the spontaneous curvature of the monolayer, where $\kappa(a_l)$ is the spontaneous curvature per molecule. The spontaneous curvature mainly depends on structural parameters, such as the composition of the membrane. It plays, however, an important role for the magnitude and the character of the lipid mediated interaction.

Depending on their chemical structures, lipids tend to curve with a slight spontaneously negative or positive curvature. Lipids with smaller acyl chain area to polar head group

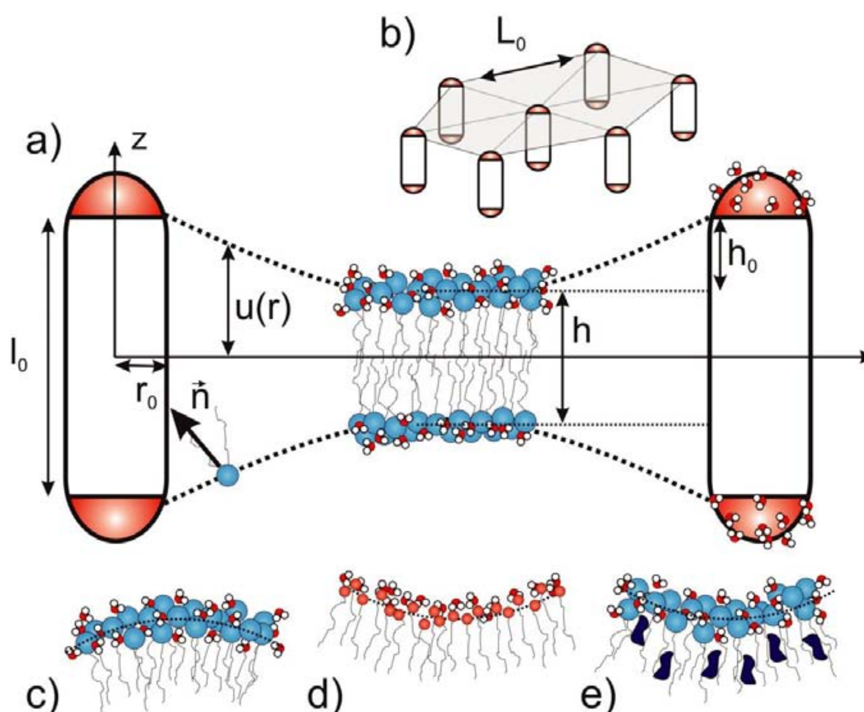

Figure 2. a) Profile of the membrane with inclusions. Hydrophobic matching leads to a distortion of the monolayer leaflets which is characterized by the thickness of the membrane as a function of the distance from the center of the inclusion (u(r)), h is the equilibrium thickness of the membrane, and l_o is the size of the hydrophobic region of the inclusion. **b**) Regular 2D arrangement of membrane inclusions with equilibrium spacing L_0. Depending on the chemical structure and membrane composition, lipid bilayers may have positive spontaneous curvature **c**) or negative spontaneous curvatures, as shown in **d**) and **e**).

area ratio, such as DMPC (dimyristoyl phosphatidyl choline), tend to exhibit positive spontaneous curvature, as shown in Fig. (**2c**). Lipids such as DOPC (dioleyl phosphatidyl choline) and DOPE (dioleyl phosphatidylethanolamine) exhibit a negative spontaneous curvature [11], as depicted in Fig. (**2d**). More complex lipid bilayers including mixtures of lipids and cholesterol can be expected to show a more complex behaviour, as sketched in Fig. (**2e**). So insertion of the inclusion as depicted in Fig. (**2a**) would be energetically favourable for lipids or mixtures with a negative spontaneous curvature, such as in Fig. (**2d** and **2e**).

Using Equation (**1**), the membrane perturbation profile and the membrane-induced interactions between an array of inclusions embedded in a two-dimensional membrane were calculated [9, 12, 13]. The inclusions arrange naturally in a tightly packed structure, which can often be described in terms of a 2D hexagonal lattice as shown in Fig. (**2b**).

