Biochimica et Biophysica Acta 1859 (2017) 1242–1253

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Choosing the right fluorophore for single-molecule fluorescence studies in a lipid environment

Zhenfu Zhang, Dan Yomo, Claudiu Gradinaru *

Department of Chemical and Physical Sciences, University of Toronto Mississauga, Mississauga, Ontario L5L 1C6, Canada
Department of Physics, University of Toronto, Toronto, Ontario M5S 1A7, Canada

ARTICLE INFO

Article history:
Received 30 December 2016
Received in revised form 14 March 2017
Accepted 5 April 2017
Available online 06 April 2017

Keywords:
Adsorption
Lipids
Fluorescent dyes
FCS
Single-molecule spectroscopy

ABSTRACT

Nonspecific interactions between lipids and fluorophores can alter the outcomes of single-molecule spectroscopy of membrane proteins in live cells, liposomes or lipid nanodiscs and of cytosolic proteins encapsulated in liposomes or tethered to supported lipid bilayers. To gain insight into these effects, we examined interactions between 9 dyes that are commonly used as labels for single-molecule fluorescence (SMF) and 6 standard lipids including cationic, zwitterionic and anionic types. The diffusion coefficients of dyes in the absence and presence of set amounts of lipid vesicles were measured by fluorescence correlation spectroscopy (FCS). The partition coefficients and the free energies of partitioning for different fluorophore-lipid pairs were obtained by global fitting of the titration FCS curves. Lipids with different charges, head groups and degrees of chain saturation were investigated, and interactions with dyes are discussed in terms of hydrophobic, electrostatic and steric contributions. Fluorescence imaging of individual fluorophores adsorbed on supported lipid bilayers provides visualization and additional quantification of the strength of dye-lipid interaction in the context of single-molecule measurements. By dissecting fluorophore-lipid interactions, our study provides new insights into setting up single-molecule fluorescence spectroscopy experiments with minimal interference from interactions between fluorescent labels and lipids in the environment.

1. Introduction

Lipids play a major role for determining the structure and the function of membrane proteins; as such, lipid-protein interactions have been widely studied using biophysical, biochemical and computational approaches [1–4]. However, the mechanisms by which the cell membrane modulates the structure, the dynamics and the function of large families of transmembrane proteins, e.g., G protein coupled receptors, to sustain important cellular processes, e.g., signaling, are still not fully understood [3]. With the development of various fluorescence microscopy and spectroscopy techniques and the advent of brighter and photostable dye labels, investigations of protein-lipid interactions using fluorescently labeled proteins have become common [5–8]. This enabled quantitative studies of conformations and dynamics of proteins in lipid vesicles, lipid nanodiscs and in live cells via fluorescence techniques [9–12].

Haran and co-workers pioneered the encapsulation of cytosolic proteins within small (~100 nm diameter) lipid vesicles for single-molecule fluorescence (SMF) studies [9]. These vesicles were immobilized on surfaces via the biotin-avidin interaction. This immobilization-confinement scheme helped prevent and control non-specific protein-surface interactions in experiments requiring extended observation times of single-molecule fluorescence. Subsequently, trapping fluorescently labelled proteins into lipid vesicles to study folding states, conformational dynamics and binding interactions became increasingly popular [10,13–16]. Recently, the vesicle encapsulation protocol was also implemented for studying the conformations of intrinsically disordered proteins [17].

Single-molecule methods have the unique feature to identify heterogeneous, rare and transient states of biological systems. However, one of the frequently raised questions is about the role of the fluorescence labels. Chemically-conjugated fluorophores may exhibit non-specific interactions with the protein of interest or with the immediate environment. This may include the lipid membrane in the studies of membrane proteins and of soluble proteins encapsulated within lipid vesicles. We refer to non-specific interactions as any interactions, typically non-covalent, that introduce unwanted artifacts in SMF experiments. These could be π-π interactions between the aromatic ring(s) of the fluorophore and those from amino acids in the protein, hydrophobic interactions between the fluorophore and hydrophobic residues of the protein, or hydrophobic/electrostatic interactions between the fluorophores and the lipids in the environment.

