Colloids and Surfaces B: Biointerfaces 161 (2018) 375–385

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Protocols

Study of rabbit erythrocytes membrane solubilization by sucrose monomyristate using laurdan and phasor analysis

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ARTICLE INFO

Article history:
Received 5 August 2017
Received in revised form 6 October 2017
Accepted 30 October 2017
Available online 1 November 2017

Keywords:
FLIM phasor
Spectral phasor
Laurdan GP
Solubilization
Surfactants
Sucrose ester
Membrane heterogeneity
Membrane fluidity

ABSTRACT

The study of surfactant and bio membranes interaction is particularly complex due to the diversity in lipid composition and the presence of proteins in natural membranes. Even more difficult is the study of this interaction in vivo since cellular damage may complicate the interpretation of the results, therefore for most of the studies in this field either artificial or model systems are used. One of the model system most used to study biomembranes are erythrocytes due to their relatively simple structure (they lack nuclei and organelles having only the plasma membrane), their convenient experimental manipulation and availability.

In this context, we used rabbit erythrocytes as a model membrane and Laurdan (6-lauroyl-2-dimethylaminonaphthalene) as the fluorescent probe to study changes promoted in the membrane by the interaction with the sucrose monoester of myristic acid, β-D-fructofuranosyl-6-*O*-myristoyl-α-D-glucopyranoside (MMS). Surfactant and erythrocytes interaction was studied by measuring hemoglobin release and the changes in water content in the membrane sensed by Laurdan. Using two-photon excitation, three types of measurements were performed: Generalized Polarization (analyzed as average GP values), Fluorescence Lifetime Imaging, FLIM (analyzed using phasor plots) and Spectral imaging (analyzed using spectral phasor). Our data indicate that at sublytical concentration of surfactant (20 μM MMS), there is a decrease of about 35% in erythrocytes size, without changes in Laurdan lifetime or emission spectra. We also demonstrate that as hemolysis progress, Laurdan lifetime increased due to the decrease in hemoglobin (strong quencher of Laurdan emission) content inside the erythrocytes. Under these conditions, Laurdan spectral phasor analyses can extract the information on the water content in the membrane in the presence of hemoglobin. Our results indicate an increase in membrane fluidity in presence of MMS.

1. Introduction

Solubilization of membranes by surfactants is a well-known method used to understand the nature and properties of the cellular bilayer and its components. The use of micelle-forming surfactants is one of the preferred methods for isolation of specific membrane constituents [1–3]. Several articles in the literature describe in detail the interaction of surfactants with artificial membranes [4–7]; however, this is not the case concerning the interaction of surfactants with biological membranes, where the lipid composition diversity and the presence of proteins make the scenario more complex.

Biological bilayers are complex, with membrane lateral heterogeneity being accepted as a requirement for their function and the notion of lipid rafts supporting that concept [8]. The operational definition of rafts came from the observation of membrane resistance to solubilization with nonionic detergents like Triton X-100 at 4 °C. Since in these assays a fraction of the membranes remained insoluble, the suggestion was made that these detergent-resistant membranes (DRMs) corresponded to supramolecular entities floating in fluid biological membranes as *rafts* [9]. However, it is commonly accepted that DRMs are produced by the reorganiza-

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https://doi.org/10.1016/j.colsurfb.2017.10.068
0927-7765/

tion of lipids and proteins upon the addition of detergent and they do not correspond to rafts in vivo [10,11]. Because of these observations, the concepts of rafts and DRMs should be used with extreme caution [12]. The actual definition of *rafts* correspond to structures existing in vivo that are sterol and sphingolipid-enriched, with small size (10–200 nm), heterogeneous and highly dynamic [13]. The solubilization process in bio membranes may be different depending on the state of this structures, in fact, the presence of cholesterol in lipid mixtures makes the bilayers more resistant to solubilization [14], and in equimolar mixtures with phosphatidylcholine, cholesterol decreased the stability of the bilayer towards Triton X-100 [15].

Erythrocytes are the most commonly used model system to study bio membranes due to their relatively simple structure: they lack nuclei and organelles, having only the plasma membrane. However, the presence of membrane lipid domains in erythrocytes has been reported using different approaches [16–20]. In studies involving erythrocyte/surfactant interaction, two parameters are normally studied: permeability and structural changes of the membrane. Permeability changes are followed by hemoglobin release, and membrane structural properties are monitored by different techniques such as fluorescence of specific dyes [21,22], spin labeling [23], scanning calorimetry, NMR and Fourier-transform infrared spectroscopy [24] among others. In erythrocytes, membrane solubilization may occur in parallel with hemolysis. Preté et al. reported [25] the use of the hemolysis curve (obtained from the interaction of Triton X-100 with erythrocytes) to obtain membrane structural parameters by applying the analysis of membrane solubilization proposed by Lichtenberg [26–28]. Thus, the beginning of hemolysis would be an indication of achievement of bilayer saturation (C^{sat}) and the end of hemolysis would correspond to the concentration of detergent for total membrane solubilization (C^{sol}).