Fig. (**3a**) depicts the free energy as a function of the distance between the inclusions for a chosen set of parameters corresponding to the example sketched in Fig. (**2a**). In the case of vanishing spontaneous curvature, the global energy minimum is obtained at r = 0, which favours aggregation. A metastable ordered state with a finite separation between the inclusions may exist, separated from the aggregated state by an energy barrier. Aggregation becomes unfavourable for nonzero spontaneous curvature and the energy becomes minimal at a finite spacing, L_o. The asymptotic limit $r \rightarrow \infty$ represents the total energy gain or loss of the membrane by the incorporation of a single inclusion. Only for negative spontaneous curvature is this process energetically favourable and the inclusion will not be rejected. While the compression-expansion term and the bending term would always favour an unperturbed membrane and aggregation of inclusions, the spontaneous curvature may favour incorporation and regular arrangement of inclusions at a certain distance. It was further observed that the spontaneous curvature of the monolayer determines the shape of the membrane deformation profile. The elastic properties of the membrane, such as compressibility and bending energy, set the perturbation length, i.e., the distance at which the membrane returns to its undistorted equilibrium thickness. A different approach involves using a sandwich model to calculate the perturbation profile, the interaction energy and the lateral capillary force between two isolated inclusions [14, 15]. The parameters used in this model were shown to relate to the above mentioned Helfrich parameters. The sandwich model also predicted non-monotonic lateral capillary forces and interaction between two inclusions.

In these mean field elasticity theories, membrane properties are taken into account using uniform phenomenological constants. Membrane properties are assumed to remain unaffected by the inclusion. By employing a "director model", conformational constraints of the flexible lipid chains by the presence of the inclusion were taken into account [16, 17]. The elastic free energy Equation (**1**) is expanded by a second order parameter, $n(r)=(n_x, n_y)$, the director field, that characterized the tilt of a given lipid chain at position r. $n(r)$ is also exemplary depicted in Fig. (**2a**). The director field $n(r)$ represents the average orientations of the corresponding lipid chains. In the vicinity of the rigid inclusion, the number of

available conformations is reduced because the lipid chains are not able to penetrate into the inclusion interior. The closer the average distance of a lipid chain to the inclusion, the larger the number of inaccessible chain conformations. The corresponding calculations yield free energies similar to those in Fig. (**3a**), though the results are presently strictly valid only for the interaction between two individual inclusions, i.e., for very low inclusion densities. Also here, the structure of the free energy was found to strongly depend on the spontaneous curvature of the bilayer.

2.2. Computer Supported Theories

An extension to the mean field approach presented above, which uses phenomenological free energy functionals, explicitly includes the microscopic structure and dynamics of the bilayer lipid molecules using all-atom molecular dynamics (MD) simulations [18-20]. This theory offers an intermediate approach, combining aspects of mean field theories and fully atomic simulations. The protein inclusions were modeled as hard, vertical, straight, repulsive cylinders that interact only with the hydrocarbon chains. The polar head groups were assumed to not to be directly affected by the proteins. The key quantity is the average density of the carbon atoms projected in the 2D membrane plane, i.e., the lateral density-density response function of the hydrocarbon core. The net average lipid-mediated force acting on two protein inclusions, in this model, arises from the asymmetry in the hydrocarbon density around the protein inclusion. Three different inclusions embedded in a DPPC (dipalmitoyl-*sn*-glycero-phosphocholine) bilayer were modeled: a small cylinder, corresponding to an aliphatic chain, has a radius of 2.5 Å; a medium cylinder, with a radius of 5 Å, corresponds to an α-helical polyalanine molecule; and a large cylinder with a radius of 9 Å, representing a small peptide such as a gramicidin channel. The lipid density next to the edge of a protein was found to be lower than its bulk value. This depletion layer was followed by a crowded region with high lipid density. The lateral perturbation of the lipid density was found to extend up to 25 Å from the edge of the cylinder. Lipid molecules next to the cylindrical inclusion therefore have a greater area per lipid molecule than lipid molecules in the unperturbed membrane. Results for the lipid-mediated free interaction energy for the three different inclusion sizes are sketched in Fig. (**3b**). While incorporation of the largest inclusion is energetically unfavourable, aggregation of the smaller cylinders reduced the free energy. However, the global energy minimum at zero distance is separated by an energy barrier, which may allow for a metastable state, where inclusions arrange on a regular grid with defined lattice spacing. In contrast to the mean-field approach where interaction stems from the minimization of an elastic free energy functional, the origin of an interaction between proteins was found to be entropic in computer simulations. It is driven by the fact that the lipid chains have to adopt a more ordered configuration close to the hard cylinder inclusions. This model results in a metastable dispersed state for several phospholipids, such as POPC, DPPC, DMPC and DOPC, as part of an effort to investigate the effect of lipid diversity [20].

