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New molecular rods — Characterization of their interaction with membranes

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ABSTRACT

Molecular rods are synthetical molecules consisting of a hydrophobic backbone which are functionalized with varying terminal groups. Here, we report on the interaction of a recently described new class of molecular rods with lipid and biological membranes. In order to characterize this interaction, different fluorescently labeled rods were synthesized allowing for the application of fluorescence spectroscopy and microscopy based approaches. Our data show that the rods are incorporated into membranes with a perpendicular orientation to the membrane surface and enrich preferentially in liquid-disordered lipid domains. These characteristics underline that rods can be applied as stable membrane-associated anchors for functionalizing membrane surfaces.

1. Introduction

Typically, molecules which span the bilayer of biological membranes are characterized by an amphiphilic structure. For phospholipids and transmembrane proteins as the main components of biological membranes, fatty acyl chains and hydrophobic α helices or β barrel structures, respectively, constitute the core hydrophobic region of the membrane whereas lipid head groups and hydrophilic amino acids, respectively, form the polar region that is exposed to the intra- and extracellular solutions. Artificial molecules which are aimed to be stably inserted into membranes have to have such an amphiphilic structure. In the last years, amphiphilic molecular rods have been designed and synthesized that gained interest for applications in membrane research.

Molecular rods are synthetic molecules, which are characterized by a high rigidity and a large aspect ratio. They offer the opportunity to position two functional moieties at a defined distance apart and connect them by a molecular structure of desired properties. Compared with the above mentioned membrane associated biomolecules, the molecular rods combine a high stiffness with a relatively low molecular weight.

A couple of different rods and their potential applications in material and in life sciences have been described [1–9]. For the former, rods have been synthesized which are e.g. able to conduct and switch electrical currents and which are designed for a use as molecular wires [4]. In life sciences, rods have been used to mimic biological functions e.g. they were applied as synthetic ion channels and pores in bilayer membranes to mediate a specific transport of ions or electrons across membranes [5,6,8,9]. For that the interaction and orientation of rods within lipid membranes have been characterized [5,6].

We have recently described a new class of molecular rods which are characterized by six-membered saturated rings joined in a spirocyclic manner, and are called oligospiroketal (OSK) rods due to their periodic ketal moieties [10,11]. Based on this work, we developed OSK rods, which have a rather hydrophobic backbone, with different more or less hydrophilic terminal functionalities. The modular synthetic strategy to OSK rods allows creating a large variety of rods having different properties with regard e.g. to length and amphiphilicity. Rods with hydrophilic terminal groups were designed for their incorporation into membranes in order to functionalize the membrane, e.g. by linking an enzymatic activity covalently or noncovalently to the rods.

By using a fluorescent rhodamine-labeled rod (RR), we could recently demonstrate that these rods can be integrated into membranes [12]. However, important properties of the membrane-embedded rods as the orientation in the lipid bilayer and lateral organisation remained unknown. Here, we present chemical strategies for covalent attachment of different fluorophores to the rods.

Abbreviations: Chol, Cholesterol; DOPC, 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine; GUVs, Giant unilamellar vesicle(s); HBS, HEPES buffered salt solution; LUVs, Large unilamellar vesicle(s); MLVs, Multilamellar vesicles; NBD, 7-nitro-2-1,3-benzoxadiazol-4-yl; NBD-PC, 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphocholine; NR, NBD labeled rod; N-Rh-DOPE, 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); OSK, Oligospiroketal; PC, Phosphocholine; POPC, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PR, Pyrene labeled rod; RBC, Red blood cell(s); RR, Rhodamine labeled rod; SL-POPC, 1-Palmitoyl-2-oleoyl-(16-doxyl)-*sn*-glycero-3-phosphocholine; SSM, N-Stearoyl-D-sphingomyelin; TCSPC, Time correlated single photon counting

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Using those labeled rods, we can now show that the rods are orientated perpendicular to the membrane surface and enrich preferentially in a liquid-disordered lipid environment.

2. Materials and methods

2.1. Synthesis

The chemicals and procedures for the synthesis of labeled rods are described in the Supplementary data.

2.2. Materials

Cholesterol (Chol), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), N-stearoyl-D-sphingomyelin (SSM), 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (N-Rh-DOPE) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). The spin labeled PC analog SL-POPC was synthesized as described recently [13]. All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). HEPES buffered salt solution (HBS) contained 10 mM HEPES and 145 mM NaCl, pH 7.4. Stock solutions of rods were prepared for liposome experiments. PR was dissolved in chloroform. NR was presolved with a small quantity of DMF and finally dissolved in chloroform. RR was presolved with a small quantity of DMSO and finally dissolved in chloroform (for preparation of large unilamellar vesicles (LUVs)) or in methanol:chloroform (1:1, v/v) (for preparation of giant unilamellar vesicles (GUVs)).

2.3. Preparation of multilamellar vesicles (MLVs) and LUVs

Aliquots of lipids and rods in organic solution (at the concentrations and compositions given in the text) were transferred to a glass tube, dried under nitrogen and high vacuum and resuspended in a small volume of ethanol (to resolve the lipid-rod film from the glass tube) and HBS (final ethanol concentration was below 1% (v/v)). For preparation of MLVs, this suspension was vigorously vortexed. To prepare LUVs, the suspension was subjected to five freeze–thaw cycles followed by extrusion of the lipid solution eleven times through a 0.1 μm polycarbonate filter at 45 °C (extruder from Avanti Polar Lipids, Alabaster, AL, filters from Costar, Nucleopore, Tübingen, Germany).