* Corresponding author at: Department of Chemical and Physical Sciences, University of Toronto Mississauga, Mississauga, Ontario L5L 1C6, Canada.
E-mail address: claudiu.gradinaru@utoronto.ca (C. Gradinaru).

http://dx.doi.org/10.1016/j.bbamem.2017.04.001
0005-2736/

These effects must be carefully measured and well understood using proper controls, and a cautionary approach must be taken when analyzing and interpreting the SMF experimental data. Molecular dynamics simulations were performed for two commonly used fluorophores (Alexa 488 and Alexa 594) to address the fluorophore-protein interaction [18]. The vesicle-fluorophore dialysis method [19] has been used to characterize the fluorophore-lipid interaction. However, a systematic quantification of the interaction between popular SMF fluorophores and various type of lipids has not been done using single-molecule techniques.

Here we focus on measuring the interaction between fluorophores and lipids. We used nine commonly used fluorophores in SMF studies and six different lipids that are widely used for making lipid vesicles. The fluorophores have different chemical structures, electrostatic charges and hydrophobicity; the lipids selected also have different charges, head groups and degrees of chain saturation.

Fluorescence correlation spectroscopy (FCS) was used to quantify the partition of each fluorophore into lipid membranes. FCS is a technique based on the analysis of fluorescence intensity fluctuations caused by molecules diffusing in and out of a small (~fL) sized sampling volume [20]. FCS is a powerful tool to measure local concentration, translational diffusion and chemical reactions [20–22]. Since large unilamellar vesicles (LUVs) are much larger than the typical size of a fluorophore, FCS is an ideal tool to resolve the diffusion times of free and vesicle-bound fluorophores. The data analysis thus provides accurate estimations of lipid-bound fractions even when the lipid-fluorophore interaction is very weak. A similar approach has been applied to study the interaction between small peptides or proteins and lipid vesicles [23–26].

In addition, total-internal reflection fluorescence (TIRF) imaging of individual fluorophores adsorbed on supported lipid bilayers (SLBs) provided visualization and an independent quantification of the interaction between fluorophores and lipid bilayers. This study provides helpful insights about selecting optimal fluorophore-lipid pairs to study the conformations of proteins trapped in lipid vesicles and the interactions between proteins and lipids at single-molecule resolution.

2. Experimental section

2.1. Materials

Alexa dyes were purchased from Thermo Fisher Scientific (A488 (cat. # A10254) and A647 (cat. # A20347)), Atto dyes from ATTO-TEC (Atto 488 (cat. # AD 488-41), Atto 532 (cat. # AD 532-41), Atto 565 (cat. # AD 565-41), Atto594 (cat. # AD 594-41) and Atto 647N (cat. # AD 647-41)), fluorescein (FL) from Sigma-Aldrich (cat. # F2456) and Cyanine 5 (Cy5) was purchased from Lumiprobe (cat. # 23080). All the fluorophores except FL have a maleimide conjugation group, which is used for thiol chemistry to label cysteine residues in the proteins. The original dye powders were dissolved in anhydrous dimethyl sulfoxide (DMSO) to 20 mM stock solutions. A small volume from the stock solution was diluted to nanomolar concentrations into a phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2SO_4, 1.8 mM KH_2PO_4, pH 7.4) for FCS experiments. The final concentration of the fluorophore was confirmed by FCS analysis prior to each lipid-fluorophore interaction series to guarantee that fluorophore concentrations in all FCS experiments were identical, unless stated otherwise. All FCS experiments were performed under fluorophore-hydrolysis conditions, in which the fluorophores were kept at 4 °C for 3 days after dilution from the stock solutions to PBS buffer, in order to mimic the conditions of real single-molecule experiments.