Protein dynamics have also been modelled using all-atom molecular dynamics computer simulations, see e.g [21-24]. Recently, evidence for collective motions between proteins was observed from the phonon spectrum of the excitations [25, 26].

3. EXPERIMENTAL TECHNIQUES

There has been a tremendous interest in the experimental determination of protein-protein interactions recently [27]. While emphasis has been on high-throughput screening techniques, such as mass spectroscopy, modern techniques are also capable of directly accessing molecular interactions in biological or biomimetic systems. Experimental evidence for lipid-protein and protein-protein interactions has been reported on for decades. Lewis and Engelman [28] investigated the relation between hydrophobic matching and protein organization in membranes by freeze-fracture studies. Here, the organization of bacteriorhodopsin monomers in a membrane was not found to be particularly sensitive to changes of the equilibrium thickness of lipid bilayer environment. The proteins remained dispersed over a large range of hydrophobic thicknesses. Only much later, Harroun *et al.* [29] reported X-ray scattering results which demonstrated hydrophobic matching and membrane mediated interactions in lipid bilayers containing Gramicidin. It was observed that the thickness of the lipid bilayers adapted to the size of the embedded peptide. The hydrophobic matching was concluded

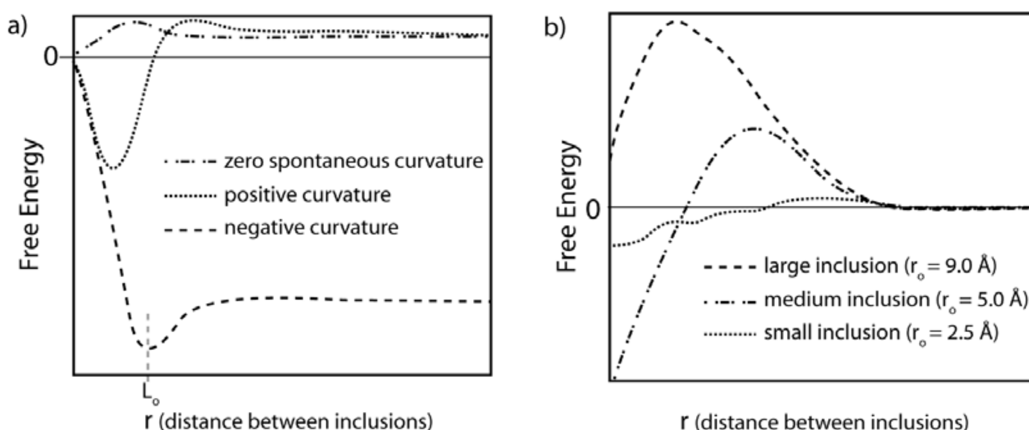

Figure 3. Free energy as a function of protein spacing. **a**) demonstrates the effect of spontaneous membrane curvature (adapted from Aranda-Espinoza, *et al.* (1996)) and **b**) shows the effect of protein size (adapted from Lagüe, *et al.* (2000)).

to create a strain field that gives rise to an attractive membrane mediated potential between the inclusions. By using videomicroscopy to study long wavelength fluctuations in giant unilamellar lipid vesicles containing bacteriorhodopsin proteins, Faris *et al.* [30] reported lowering of the membrane tension when the proteins were activated. The protein activity was observed to enhance membrane fluctuations and thus pointing to an effective lipid-protein interaction.

In experiments, the fingerprint of molecular interactions can be found in dynamical properties. However, very few approaches and techniques are capable of directly accessing collective molecular motions and molecular interactions. Inelastic X-ray and neutron scattering experiments can directly access collective dynamics in membranes and proteins by measuring the corresponding excitation spectrum. Phonon-like excitations of proteins in hydrated protein powder were reported from X-ray scattering experiments using synchrotron X-ray radiation [31]. Experimental evidence for a protein-protein interaction in a purple membrane under physiological conditions was presented recently from neutron scattering experiments [2]. From high speed atomic force microscopy (AFM) experiments cooperative protein activity was reported from in situ observations in real space [3].

