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Effects of protein crowding on membrane systems☆

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ABSTRACT

Cellular membranes are typically decorated with a plethora of embedded and adsorbed macromolecules, e.g. proteins, that participate in numerous vital processes. With typical surface densities of 30,000 proteins per μm^2 cellular membranes are indeed crowded places that leave only few nanometers of private space for individual proteins. Here, we review recent advances in our understanding of protein crowding in membrane systems. We first give a brief overview on state-of-the-art approaches in experiment and simulation that are frequently used to study crowded membranes. After that, we review how crowding can affect diffusive transport of proteins and lipids in membrane systems. Next, we discuss lipid and protein sorting in crowded membrane systems, including effects like protein cluster formation, phase segregation, and lipid droplet formation. Subsequently, we highlight recent progress in uncovering crowding-induced conformational changes of membranes, e.g. membrane budding and vesicle formation. Finally, we give a short outlook on potential future developments in the field of crowded membrane systems. This article is part of a Special Issue entitled: Biosimulations edited by Ilpo Vattulainen and Tomasz Róg.

1. Introduction

Membranes are the defining envelopes of cells and intracellular compartments in eukaryotes. Acting as tunable barriers between reaction spaces but also as meeting point for vital biochemical reactions, cellular membranes appear to be indispensible for the emergence of advanced organisms. Essential ingredients for membranes are lipids that spontaneously assemble into a bilayer in an aqueous solvent [1]. While artificial lipid systems in the test tube mostly are composed of only a few lipid species, biomembranes typically consist of some hundred different lipid species that often are distributed asymmetrically between both leaflets of the bilayer [2]. Hence, even without the influence of any protein one can observe non-trivial membrane shapes and domain formation [3,4] that can provide first clues about observations in cellular membrane systems. In a cell, however, lipid bilayers hardly occur 'naked'. About one third of a mammalian cell's proteome is predicted to be membrane-associated proteins [1,5]. Expression levels are indeed so high that membrane proteins contribute about 50% of the mass of a typical mammalian plasma membrane and the protein-to-lipid number ratio is about 1 : 50 [2]. It is estimated that membrane proteins occupy 30-50% of the membrane area, meaning that each protein has only a few nanometers of private space on a membrane [6]. Therefore, the long anticipated view of a large lipid sea around few proteins has overturned and biomembranes appear today "more mosaic than fluid" [7].

It is worth noting that cellular fluids are also highly crowded with concentrations of macromolecules reaching up to 400mg/ml [8]. Here, the term 'crowded' is typically used instead of 'dense' or 'concentrated' to highlight that not only a single molecular species but rather a variety of macromolecules including lipids, proteins and higher-order complexes is considered. As a consequence of crowding, biochemical reactions are frequently altered due to excluded-volume effects (see, for example, [9] for an extensive review). Moreover, diffusive transport is slowed down [10] and may even become anomalous on intermediate length and time scales [11,12] as evidenced by a nonlinear scaling of the mean squared displacement, $MSD \sim t^{\alpha}$. Relating the diffusion anomaly $\alpha < 1$ to a crowding-induced viscoelasticity of the fluid, even an enhancement of excluded-volume effects on biochemical reactions is possible [13,14]. All these phenomena can be expected to hold true also for crowded membranes, yet membranes have an additional feature that makes them particularly interesting: They are two-dimensional manifolds embedded in three-dimensional space. Not only can a membrane sheet show smooth geometrical deformations due to protein crowding but it may even undergo topological changes, e.g. when pinching off a transport vesicle. Given the complexity of membrane traffic in living cells with a variety of coat proteins that support the making of transport intermediates on crowded biomembranes, an in-depth understanding of crowded membrane systems appears mandatory to gain insights into the physico-chemical basis of cellular membrane trafficking.

In fact, the scientific community is just about to explore the wealth of phenomena that emerge due to crowding on membrane systems. Therefore, we want to discuss here recent advances in our understanding of protein crowding in membrane systems with a particular focus on diffusive transport, segregation phenomena, and changes in membrane

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conformation. Before highlighting recent advances in our understanding of these topics, we will give a brief overview of experimental and theoretical approaches that are state-of-the-art tools for studying crowded membrane systems.

2. Methods to study crowded membrane systems

2.1. Experimental approaches

Effects of protein crowding on membranes have been studied *in vivo* and *in vitro* with a variety of experimental techniques. Concomitant to the dynamic progress in labeling techniques and modern optics, fluorescence microscopy methods have emerged as versatile and perhaps most potent tools in this field during the last decade. Besides quantitative imaging methods more advanced approaches like fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), fluorescence lifetime imaging microscopy (FLIM), and fluorescence resonance energy transfer (FRET) have provided deep insights into the secret life of crowded membranes in bacteria and eukaryotes as well as in model systems like giant unilamellar vesicles (GUVs) and supported lipid bilayers. In the interest of brevity we will refrain from reviewing the multitude of modern labeling techniques that have paved the way for modern fluorescence microscopy approaches. Suffices to state that labeling of living specimen with fluorescent proteins in all colors of the rainbow with different photophysical properties is by now a straight-forward approach [15]. Also smaller dyes that can be linked specifically to lipids and proteins at various positions without introducing major perturbations have been engineered [16] and support the application of quantitative fluorescence methods.

