Molecular Rods

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Molecular Rods with Oligospiroketal Backbones as Anchors in Biological Membranes**

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Phospholipid bilayers organized as supported layers or as vesicles find wide application as biocompatible assemblies in biotechnology and medicine.[1] For example, supported bilayers are used as inert surfaces on which enzyme reactions can proceed efficiently at the nanoscale.[2] Furthermore, liposomes are used as carriers for bioactive substances such as drugs which affect the metabolism and/or signal transduction cascade of cells upon their release into the cytoplasm.[1a,3] To functionalize bilayers with enzymes or to cover them with ligands recognizing specific target cells, the components and functional molecules, respectively, have to be stably anchored to the bilayer surface. This can be realized by the covalent linkage of molecules to lipids.[4] However, in the case of large, rather hydrophilic functional entities, anchoring to the lipid may not provide a stable association with the membrane.

Hydrophobic structures which span the whole bilayer should be more suitable for anchoring functional entities stably to the membrane. Peptide sequences derived from transmembrane domains of integral membrane proteins are candidates for those structures. An alternative would be hydrophobic molecular rods which can be synthesized with various lengths and conformational rigidity and equipped with reactive groups or ligands at the terminus which enable covalent or noncovalent association of the desired functional entity. The development of molecular rods, that is, relatively rigid molecules with a large aspect ratio, has been an intensively studied research area for several years.[5] It should be noted that anchoring molecular rods consisting of oligo(*p*-phenylene)s in membranes was intensively investigated by Matile and co-workers.[6]

Herein we describe the synthesis of a hydrophobic molecular rod which is terminally functionalized with fluorescent groups. For the first time, a recently developed class of molecular rods was used as the hydrophobic membrane anchor.[7] The structure of these rods is based on six-membered saturated rings joined in a spirocyclic manner, as shown in Figure 1. These types of molecular rods are called

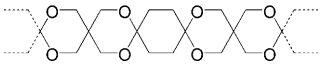

Figure 1. Structure of the oligospiroketal (OSK) rods.

oligospiroketal (OSK) rods because of the periodic ketal moieties. Compared with peptide sequences, the advantages of these rods are their straightforward and flexible preparation, their high conformational rigidity, and their proteolytic stability. By characterization of the fluorescence properties, we demonstrate that the rod can be integrated into lipid membranes during or after formation of the bilayer. In particular, we provide clear evidence that OSK rods can be inserted into intact biological membranes such as mammalian plasma membranes.

Recently, we reported an efficient synthesis of OSK rods with lateral alkyl groups, which enhance the solubility of the rods in organic solvents.[8] These groups (butyl groups in the present case) ensure that even relatively long OSK rods with lengths of more than 3 nm have satisfactory solubility and can be easily functionalized at their terminal positions. In the current study we used the previously described bisazide **1** as the central building block for the membrane anchor (Scheme 1). To facilitate the incorporation of rather hydrophobic OSK rods in biological membranes they must be tethered with hydrophilic moieties at the terminal positions, thereby giving bolaamphiphilic rods. Furthermore, fluorescence dyes have to be introduced into the hydrophilic units to enable the incorporation of the rods in biological membranes to be studied by fluorescence spectroscopy.

As hydrophilic moieties we have chosen the tripeptide Lys_3, which has been proven to be appropriate in similar cases.[9] A terminal alkyne moiety was needed to enable the peptide to be coupled with the OSK rod **1** by a copper-catalyzed azide-alkyne cycloaddition ("click" reaction),[10] and thus L-propargyl glycine (Pra) was incorporated at the C-terminal position of the peptide. After preparation of the tetrapeptide Lys_3-Pra by solid-phase peptide synthesis (SPPS), we found that direct coupling of the fluorescent dye 5-carboxytetraethylrhodamine (CR) with terminal lysine is only partially successful. This problem could be circumvented by insertion of a βAla moiety as a spacer. Deprotection, cleavage from the resin, and purification by preparative

