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Nanomechanical properties of composite protein networks of erythroid membranes at lipid surfaces

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ABSTRACT

Erythrocyte membranes have been particularly useful as a model for studies of membrane structure and mechanics. Native erythroid membranes can be electroformed as giant unilamellar vesicles (eGUVs). In the presence of ATP, the erythroid membrane proteins of eGUVs rearrange into protein networks at the microscale. Here, we present a detailed nanomechanical study of individual protein microfilaments forming the protein networks of eGUVs when spread on supporting surfaces. Using Peak Force tapping Atomic Force Microscopy (PF-AFM) in liquid environment we have obtained the mechanical maps of the composite lipid-protein networks supported on solid surface. In the absence of ATP, the protein pool was characterized by a Young's Modulus $E_{pool} \approx 5–15$ MPa whereas the complex filaments were found softer after protein supramolecular rearrangement; $E_{fil} \approx 0.4$ MPa. The observed protein softening and reassembling could be relevant for understanding the mechanisms of cytoskeleton reorganization found in pathological erythrocytes or erythrocytes that are affected by biological agents.

1. Introduction

Red Blood Cells (RBCs) contain a high concentration of hemoglobin, a carrier protein that transports molecular oxygen from the lungs to the tissues. During oxygen transport, RBCs undergo large stretching and shear deformations. The membrane skeleton of RBCs is a supramolecular structure that endows these cells with sufficient mechanical resilience to undergo deformation without failure in service conditions.

The native erythroid membrane is composed of lipids and a skeletal protein network. This protein network is formed by flexible spectrin filaments of 50 nm length that are cross-linked by junctional complexes [1] that contain short actin filaments (typically, 35 nm in length), which are capped by regulation proteins. Early electron microscopy (EM) studies with stretched membrane patches revealed a two-dimensional lattice made of straight spectrin filaments that intersect at the junctional complexes forming a nearly-hexagonal planar network [2,3] Immunolocalization of tropomodulin, tropomyosin and actin in spread human erythrocyte skeletons. The recent use of cryo-TEM tomography allowed the imaging of the three-dimensional structure of the RBC cytoskeleton in its intact native state [4]. Those studies show the presence of a tridimensional foam-like densely packed meshwork of entangled filaments instead of the quasi-regular 2D-lattice seen in the stretched membrane patches. Structural alteration due to the presence of mutated proteins in the network result in alterations of RBC deformability and plasticity, a common feature of numerous erythroid diseases as spherocytosis, sickle cell anemia and Fanconi's anemia among others [5–7].

The nanostructural characterisation of dry individual filaments of erythrocyte cytoskeleton became available with the development of single molecule techniques as Atomic Force Microscopy (AFM) [8–10]. Nonetheless, AFM turned out be a highly versatile tool to investigate biological membranes under physiological wet conditions [11,12]. AFM structural studies on native erythroid membranes have shown both classes of structures, well-defined polymerized networks [13,14] that resemble the topology of the regular skeleton revealed from EM and, alternatively, disordered

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assemblies that are constituted by unstructured protein complexes [15,16]. Reorganization of the erythroid cytoskeleton associated to biochemical or biological factors including Ca^{2+} concentration [17,18], phosphorylation state [16], mutations in cytoskeletal proteins [19] or parasite infection [20] have been also recorded using AFM. A significant amount of network rearrangement and protein aggregation occurred under those conditions, including both increased spectrin length and mesh size. Furthermore, high-resolution force distance based atomic force microscopy allows the biophysical characterization of protein structures [21], like individual filaments of polymerizing erythroid proteins [22], and the quantitative measurement of the nano-mechanical characteristics of the cytoskeleton components, to correlate local stiffness with the phosphorylation state [16].

Microscopic spectrin networks can be artificially reconstructed on giant vesicles made of native membrane of human erythroid cells (erythroGUVs) [23]. eGUVs can adsorb onto glass cover slides for surface characterization. In this work, taking advantage of AFM combined with fluorescence microscopy imaging, we map the topology and the intrinsic stiffness of erythroid membrane skeletons supported on lipid surfaces. We have differentially unveiled the polymerization and networking of actin-spectrin composite filaments of the artificially reconstructed erythroid networks using AFM in Amplitude and Peak Force modulated modes. The superposition of topographic and nano-mechanical maps allowed us to correlate structure and mechanics of the different single filaments of the network. Our results deliver new insights to better understand the mechanism that underlies the important question of cytoskeleton organization and remodeling when erythrocytes are affected by age, disease (sickle cell anemia, diabetes) or external pathogenic agents, such as venoms [24] or parasites [25].

2. Materials and methods

2.1. Chemicals

Sodium Chloride (NaCl), Potassium chloride (KCl, 99.5%), sodium phosphate (NaH2PO4), Magnesium sulphate (MgSO4), Magnesium Chloride ($MgCl_2$), Ethylenediaminetetraacetic acid (EDTA), Adenosine-5′-triphosphate (ATP) and HEPES were supplied by Sigma-Aldrich. Glucose and sucrose were from Riedel-de Haën. Ultrapure water was taken from a Milli-Q unit (Millipore, conductivity lower than 18MΩ cm, organic residuum less than 2ppb).

