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Colloidal probe microscopy of membrane–membrane interactions: From ligand–receptor recognition to fusion events

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ABSTRACT

A versatile model system to study membrane–membrane interactions in great detail is introduced. Based on colloidal probe microscopy with membrane covered spherical probes attached to tip-less cantilevers the interaction forces and adhesion energies are quantified down to single molecule resolution. Two opposing membranes equipped with ligands on one side and receptors on the other side were brought in contact at a defined load and pulled apart at constant velocity. Ni-NTA functionalized lipids served as receptors on the probe, while lipopeptides displaying short His-tags ($CGGH_6$ or $CGWH_6$) were incorporated in the planar supporting membrane on a silicon substrate. The rather intricate force distance curves were scrutinized in terms of breakthrough events upon contact of the probe with the surface, the overall work of adhesion, maximum adhesion force, as well as formation frequency, lifetime, and force of membrane tethers suggesting that hemifusion of the two opposing bilayers takes place.

1. Introduction

The mechanics and dynamics of plasma membranes play a crucial role in many cellular events such as adhesion, motility, membrane fusion, as well as exo- and endocytosis of mammalian cells. Particularly, membrane–membrane interactions display great versatility since molecular recognition, deformation, adhesion, pore formation, and fusion of lipid bilayers might occur depending on an intricate interplay between proteins, lipids, and forces. Among the considerable spectrum of processes involving membrane–membrane interactions, the understanding of cell–cell contacts [1], and membrane fusion is of particular interest. Membrane fusion occurs when two separate lipid membranes merge into a single continuous bilayer and plays a crucial role in embryogenesis, neurophysiology as well as viral infection [2–5]. Regardless of the process, the sequence of events, which is driven by subtle changes in free energy [3,4], requires almost without exception an initial specific recognition that triggers the subsequent dynamic response of the bilayer. This specific recognition between ligands on one membrane and the receptor displayed by the corresponding counterpart produces the initial molecular connection between two membranes, which eventually determines the fate of the membrane–membrane assembly, i.e. aggregation, hemifusion or full fusion [2–4].

Model systems of cellular membranes such as liposomes or supported membranes provide an excellent resource to investigate these processes in a quantitative manner under controlled conditions and defined composition. Investigation of membrane–membrane interactions in solution, however, bears the inevitable problem of aggregate formation due to cross-linked liposomes. As a consequence, precipitation and increased light scattering perturb or prevent a quantitative spectroscopic analysis.

Fusion is modulated by a number of external stimuli which control the crucial distance of the two membranes from each other and the hydration of the membranes such as force, calcium content, pH or other means that draw water from the contact zone such as polyethylene glycol [3,4]. When bilayers are separated below 1 nm, the energy of repulsion due to the pervasion of the hydration shell is assumed to drive fusion. It is widely accepted that formation of a fusion stalk and its expansion into a hemifusion diaphragm relaxes the hydration energy and hence provides the necessary driving force. Forces and, as a consequence, distances between two membranes can either be tuned by changing the interaction potential using charged lipids or varying the electrolyte or more directly using force probes such as optical/magnetic tweezers [6,7], the atomic force microscope [8] or the surface force apparatus (SFA) [9,10]. With properly functionalized probes, these methods allow to control composition of the two bilayers and the interaction force. While the SFA has been frequently used to identify and quantify van-der-Waals, electrostatic, repulsive hydration, and steric forces, it has in recent years also been applied to study more complex biological assemblies including membrane–membrane interactions [11,12]. Israelachvili and coworkers could assign hemifusion and full fusion events of two DMPC bilayers immobilized on PEI/mica to instabilities in force distances curves

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[13,14]. Full fusion was reported to occur only at very high joining pressure.

The cumbersome handling of the surface force apparatus and the lack of local information, however, prevented abundant dissemination to date. Atomic force microscopy, on the other hand, provides local information with a lateral resolution in the nanometer regime paired with an accessible force regime ranging from 10^{-11} to 10^{-6} N [8,15]. The drawback of conventional sharp tips for functionalization with lipid bilayers is their high curvature and therefore the difficulty to accomplish a successful functionalization. Particularly, the often undefined geometry of the tip renders conventional AFM cantilevers unsuitable to quantify membrane–membrane interactions.

Colloidal probe microscopy (CPM), a recent offspring of atomic force microscopy, combines the merits of a defined probe geometry and controllable surface functionalization with the potentiality to conduct site specific analysis of force distance curves [16,17]. In CPM a spherical particle is glued to a tip-less AFM cantilever essentially replacing the pyramidal tip with a spherical probe. The dimensions of the spherical (colloidal) particle are usually in between 1 and 20 μm in diameter. While CPM is frequently used for surface (friction) analysis under defined conditions, less applications are found in life sciences. Recently, Moy et al. investigated SNARE mediated fusion by membrane–protein functionalized surfaces and colloidal probes [18]. The authors interpret their results in terms of SNARE mediated full fusion of two opposing bilayers due to two consecutive instabilities observed in the approach curve. Previously, we established a setup that allows to determine the adhesion forces between the cytoskeleton and an ezrin covered membrane revealing a new route to ezrin activation via receptor binding [19].

Here, we report on a universal setup based on membrane covered silicon particles attached to tip-less cantilevers probing a solid supported membrane (SSM). The opposing membranes are either equipped with ligands or receptors allowing to study membrane–membrane interaction from the initial contact and formation of a non-covalent linkage to a possible (hemi)fusion and finally the dynamic action of the bilayers upon retraction creating membrane tethers that display the inherent mechanics of the two joint bilayers.