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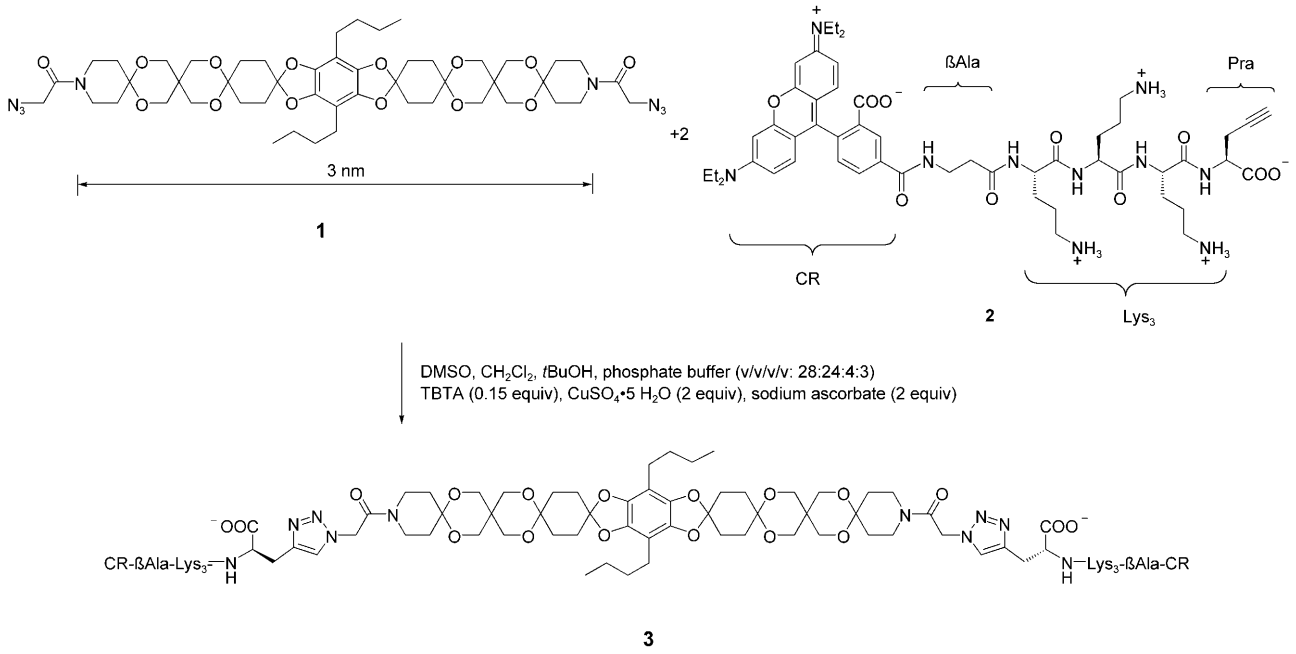

Scheme 1. Synthesis of the membrane anchor **3** from bisazide building block **1** and dye-labeled pentapeptide CR-βAla-Lys_3-Pra **2**. TBTA = tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine.

HPLC afforded the building block **2** (Scheme 1, for details see the Supporting Information). In the final step, peptide **2** and bisazide **1** were coupled in a click reaction to afford the complete membrane anchor **3** (Scheme 1).

A series of independent, complementary approaches were used to assess the insertion of **3** into membranes. First, **3** was added to the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) during the preparation of multilamellar vesicles (MLVs). Measurement of the fluorescent properties of the MLVs revealed the typical excitation and emission spectra of rhodamine, with an excitation maximum at about 560 nm and an emission maximum at about 580 nm (see the Supporting Information).

Second, fluorescence resonance energy transfer (FRET) was measured between the phospholipid-linked fluorophore NBD [*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl-*sn*-phosphoethanolamine (N-NBD-PE) as the donor] and the rhodamine moiety (acceptor) of **3** (Figure 2). For this, the fluorescence spectra of POPC/N-NBD-PE/**3** MLVs and POPC/N-NBD-PE MLVs were recorded. Comparison of the two spectra showed that energy transfer occured, as evident from the decrease in the NBD fluorescence at 535 nm and an increase in the rhodamine fluorescence at 580 nm (Figure 2, see arrows), and thus indicating the rod was embeded in the membrane. FRET between the two fluorophores was confirmed by measuring the fluorescence lifetimes (τ) of the donor NBD in both types of vesicles (Figure 2 inset). In the presence of **3**, the value of τ was significantly reduced, which is typical for FRET.