Biologically relevant materials can be thought of as "multi-scale" materials, due to the fact that relevant dynamics take place over extended length and time scales [32, 33]. To address this multi-scale behaviour experimentally, different techniques must be applied and combined. Fig. (**4**) depicts the length and time scales accessible by high speed AFM, inelastic neutron scattering, inelastic X-ray scattering, dynamic light scattering (DLS), Brillouin and Raman scattering, and dielectric spectroscopy. By combining the different techniques a large range of dynamical behaviour can be elucidated. For example, inelastic neutron and X-ray scattering access length scales from less than 1 Å to greater than 100 nm, and time scales from picoseconds to almost one microsecond Fig. (**4**). While inelastic X-ray scattering is the perfect tool to measure fast dynamics at large distances (i.e. small scattering vectors Q), inelastic neutron scattering can more easily and precisely access slow dynamics at smaller length scales (large Q). In recent years, high speed AFM has combined a high spatial resolution of about 5 Å with a time resolution of up to 20 milliseconds. MD simulations are also proving to be an invaluable tool in developing models for molecular structure and dynamics in membranes and proteins. Because of the continuous increase in computational power and the optimization of algorithms, large complex systems (i.e. many hundreds of molecules) and long simulation times can now be addressed, see e.g. [22, 23, 34-38]. The dashed rectangle in Fig. (**4**) marks the dynamic range currently accessed by computer simulations – the elementary time scale for simulations is in the order of femtoseconds.

Atomic and molecular motions in membranes and proteins can be classified as local, self-correlated, and collective, pair-correlated dynamics. Local dynamics corresponds to the motion of molecules or functional groups in local energy potentials. The force and time constants involved are determined by the local friction and restoring forces. This type of dynamics is called incoherent because the particles

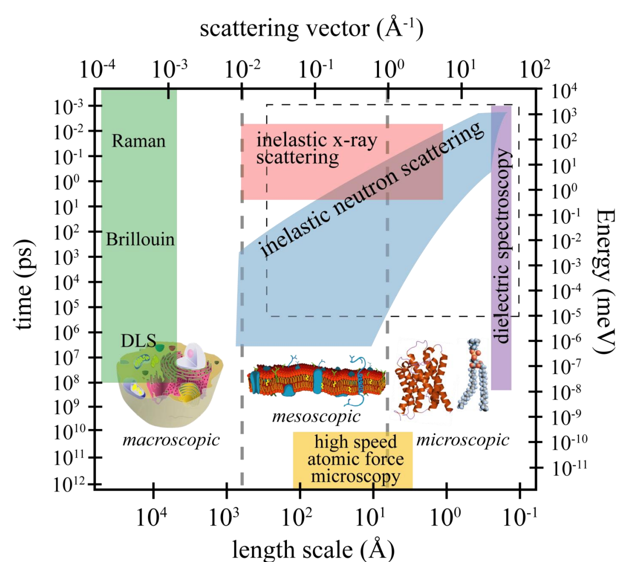

Figure 4. Length and time scales, and corresponding energy and momentum transfers, for spectroscopic techniques covering a range of dynamics from the microscopic to the macroscopic. Light scattering techniques include Raman, Brillouin, and dynamic light scattering (DLS). Inelastic X-ray and neutron scattering access dynamics on Å and nm length scales. Dielectric spectroscopy probes the length scale of an elementary molecular electric dipole, which can be estimated through the C—O bond length (~ 140 picometer). High speed AFM has a spatial resolution of Å to nm. The area enclosed by the dashed line box is the dynamical range accessible by computer simulations.