In our setup the colloidal probe with a diameter of 15 μm is coated with a phosphocholine membrane doped with phospholipids possessing a nitrilotriacetic acid headgroup (NTA) referred to as DXPC/Ni-NTA-DOGS capable of establishing strong non-covalent complexes with histidine oligomers in the presence of Ni^{2+}. Bilayers (DPPC/MCC-DPPE) deposited on flat silicon surfaces are functionalized with short His-tag moieties ($CGGH_6$ or $CGWH_6$) using recently introduced in situ coupling strategies [20–22]. By performing force–distance curves, which essentially brings the two bilayers in contact, we propose to induce hemifusion of the two membranes inferring from the occurrence of bilayer breakthrough events in the approach curve. After a short dwell time on the surface we move the cantilever backwards to separate the two membranes measuring the bonds that have been formed during the close proximity of the two membranes. Besides a typical adhesion force close to contact, we also observe formation of multiple tethers that exhibit lifetimes suggesting formation of lipid bridges.

2. Methods

Colloidal probe cantilevers were prepared by attaching a borosilicate glass microsphere ($Ø = (15 \pm 1)$ μm, Duke Scientific, Waltham, MA, USA) to an MLCT-C cantilever (Veeco, Santa Barbara, CA, USA) using epoxy resin (Epikote 1004, Shell, Hamburg, Germany). Attachment was monitored and carried out by using an upright light microscope with a 20× magnification lens equipped with a micromanipulator and a special device to attach the cantilever (MMO-203, Narishige, Tokyo, Japan). Prior to bilayer preparation on the colloidal probe, the cantilevers were cleaned in an argon plasma for 1 min.

Silicon wafers were cleaned by rinsing them with chloroform, isopropanol, and deionized water. Removal of native SiO_2 and controlled re-oxidation of the silicon wafer to an approximate oxide layer thickness of 3 nm was performed by using 1% hydrofluoric acid solution (15 min, room temperature (RT)), and an aqueous solution of ammonia and hydrogen peroxide (5:1:1; 15 min, 70 °C), respectively. For all measurements, unilamellar vesicles (SUVs) were prepared by extrusion using phosphate buffer (PB; Na_2HPO_4, 50 mM), pH 5.9 for lipid mixtures containing maleimide-functionalized vesicles, and pH 6.8 for Ni-NTA containing vesicles. SUVs were obtained by passing a suspension of multilamellar vesicles 31 times through a single polycarbonate membrane (pore diameter: 50 nm, Avestin, Mannheim, Germany). Lipid bilayers were prepared by incubating the SUV suspension on the substrates at RT followed by incubation above the corresponding main phase transition temperature (T_M) of the lipid, ($T_{M,DOPC} = -20$ °C, $T_{M,DPPC} = 41.5$ °C [23]; $c_{Lipid} = 0.1$ mg/ml of respective buffer, 30 min each step) and by thorough rinsing of the bilayer. For DOPC vesicles on the colloidal probe, incubation time was reduced to 15 min at RT. Planar bilayer formation and coverage of substrates was controlled by (confocal laser) fluorescence microscopy and atomic force microscopy imaging.

Lipid bilayers as spread on the colloidal probes were composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl] (nickel salt) (Ni-NTA-DOGS) and 2-(4.4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY C12-HPC, Invitrogen, Karlsruhe, Germany), in a molar ratio of 89:10:1. For functionalization of the flat silicon wafers, we used 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as matrix lipid, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC-DPPE) in a concentration of 10 mol% as attachment site for the His-tag. In the case of DOPC as the matrix lipid MCC-DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide) was used instead. Maleimide-functionalized DPPC bilayers were then incubated with acetylated $CGGH_6$ for 30 min at RT. $CGGH_6$-Ac ($M = 1100$ g/mol) was synthesized via Merryfield solid phase synthesis with Fmoc chemistry, purified via IEC and HPLC, and characterized by MALDI-ToF [22].

For force–distance measurements we used a commercial atomic force microscope (MFP3D, Asylum Research, Santa Barbara, CA, USA) and the above mentioned cantilevers with a nominal spring constant of 0.01 N/m. Spring constants were calibrated by using the thermal noise method and found to be approximately 0.03 N/m. Force–distance cycles were performed with a sample rate of 12.5 kHz, varying force load, dwell times, and pulling velocities. All measurements were performed in 50 mM phosphate buffer (pH = 6.8, RT) in a home-made measuring chamber. The integrity of lipid bilayers after force–distance measurements was controlled by fluorescence microscopy.

Liposome spreading and various subsequent binding events were also analyzed by extracting frequency and dissipation signals from quartz crystal microbalance measurements. We used a 5 MHz quartz with silicon dioxide coated electrodes (Qsense, Kista, Sweden). Prior to measurements, the quartz crystals were hydrophilized in oxygen plasma for 3 min. Measurements were performed in PB at 25 °C and vesicle concentrations of 0.1 mg/ml were applied. Vesicles consisting of DOPC with 10 mol% of MCC-DOPE and Ni-NTA-DOGS were spread on the resonators. Concentration of $CGWH_6$ for QCM-D measurements was 0.1 μmol/ml PB (pH = 6.8).

Texas Red-self-quenching experiments were carried out with DOPC vesicles (Ø = 50 nm) functionalized with 2 mol% MCC-DOPE, 10 mol% Texas Red DHPE (Sulforhodamine 101 DHPE, Sigma-Aldrich, Seelze, Germany) (population 1) or 2 mol% Ni-NTA-DOGS (population 2). Fluorescence spectroscopy with an excitation wavelength of