After having labeled the membrane system of interest, a first readout that can be achieved easily with light microscopy is certainly a simple image. Due to the two-dimensional character and an often almost flat arrangement of extended membrane systems, even classical epi-fluorescence microscopy may already reveal sufficient details to observe the segregation of lipids and/or proteins. A prime example in this context is the emergence of membrane domains on GUVs with a ternary lipid composition in the fluid-fluid coexistence regime [4] that can be readily observed by epi-fluorescence microscopy. Confocal laser scanning microscopy and its sibling, spinning disc confocal microscopy, go beyond this approach as they allow one to also resolve the three-dimensional arrangement of membranes via optical sectioning. As a result, three-dimensional images with a typical voxel volume of less than a femtoliter can be acquired routinely with commercial instruments with a temporal resolution of some 100ms. Hence, membrane deformations (induced, for example, by protein crowding) can be observed in excellent spatiotemporal resolution, albeit still being restricted by the diffraction limit. Super-resolution imaging approaches like STED, PALM, STORM etc. may even provide a spatial resolution down to some 10nm [17,18] yet mostly at an at-least equivalent loss in temporal resolution. It is therefore up to the researcher to find the best trade-off between spatial and temporal resolution for the particular membrane system that is to be studied.

Aiming at insights on the molecular level, mere imaging may soon become insufficient. Elucidating, for example, how diffusion of lipids and/or proteins is locally altered in crowded membrane systems requires considerably more effort. Here, a number of techniques, all equipped with certain features and limitations, are available. Tracking of single particles or molecules certainly is the most straightforward technique as it relies basically on imaging sparsely labeled samples. With reasonable effort, positions of particles can be determined with some 10nm accuracy with a temporal resolution in the millisecond range [19]. In principle, the trajectory yields all necessary information about the diffusion process and can be evaluated with advanced methods to reveal whether a certain stochastic process is underlying the molecule's motion (see [20] for a recent review). Yet, unavoidable uncertainties in determining the positions within the experimental trajectory may hamper this analysis: Determining an accurate position of molecules or particles requires the acquisition of some hundred photons, since the positional error depends on the reciprocal square-root of the number of photons. Yet, acquiring photons takes a finite time during which the particle continues its diffusional motion, i.e. the position becomes increasingly blurred the more time is spent for photon acquisition. The latter relates to an intrinsic problem of tracking approaches in that transport coefficients are determined from a spatially extended trajectory rather than from a tiny locus. For strongly heterogeneous systems, one might hence be confronted with systematic errors. Moreover, in particular for single-molecule tracking trajectory lengths are typically limited to some 10-100 positions so that ensemble averaging, and hence an even stronger loss of information on the local scale, is needed.

As an alternative to tracking approaches one may use FRAP or FCS which include averaging steps by their very definition [12]. In FRAP, the fluorescence in a region of interest is rapidly bleached and the recovery reports on the diffusive influx of non-bleached molecules into this region. By solving the diffusion equation with appropriate initial values and boundary conditions one can obtain fitting functions for the FRAP experiment that eventually reveal the ensemble average of the diffusion coefficient on length scales of the diffraction limit. In contrast, FCS is a less invasive method that avoids bleaching but only records the fluctuating fluorescence due to the diffusive walk of labeled molecules into and out of the focus. Using an autocorrelation function of the fluctuations, one can determine the local diffusion properties of the ensemble of molecules that has visited the diffraction-limited focus [12]. Using stimulated emission depletion in conjunction with FCS (STED-FCS) spatial resolutions well below the diffraction limit can be achieved [21], i.e. time and length scales of current simulation approaches (some 10μs and tens of nm) are within reach. Yet, similar to single-particle tracking, both FRAP and FCS are limited by experimental uncertainties. While evaluating FRAP curves may suffer from the inappropriate assumption that bleaching has been performed instantaneously [22], FCS curves often can be fitted equally well with several fitting functions that have been derived for different physical processes. An example for the latter is the ambiguity whether a superposition of two normally diffusing species has been observed or if it was rather one species that underwent anomalous diffusion [23]. Thus, due to their individual advantages and limitations, a combination of techniques and proper controls are essential when trying to elucidate transport processes in crowded membrane systems. With this caveat in mind the recent addition of high-speed atomic force microscopy as an alternative tool to quantify the mobility of unlabeled membrane proteins [24] is an important step forward.

Another question that often needs to be addressed when studying crowded membrane systems is the proximity of molecules and the formation of (transient) complexes. Here, FRET and FLIM have emerged as powerful methods. Probing whether a green-labeled protein can interact with another, red-labeled protein is straightforward with FRET/FLIM. Due to a dipole-dipole coupling, the excitation of the green dye may be transferred without radiation to the red dye if both molecules are located only few nanometers apart. Indeed, the dipole-dipole coupling decays with the sixth power of the distance, hence providing a means to measure nanometric distances and complex formation even with diffraction-limited fluorescence microscopy. Several variants for quantitative FRET are available with the sensitized-emission method being arguably the easiest and least error-prone [25]. On a par, yet more demanding in terms of the experimental setup is FLIM as it detects reliably the additional decay channel of the excited state of the green dye in the case of FRET [26]. Therefore, FRET/FLIM allows one to experimentally quantify complex formation and/or typical distances of proteins on crowded membranes with reasonable effort.