move independently in their local environments and examples are vibration, rotation, libration (hindered rotation) and diffusion of individual lipid molecules. Coupled, collective molecular motions arise due to an interaction between particles or functional groups (which can be pictured as a spring). This type of dynamics is called coherent. Collective molecular motions determine, for instance, elasticity of membranes and, in particular, interactions between membrane embedded proteins. In biology, any dynamics will most likely show a mixed behaviour of particles moving in local potentials but with a more or less pronounced coherent character. Motions in lipid bilayers, for instance, range from long wavelength undulation and bending modes, with typical relaxation times on the order of nanoseconds and lateral length scales of several hundred lipid molecules (i.e. tens of nanometers), to short wavelength density fluctuations in the picosecond range and nearest neighbour length scales [39-51]. Different techniques have been used to study the different types of motions. For example, local dynamics in lipid bilayers (i.e. individual lipid molecules), such as vibration, rotation, libration (hindered rotation) and diffusion, have been investigated by incoherent neutron scattering [34, 40-43] and Nuclear Magnetic Resonance (NMR) [52, 53] in order to determine the short wavelength translational and rotational diffusion constants. On the other hand, collective bilayer undulations have been examined by coherent scattering experiments using neutron spin-echo spectrometers [34, 41, 49, 54] and DLS [55-57], while collective short wavelength dynamics have been studied by inelastic X-ray [58] and neutron [48] scattering using the ubiquitous triple-axis spectrometer in-

vented by B.N. Brockhouse in the 1950s. In contrast to other spectroscopic techniques, such as, dielectric spectroscopy, inelastic X-ray and neutron scattering result in wave vector resolved access to molecular dynamics. For example, excitation frequencies and relaxation rates are measured at the different internal length scales of the system. A typical dynamical scattering experiment measures (Q,ω) pairs, resulting in a frequency along with a corresponding length scale, and possibly a corresponding direction (e.g. parallel or perpendicular to a protein's axis). This additional information is of paramount importance when it comes to relating dynamical information to structure. In short, the suite of inelastic instruments used to study soft and biologically relevant materials is comprised of time-of-flight, backscattering, triple-axis and spin-echo spectrometers [49, 59, 60].

Lipid-protein and direct protein-protein interactions can also be detected using NMR techniques. The fingerprint of interactions can be found in changes of the chemical environment of atoms or functional groups, which then involve chemical shift changes with conformational changes [61-64]. NMR studies can therefore provide important information about specific molecules or groups involved in interactions or binding.

Scanning probe microscopic techniques, such as AFM [65] and Scanning Tunneling Microscopy, on the other hand, give high resolution real space pictures but are essentially surface sensitive. The combination with scattering techniques is therefore a powerful tool to gain information about bulk *and* surface structure. As is well known, AFM can be used to monitor biological processes under physiological conditions [66] on the cellular [67] and the molecular scale [68]. In addition to topographical maps of the sample surface, the local mechanical properties of soft samples can be measured by the application of very small loading forces [69-74]. By using the Hertz model for elastic indentations [75, 76] the Young's modulus E can be determined. Phospholipid membranes have been extensively studied by AFM techniques. AFM was used to reveal information on the morphology and topology of membranes, bilayers, domains [77-81] and ripple phases [82, 83] in real time using controlled environments [84, 85]. In addition, Fourier transformation was used to corroborate the periodicity of the ripple structure [86].

Experiments to study protein-protein interactions were conducted using Purple membrane (PM). PM is a well-characterized native membrane system and a simple example of a bio-energetic device. Due to the highly organized arrangement of its proteins, and easy spectroscopic access to its functional state, PM is one of the best suited model systems for the experimental study of biological function. A possible interaction between membrane embedded proteins may lead to dynamic coupling between proteins, i.e., concerted protein dynamics. As discussed above, the proteins may communicate by using the membrane as a signal transmitting medium.

PM occurs naturally in the form of a two-dimensional crystal, consisting of 75% (wt/wt) of a single protein, bacteriorhodopsin (BR) (that functions as a light-activated proton pump), and 25% various lipid species, mostly phospho- and glycolipids [87]. BR is a proton transporting membrane protein, composed of seven transmembrane alpha helices arranged around the photosensitive retinal molecule. PM structure has been well established by electron microscopy, and neutron and X-ray diffraction experiments e.g. [87-92]. The protein in the lipid matrix is organized into trimers that form a highly ordered 2D hexagonal lattice with lattice parameter a = 62 Å. A protein coupling may be relevant for the photo cycle in PM. The BR proteins undergo structural changes during the photo cycle, involving displacements of up to 1.7 Å [93]. Because of the elastic coupling of the BR proteins, those distortions can propagate to neighbouring proteins. It can therefore be speculated that there is a protein-protein communication during the photocycle in PM.