In the present study, we report the interaction of rabbit erythrocytes with the sucrose monoester, β-D-fructofuranosyl-6-*O*-myristyl-α-D-glucopyranoside (Mono myristoyl sucrose, MMS) using two-photon excitation fluorescent microscopy. Sucrose esters correspond to a class of nonionic detergents that are non-toxic, skin compatible, non-polluting and biodegradable. Derivatives of stearic acid had been employed for the extraction of cytochrome and lysozyme [29–31] and their capability to solubilize different synthetic membranes has been reported by our group [2,6]. As a fluorescent dye we used Laurdan ((6-lauroyl-2-dimethylaminonaphthalene)), a molecule widely used to study membrane properties both in artificial [32,33] and natural systems, in cuvette [7,22] and under the microscope [2,34]. Laurdan Lifetime and spectral images were analyzed using the phasor method [35–37]. We followed hemoglobin release and changes in the membrane properties of erythrocytes after interaction with MMS. Hemoglobin release was measured by colorimetric and biochemical methods and membrane properties were observed by bright-field and two-photon excitation fluorescence microscopy.

2. Material and methods

2.1. Samples

Rabbit blood samples were purchased from Colorado Serum Company (Denver, CO, USA). Sucrose monoester, β-D-fructofuranosyl-6-*O*-myristyl-α-D-glucopyranoside (MMS, Mono myristyl sucrose, C14) was synthesized by a modification of the Vlahov method [38] where the relation between sucrose complex and acyl chloride was changed from two to one. The reaction yields a mixture of monoesters (mainly 6-O and presumably a small quantity of 1-O), accompanied with low amounts of di- and tri-esters. Pure 6-O monoester was obtained by chromatography on silica column. For solubilization experiments with erythrocytes, a stock solution of MMS in PBS-saline buffer (10 mM Na_2PO_4 pH 7.5, containing 147 mM NaCl and 3 mM KCl) was prepared. Laurdan was purchased from Invitrogen (Thermos Fisher Scientific).

2.2. Preparation of erythrocytes and ghosts

Freshly defibrinated rabbit blood (Colorado Company) was used in all the solubilization experiment. Blood, received the same day it was drawn, was stored at 4 °C and the whole erythrocyte experiments were performed the same day. Rabbit erythrocytes (RRBC), were centrifuged at 4000 rpm (1800*g*), rinsed and suspended to the original hematocrit (40% vol/vol) in PBS-saline buffer. Erythrocytes (at final hematocrit of 5% vol/vol) were incubated with different concentrations of the detergent in PBS for 1 h at 37 °C. After incubation, erythrocytes were centrifuged at 4000 rpm (1800*g*), and hemoglobin determination (absorbance at 410 nm) and protein released from the membrane (gradient 5–20% native PAGE) were determined in the supernatant. Pellet was rinsed three times with PBS for the fluorescence imaging measurements. Detergent-treated erythrocytes were diluted with PBS to hematocrit 0.2% vol/vol and incubated with 1 μM Laurdan for 30 min at 37 °C. For microscope observation, samples were deposited in a microscope dish coated with poly-lysine (MatTek Co. Ashland, MA, USA) to allow the adhesion of the erythrocytes to the dish. Measurements under the microscope (fluorescence and transmission) were obtained at 37 °C in a temperature-controlled stage.

Ghost erythrocytes were prepared using a modified protocol published by Dodge et al. [20]. Briefly, erythrocytes were lysed with an hypotonic buffer (1 mM PBS pH 7.4) during 30 min at 4 °C. Then ghost membranes were separated by centrifugation at 14,000 rpm (22000*g*) for 10 min at 4 °C. This procedure was repeated until the samples were free of hemoglobin.

2.3. SDS – PAGE to study protein released from treated-erythrocytes membranes with MMS

Fifteen μl of the supernatant obtained after centrifugation of treated-RRBC with different MMS were analyzed by 5–20% (w/v) gradient SDS-PAGE. Proteins were stained with Coomassie blue R-250 [39].

2.4. Hemolysis curve

The hemolysis curve of erythrocytes treated with different concentrations of MSS was obtained by measuring hemoglobin content in the supernatant. Absorbance at 412 nm of supernatant was measured in a Shimadzu, UV- Spectrophotometer, UV-1800. Data was normalized to hemoglobin content in samples treated with 100 μM MMS.

2.5. Changes in membrane properties using 2-photon excitation microscopy

The Laurdan molecule possesses a *dipole moment* (due to the partial charge separation at the naphthalene head) at the ground state. This dipole moment increases after excitation and the reorganization of the solvent molecules around the Laurdan molecule is responsible for the shift of the emission spectra towards longer wavelengths. In lipid bilayer, Laurdan is used to detect membrane heterogeneity since its emission spectrum depends on the phase state of the bilayer: the spectrum presents a maximum at 440 nm when the phospholipids are in gel phase and a maximum at 490 nm when they are in liquid/fluid phase. The naphthalene moiety of Laurdan locates in the membrane at the level of the glycerol backbone of the phospholipids and the shift of the emission spectrum