2.4. Preparation of GUVs

GUVs were prepared by the electroformation method using titanium chambers [14,15]. Lipid mixtures were made from stock solutions in chloroform. Finally, lipids (100 nmol, DOPC or DOPC:SSM:Chol, 1:1:1, molar ratio) were dissolved in chloroform (50 μl) along with either 1 mol% of NBD-PC or N-Rh-DOPE as a marker for the liquid disordered domains. For a reconstitution of the respective rod during GUV formation the rod was added to the lipid solution. This solution was spotted onto two hollowed titanium plates which were placed on a heater plate at approx. 50 °C to facilitate solvent evaporation, and subsequently put under high vacuum for at least 1 h for evaporation of remaining traces of solvent. Lipid-coated plates were assembled using one layer of Parafilm for insulation [16]. The electroswelling chamber was filled with 1 ml preheated sucrose buffer (250 mM sucrose, 15 mM NaN_3, and osmolarity of 280 mOsm/kg) and sealed with modeling clay. An alternating electrical field of 10 Hz rising from 0.02 V to 1.1 V in the first 30 min was applied for 2.5 h at 55 °C followed by 54 min of 4 Hz and 1.3 V to detach the formed liposomes. For a subsequent incorporation of the rod, it was added to GUVs and incubated for approx. 1 h. Finally, GUVs with reconstituted or subsequently incorporated rod were mixed to glucose buffer (250 mM glucose and 11.6 mM potassium phosphate, pH 7.2) with an osmolarity of 300 mOsm/kg at a ratio of 1:1 to 1:2 for microscopy.

2.5. Preparation of red blood cell (RBC) ghosts

Citrate-stabilized blood samples of healthy donors were purchased from the local blood bank (Berlin, Germany). RBC were washed twice with HBS (10 min, 2000 g) at 4 °C. RBC ghosts were prepared according to [17]. Rod was added to RBC ghosts from stock solution of DMSO (RR) and DMF (NR), respectively.

2.6. Measurement of fluorescence spectra

Fluorescence spectra were recorded using an Aminco Bowman Series 2 spectrofluorometer (SLM-AMINCO, Rochester, NY) between 350 and 600 nm ($\lambda_{ex} = 340$ nm) (for PR), between 480 and 600 nm ($\lambda_{ex} = 470$ nm) (for NR) and between 560 and 650 nm ($\lambda_{ex} = 550$ nm) (for RR) and slit width for excitation and emission of 4 nm.

2.7. Measurement of fluorescence lifetimes

Fluorescence lifetimes were measured using a FluoTime 200 (PicoQuant, Germany) or a Fluoromax 4 (Horiba Jobin Yvon, Germany). Pyrene was excited with a NanoLED-250 (254 nm, repetition rate 1 MHz, Horiba Jobin Yvon, Germany) and emission was detected at 375 nm. NBD was excited with a NanoLED-450 (447 nm, repetition rate 1 MHz, Horiba Jobin Yvon, Germany) and emission was detected at 523 nm. Rhodamine was excited with a pulsed laser diode (LDH-P-C, PicoQuant, Germany) of 470 nm and a pulse frequency of 8 MHz and emission was detected at 570 nm. Single photons were recorded with a time correlated single photon counting (TCSPC) setup and time resolution was set to 33 ps. The spectral bandwidth was set to 4 nm in all cases. Mean photon count rates were ~$1–4 \times 10^4$ counts/s. The decay curves were fitted with one or two exponential terms and the average lifetime τ_{AVG} was calculated as:

$$\tau_{AVG} = \frac{\sum_{i=1}^{n} \alpha_i \tau_i^2}{\sum_{i=1}^{n} \alpha_i \tau_i}$$

where τ_i is the lifetime of the component i and α_i represents the normalized pre-exponential coefficient relative to the fraction of molecules showing the decay time i.

2.8. Reduction of NBD fluorescence by dithionite

POPC-LUVs were prepared in the presence of NR or NBD-PC. For NBD-PC, the LUVs are symmetrically labeled, i.e. the fluorescent lipid analog is localized in both membrane leaflets. Labeled LUVs were suspended in HBS (additionally containing 50 mM HEPES to keep the pH constant after addition of dithionite). Final concentrations were 17 μM for POPC and 0.3 μM for NR or NBD-PC. For investigating the incorporation of NR with preformed membranes, 5 mM POPC-MLVs were incubated with 50 μM NR for 1 h at 20 °C. 100 μl of this solution was washed twice with HBS (10 min, 13,000 g) to remove non-incorporated NR. The final pellet was suspended in 1.5 ml HBS (additionally containing 50 mM HEPES). The fluorescence of the NBD moiety was monitored continuously ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 540$ nm; slit width = 4 nm for excitation and emission) at 4 °C. At time 0, sodium dithionite was added from a freshly prepared 1 M stock solution in 100 mM Tris (pH 10) to give a final concentration of 25 mM. After 250 s, Triton X-100 was added to a final concentration of 0.5% (w/v), enabling access of the remaining analogs on the inner side