3.1. Protein-Protein Interaction in Purple Membranes

The interaction between membrane embedded proteins at physiological temperatures was recently reported from inelastic neutron scattering experiments. The magnitude of the coupling between proteins was determined by measuring the spectrum of the acoustic phonons in the 2D protein lattice [2]. The experiments were performed on the IN12 cold triple-axis spectrometer at the Institut Laue-Langevin in Grenoble, France, with an energy resolution of $\Delta\hbar\omega$= 25 μeV. This apparatus allowed the simultaneous measurement of diffraction and inelastic scattering. The excitation spectrum of the 2D protein lattice was also modeled analytically. In this model, the protein trimers were taken as the centres of a primitive hexagonal lattice, and the spectrum of the acoustic phonons was calculated. The model is depicted in Fig. (**5a**). The interaction between protein trimers is contained within the springs with an effective (longitudinal) spring constant k. The calculated longitudinal excitation spectrum $C_l(q, \omega)$ as is defined by $C_l(q, \omega) = (\omega^2/q^2)S(q, \omega)$, is shown in Fig. (**5b**). The statistical average in the plane of the membrane leads to a superposition of the different phonon branches which start and end at the hexagonal Bragg peaks (at $\hbar\omega = 0$). The data points are the excitation positions as determined from the inelastic neutron triple-axis experiment [2].

The effective protein-protein spring constant k was determined to be 54 N/m. The amplitude of this mode of vibration can be estimated from the equipartition theorem to

$\langle x^2 \rangle = \sqrt{k_B T/k}$ = 0.1 Å, and the interaction force between two neighbouring trimers to $F = k\sqrt{\langle x^2 \rangle}$ = 0.5 nN. For comparison, using the same approach, the spring constant for graphite was calculated to be 27,000 N/m for the in-plane interaction, and 3.5 N/m for out-of-plane interactions. The protein interaction force constant for PM is therefore 1-2 orders of magnitude larger than the effective van-der-Waals force constant found in graphite, but 2-3 orders of magnitude weaker than a C-C bond. In contrast, the amplitudes of diffusive, self-correlated motions are usually about one magnitude larger than those of collective pair correlated motions [1, 90]. From this, one can conclude that the spectrum of thermal fluctuations in a biological membrane is most likely dominated by the diffusive motions of lipids and proteins. Interactions, however, may become more relevant for certain functions, such as photo cycle [92] and signal transmission. To establish a clear relationship with protein function, protein

dynamics of activated proteins, i.e., the collective dynamics of proteins undergoing the photo cycle, can be studied using recently developed laser-neutron pump-probe experiments [94].

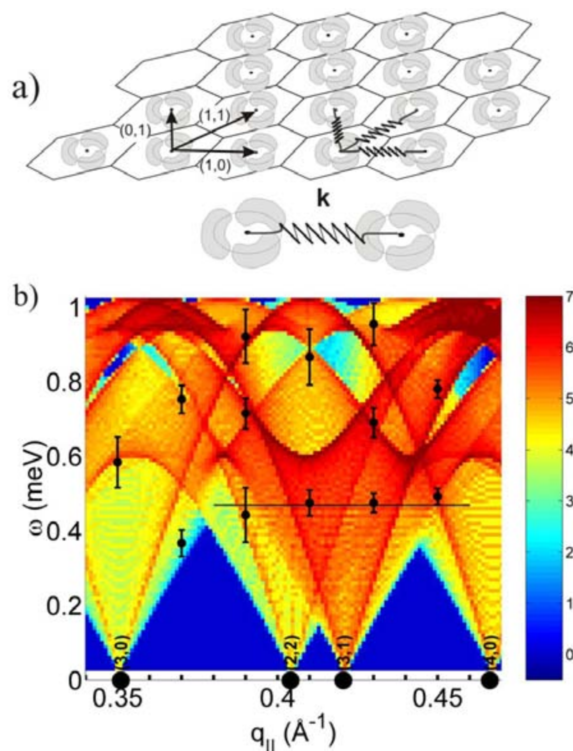

Figure 5. a) BR trimers are arranged on a hexagonal lattice of lattice constant a = 62 Å, where the basic hexagonal translations are indicated by arrows. The interaction between the protein trimers is depicted as springs with effective spring constant k. **b)** The calculated excitation spectrum $C_l(q, \omega)$ in the range of the experimental data. Data points mark the positions of excitations. The horizontal line at $\hbar\omega$ =0.45 meV marks the position of a possible optical phonon mode, not included in the calculations. adapted from Rheinstädter *et al.*, 2009 [2].