Third, confocal laser scanning microscopy was used to visualize the incorporation of **3** into membranes. Thus, **3** was either incorporated into model 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) membranes of giant unilamellar vesicles (GUVs, not shown) during formation of the GUVs or

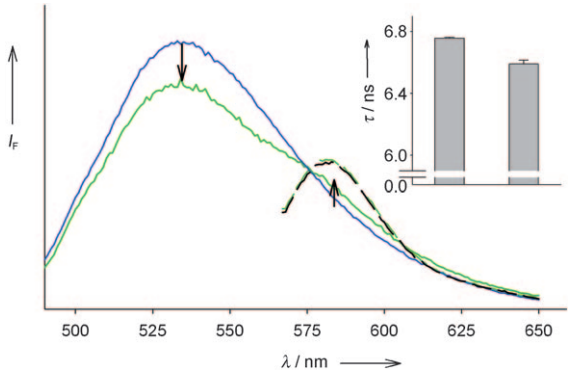

Figure 2. FRET from N-NBD-PE to **3**. 0.5 mM POPC MLVs were labeled with N-NBD-PE (10 μM) and **3** (50 μM). The fluorescence spectra of POPC/N-NBD-PE/**3** MLVs were recorded at 37 °C with an excitation at 470 nm (——) and 560 nm (-----). As a control, the spectra of POPC/N-NBD-PE MLVs (excitation at 470 nm; ——) and of POPC/**3** MLVs (excitation at 560 nm; -----) were recorded. Inset: Fluorescence lifetimes τ of N-NBD-PE in POPC/N-NBD-PE MLVs (left column) and in POPC/N-NBD-PE/**3** MLVs (right column) measured at 37 °C. The data represent the mean ± standard error of two samples which were measured three times. (I_F = fluorescence intensity).

added to preformed GUVs (Figure 3a). In both cases, a bright uniform fluorescence was observed, which shows there is a homogeneous lateral distribution of **3** in the membrane.

Fourth, since the experiments on the GUVs demonstrated that **3** can be inserted into preformed membranes, we incubated human red blood cells (RBCs) with **3**. We found that the rods efficiently incorporated into the plasma membrane of RBC ghosts (an erythrocyte membrane that remains intact after hemolysis; Figure 3b) and intact RBC (not shown), again indicated by a homogeneous fluorescence.

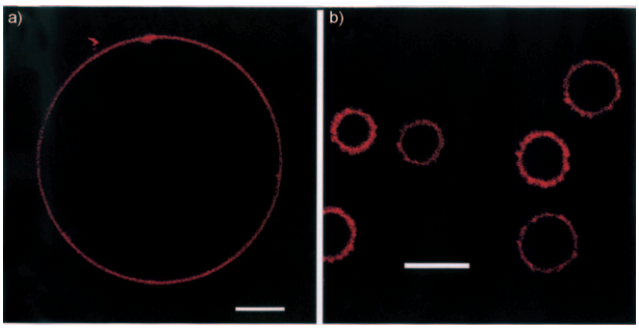

Figure 3. Incorporation of **3** into GUVs and erythrocyte membranes visualized by the detection of rhodamine fluorescence. **3** was added to preformed DOPC GUVs (a) or to RBC ghosts (b; see text). Scale bar = 5 μm. The uniform fluorescence indicates a homogeneous lateral distribution of **3** in membranes.

Here, we have shown that hydrophobic molecular rods can be efficiently integrated not only into model membranes but also into intact biological membranes without using any agents (for example, detergents, cyclodextrins) that mediate the incorporation in the membrane. Hence, such rods can potentially be used as stable membrane-associated anchors for the functionalization of membrane surfaces. Functionalization can be undertaken before and also after insertion of the rods into the membrane. For example, receptors, enzymes, or other bioactive molecules can be linked covalently to surface-exposed reactive groups of the rods. Alternatively, a reversible functionalization can be achieved with rod-linked oligonucleotides (DNA, peptide nucleic acids (PNAs)), thus allowing the binding of molecules through a complementary oligonucleotide.[11]

A challenging aspect for further study is to explore how the chemical and geometrical structure as well as the physical properties of such molecular rods can modulate specific interactions with lipids. It is known that biological membranes, such as the plasma membrane of mammalian cells, form small lipid domains at the nanoscale which differ in their lipid composition.[12] Membrane proteins can accumulate specifically at those domains, depending also on the properties of their membrane anchor. Hence, such domains are considered to serve as platforms for efficient cell signaling and/or for formation of protein-enriched membrane areas that are transported to distinct intracellular sites by vesicles. For eukaryotic cells, in particular mammalian cells, besides the plasma membrane, intracellular membranes may also represent targets for functionalized rods. After endocytosis, the internalized rods can be shuttled by vesicular transport to organelles enveloped by membranes. Notably, directing rods to distinct lipid domains in the plasma membrane offers the opportunity to regulate the uptake of functionalized rods. Future studies should be directed to elucidating the mechanism of cellular uptake and the subsequent intracellular transport of functionalized rods.

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