The fluorescent lipid Bodipy-FL C5 was purchased from Thermo Fisher Scientific (cat. # D3834). The lipids, i.e., 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (cat. # 850375), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) (cat. # 840475), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) (cat. # 840035), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (cat. # 850457), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (cat. # 850355), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (cat. # 890890), and the cholesterol (CHOL) (cat. # 700156) were purchased from Avanti Polar Lipids. Each lipid was dissolved in 95% chloroform and 5% methanol to be 20 mg/mL, unless stated otherwise. All phospholipid concentrations were assessed via a phosphorus assay [27,28]. All lipid solutions were stored in parafilm-wrapped glass vials at −20 °C.

2.2. Sample preparation

A desired lipid quantity from the lipid stock was transferred to a new vial. The lipid solution was dried using nitrogen gas to form lipid films and was left in a vacuum desiccator overnight to remove residual chloroform and methanol. The lipid films were hydrated for 2 h in PBS. Large unilamellar vesicles (LUVs) with average diameters around 50 nm and around 100 nm were prepared using the extrusion method [29]. Using a Mini-Extruder apparatus (cat. # 610000, Avanti Polar Lipids), the hydrated vesicle solution was pushed 35 times through a porous polycarbonate membrane filter with a pore diameter of 50 nm or 100 nm. DPPC vesicles were extruded at temperature of around 55 °C. The other lipid vesicles were prepared at room temperature. Fluorescent LUVs were prepared by mixing 0.01 mol% Bodipy-FL lipid with DOPC. The vesicle size was measured by dynamic light scattering (DLS). When using the 50-nm membrane filter, the average diameter for DOPC vesicles measured by DLS was 60.0 ± 4.4 nm. When using the 100-nm filter, the average diameters for DOTAP, POPC, DOPC, DPPC, DOPS and DOPG LUVs were 104.3 ± 10.9 nm, 118.9 ± 7.0 nm, 115.6 ± 11.1 nm, 114.8 ± 5.8 nm, 110.0 ± 14.8 nm, 108.3 ± 19.7 nm, respectively. Here, the error margins were derived from the standard deviation of several mean values from repeat measurements. The highest lipid stock concentration prepared in PBS buffer was 80 mg/mL. The lipid vesicles were stored at 4 °C and were used within 3 days after preparation.

Lipid bilayers were formed by sandwiching a solution containing 10 mg/mL DOPC vesicles in a PBS buffer containing 4 mM $CaCl_2$ between closely spaced plasma-cleaned coverslips. After incubation for about 2 h at room temperature, the coverslips were separated and rinsed with PBS buffer thoroughly [30]. The formation of supported lipid bilayer (SLB) on the glass coverslip was verified by fluorescence recovery after photobleaching (FRAP) experiments using a mixture of 90 mol% DOPC and 10 mol% Bodipy-FL lipids (Movie S1, Supporting Information (SI)). In single-molecule imaging experiments, 50 μL fluorophore solutions were incubated on SLBs for ~10 min in the dark. Subsequently, the SLBs were rinsed at least 3 times with PBS buffer and then incubated with a 100 mL PBS buffer solution for imaging on a TIRF microscope.

Polyethylene glycol (PEG) was used to coat microscope coverslips according to a published protocol [16]. Briefly, the coverslips (cat. # 48366-249-1, VWR) were rinsed and sonicated for 30 min in each step in the following sequence: i) Milli-Q water, ii) methanol (cat. # 34860-1L-R, Sigma Aldrich), iii) acetone (cat. # 270725-1L, Sigma Aldrich), iv) fresh 1 M KOH solution. The coverslips were then rinsed with Milli-Q water and dried with argon gas. A fresh 230 g/L m-PEG-silane (cat. # mPEG-silane, MW 5000, Lysan Bio.) solution in 10 mM sodium bicarbonate buffer (pH 8.5) was used to coat the coverslips. Incubation was performed in the dark overnight in a humidified PEG incubation container at room temperature. After incubation, each coverslip was washed by 10 mM sodium bicarbonate buffer, followed by Milli-Q water and then dried with argon gas. The PEG-coated coverslips were stored in a vacuum desiccator in the dark before use. The mixture of fluorophore and lipid solution was first incubated for 5 min and then dropped onto the PEG-coated coverslip to prevent non-specific adsorption during FCS experiments.