3.2. Lateral Coupling and Cooperative Dynamics in the Function of the Native Membrane Protein Bacteriorhodopsin

A high-speed AFM [95] was used to study protein dynamics of the BR proteins in PM under native conditions using a native membrane in a saline solution [3]. A green laser was focused near the tip of the cantilever to study structure and dynamics of activated proteins undergoing the photocycle. A sketch of the experimental setup is shown in Fig. (**6a**). The spatial resolutions achieved by this new technique were 4 Å laterally and 1 Å vertically.

Fig.(**6c** and **d**) depict the photo-induced conformational changes of the BR protein with c) and without illumination by the green laser d), in the dark state). The monomer surface split into two protrusions when the membrane was illuminated, resulting in a shift of 7 Å, which leads to a distinct distortion of the protein trimer. However, the amplitude of the shift observed by high-speed AFM is significantly (2-3 times) larger than previously reported from X-ray and electron diffraction structure determinations [92, 96]. A possible explanation may be the fact that the sample was frozen to cryogenic temperatures in these studies, while the AFM images were taken at an almost native state at room temperature making the system more flexible.

Cooperative protein dynamics during the photo cycle were observed. Monomer isomerisation (a rearrangement of the atoms inside of a molecule) was found to correlate with the successive isomerisation of the other two monomers of the trimer within about 50 ms. Only one photo-isomerization per trimer at a time was observed. This implies a cooperative protein dynamics may enhance the pumping efficiency of the protein. A coherent lateral movement of the protein trimers within the membrane was also observed when the membrane was illuminated. The relaxation time at which the proteins returned to their initial positions was determined to be $\tau = 119$ ms. This process was associated with the membrane's elastic properties and the work necessary for the photo-induced tilt of the helices inside of a protein see Fig. (**6b**) was estimated to $W = 5.4 \cdot 10^{-20}$ J.

The inelastic neutron and the high-speed AFM study present the first experimental evidence for a protein-protein interaction in purple membrane and a cooperative protein dynamics in a biological membrane. The interactions between the protein trimers and between the monomers within a trimer are most likely lipid mediated and depend on the elastic properties of the membrane. The force constant of the monomer-monomer interaction can be estimated from the AFM results to $k_M = 2W/\langle x^2 \rangle = 0.2$ N/m. The force constant for the interaction between trimers of k=54 N/m, as determined by neutron scattering, is therefore about two orders of magnitude larger than k_M. One should, however, take into account the different time scales which were accessed in the experiments. While the neutron experiment studied dynamics of pico- to nanoseconds, the AFM time resolution is in the order of milliseconds. The elastic behaviour and properties of the PM membrane and also the proteins may strongly depend on the time scale at which they are observed. The membrane may appear much stiffer when studied at high frequencies as molecular reorientations, which occur on pico-microsecond time scales, most likely relax, also due to diffusion between two AFM images. This finding may also be relevant if one wants to compare the experimental findings to the previously discussed theories as elastic constants in these visoelastic materials critically depend on the length scale at which they are observed.

4. DISCUSSION AND CONCLUDING REMARKS

Theoretical models can be divided into two categories. The first model mainly considers the elastic properties of bilayer and monolayers. In this case, the protein interaction results from the minimization of an elastic free energy functional. The second model assumes that protein interactions are entropy driven. The number of conformational states available to the lipid tails in the hydrophobic membrane core is constrained by the presence of the inclusions. This can result in an attractive force between proteins that can lead to protein aggregation as the global energy minimum of the system. However, depending on the choice of parameters, there may exist a metastable state which causes the protein to