2.2. Lipids, antibodies and fluorescent probes

The fluorescent lipid 1,2-dioleoyl-sn-*glycero*-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE) was supplied by Avanti Polar Lipids and dissolved in chloroform at 1 mg/ml after receipt. Monoclonal anti-human spectrin (α and β) (clone SB-S1) was purchased from Sigma-Alrich. The secondary fluorescent antibody Alexa Fluor 488 goat antimouse IgG, IgA, IgM (H + L) was supplied by Invitrogen. Alexa Fluor® 594 Phalliodin (300 U) was purchased from Invitrogen and dissolved in 1.5 mL ethanol after receipt. Molecular solutions were stored at −20 °C.

2.3. Erythrocyte membrane extraxtion

Human blood was obtained from healthy donors and stored in EDTA-containing tubes to avoid coagulation. Immediately after, the erythrocyte concentrate is obtained by washing out three times in PBS buffer (1 blood/5 PBS (vol/vol); NaCl 150 mM, sodium phosphate 5 mM, pH = 8) following the Steck and Kant protocol [26]. Then, cytoplasm contents are removed under hemolytic conditions in a hypotonic buffer (1 erythroid/40 buffer (vol/vol); 1 mM MgSO4, sodium phosphate 5 mM, pH = 8). Sealed ghosts were carefully recuperated after centrifugation (22,000*g*, 10 min; 4 °C), discarding the hard button enriched in proteases obtained at the tube bottom. The buffer washing out was repeated twice again in a dilution ramp at constant pH = 8 (1st: 1 mM $MgSO_4$, sodium phosphate 2.5 mM; 2nd: 1 mM $MgSO_4$, sodium phosphate 1.25 mM) [27]. The procedure was carried out at 4 °C. The erythroid membrane is vesiculated by passing the extract suspension 5 times through a gauge needle (No. 23) using a 50 mL syringe. The final protein concentration of the erythrocyte membrane is ca. 0.5 mg/ml (see SI) as estimated by the RC DC Protein Assay kit (BIORAD) using BSA as a reference. Aliquots (20 μL) of the RBC membrane concentrate were stored at −20 °C.

2.4. ErytroGUVs preparation

Giant vesicles made of erythrocyte membrane (ErytroGUVs) were prepared by electro-swelling [28] and following the same protocol described in [22]. Prior to spreading on the electro-swelling chamber erythroid membranes were labeled with fluorescent Rho-PE. For that, erythroid membrane aliquots were incubated for 30 min in an Eppendorf tube previously containing a lipid film. These films are prepared by solvent evaporation from a chloroform solution containing 1 mg/ml of Rh-PE.

2.5. Indirect inmunofluorescence

Freshly prepared eGUVs were incubated for 1 h in isosmolar buffer solution containing primary monoclonal anti-human α,β-spectrin (anti-body final concentration, 4 μg/ml, 75 mM NaCl, 40 mM glucose, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM KCl, pH = 7,4). The secondary fluorescent antibody Alexa Fluor 488 goat anti-mouse IgG, IgA, IgM (H + L) (ca. 4 μg/ml, final) was subsequently incubated for 1 h more. Actin staining was performed by adding 5 uL of Alexa Fluor® 594 Phaolloidin solution to 100 μL of eGUV suspension (80 μL + 20 μL of eGUV containing ATP (>1 mM final) when necessary) and incubated for 1 h.

2.6. Simultaneous fluorescence and AFM imaging in amplitude modulated dynamic mode (abbrev. AM)

100 μL of eGUV suspension (at different ATP concentration) were incubated on glass cover slides (Menzer-gläser, #2 Ø 24 mm) for 30 min. The hydrophilic interaction between the negative glass and erythrocyte membranes is mediated by the presence of divalent ions and resulted in the formation of supported membranes spread onto the glass surface. Prior to AFM visualization, the spread membranes were washed with abundant ATP containing buffer. AFM characterization of Figs. 2 and 5 was performed using a JPK NanoWizard II, coupled to a Nikon Eclipse Ti inverted fluorescence optical microscope. The AFM was operated in amplitude modulated dynamic mode in aqueous solution, an intermittent contact mode where the cantilever oscillates at its resonant frequency. Imaging is performed when a setpoint of amplitude is chosen, and the height adjusted to match this amplitude through the feedback system. Height, error, and phase signals were simultaneously recorded. The amplitude error signal is very sensitive to the in plane derivative of the topography whereas the phase is related to the energy dissipation. An Olympus commercial silicon nitride cantilever tip with a force constant of 0.76 N/m (calibrated by thermal tuning), 15 nm of nominal radius, and a free resonant frequency of 71 kHz was employed.