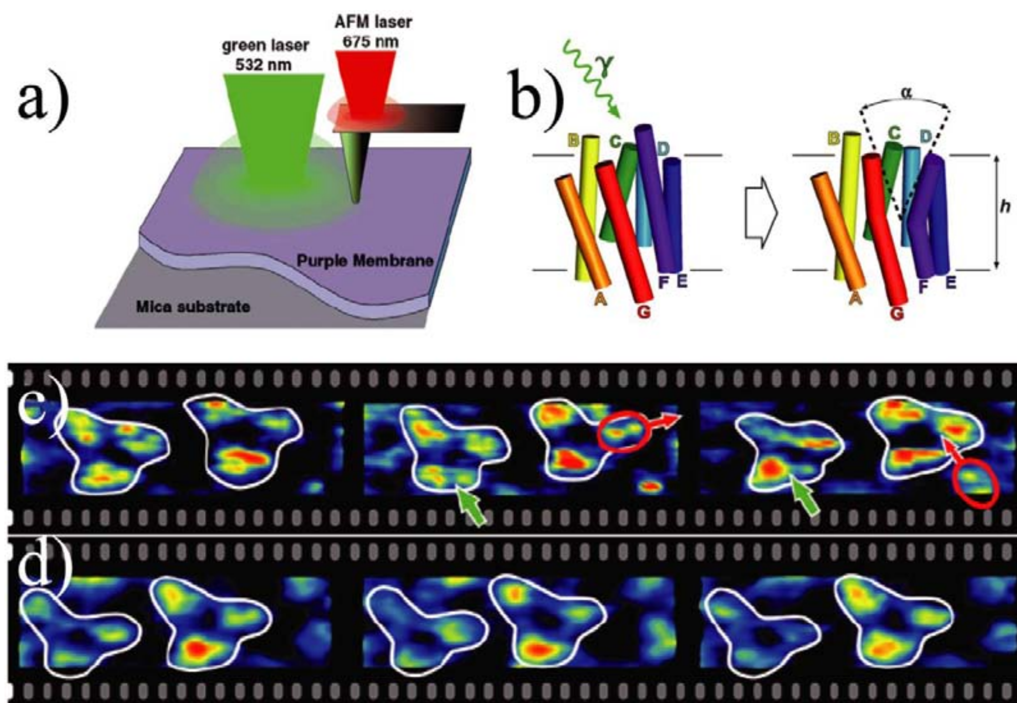

Figure 6. a) Experimental setup of the high speed AFM experiment. **b**) Conformation change of the bacteriorhodopsin protein during the photocyles. The photo induced tilt of helicies f and g opens a channel in the cytoplasmic part of the protein. **c**) High speed AFM image of the PM surface in the activated state after photoisomerization of the proteins. **d**) Dark state image of the PM surface. Adapted from Voïtchovsky *et al.* [3].

stay dispersed in the membrane with a finite spacing. Only one theory, which explicitly includes the effects of spontaneous membrane curvature as well as the incorporation of a 2D protein structure, finds the distributed state as the global energy minimum of the system. This result may be relevant in explaining the observed structure of Purple membrane, in which the proteins are arranged on a regular 2D hexagonal lattice. While all of the above mentioned parameters, such as elastic parameters, lipid density, number of conformational states, most likely play a role in protein arrangement, it is at this point difficult to unambiguously decide which parameter is the dominant factor.

A quantitative comparison between theory and experiment remains challenging because of current limitations in both areas. Theoretical techniques are able to access properties of the system on a molecular level, while experimental methods are more adept at measuring the macroscopic features. Only recently have experimental techniques begun to access the relevant lengths and time scales to achieve the microscopic resolution necessary to close that the gap between theory and experiment. Modern super computers allow for increased system sizes in simulations and access biologically relevant time scales. It can be speculated that both fields will continue to converge such that a quantitative comparison can be envisioned in the near future. Experiments will be important to refine force fields used in simulations. Analytical theories and simulations are already an invaluable tool for the interpretation of experimental data.

Neutron scattering and AFM will most likely be used in the future to determine the protein-protein interactions in membranes of different composition. By changing the lipid composition of the membrane, the spontaneous curvature of the monolayers can be modified. This may change the system from a protein-aggregated to a protein-distributed state, as well as alter strength of the interaction and the distance between inclusions. Saturated and unsaturated lipids may be used to investigate the effects of lipid-hydrocarbon density on protein interaction. The elastic parameters of membranes can be controlled by the incorporation of cholesterol to understand the impact of elasticity on the interaction force. By identifying the driving force behind membrane mediated interactions between membrane inclusions, present theories could be refined and eventually quantitatively compared to experimental results. A quantitative theory may eventually be used to control protein interaction to trigger protein aggregation or distribution, or impact on protein function and to tailor complex membranes with specific properties. This research is likely to have a great impact on Alzheimer's and cancer research as in both fields protein-protein interactions play an important role.

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