Luminescent Probes

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Intermolecular Sensitization of a Terbium-Containing Amphiphile by an Integral Membrane Protein**

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Dedicated to Professor Bernt Krebs on the occasion of his 70th birthday

Due to their distinct emission spectra and long lifetimes, lanthanide-based luminescent probes are particularly suited to biological applications.[1–3] To emit strongly, lanthanide ions require an antenna chromophore to absorb the energy of the light and transfer it to the emitting level of the lanthanide. Here we describe the development of a new type of luminescent probe for the visualization of integral membrane proteins (IMPs), which uses the tryptophan and tyrosine residues in the aromatic collar of an IMP as antenna chromophores (Figure 1 a). The luminescence of the terbium center bound in the head group of our amphiphilic probe is sensitized to those probe molecules that are in close proximity to the aromatic collar at the hydrophobic boundary of an IMP. Since aromatic collars are structural features that are specific to IMPs, our approach offers the opportunity to visualize these proteins selectively. In addition, this noncovalent labeling is potentially reversible, flexible, and minimally invasive.

In conventional probes, the chromophore is usually covalently attached to a ligand that chelates the lanthanide or is directly coordinated to the lanthanide ion. In the context of cyclodextrin[4,5] and peptide chemistry[6,7] it has been demonstrated that noncovalent interactions can also be used to achieve sensitization, provided that the interactions increase the effective concentration of the antenna in the vicinity of the lanthanide ion. Similarly, micelles can be used to bring the energy-transfer components into close proximity. Fendler et al. demonstrated that naphthalene, if solubilized in the hydrophobic interior of sodium dodecyl sulfate (SDS) micelles, sensitizes Tb^{3+} cations that are attracted to the head groups of the anionic surfactant.[8] Metallosurfactants offer the additional advantage of incorporating the metal ion into their polar head group, thereby localizing it at a micellar interface.[9]

Our aim was to position the terbium-containing head group of our amphiphilic probe close to the aromatic collar of a surfactant-solubilized IMP to facilitate intermolecular energy transfer at the hydrophilic/hydrophobic interface. As gateways to the cell, IMPs are important drug targets. In current research, however, IMPs present a bottleneck due to difficult purification and crystallization. The IMPs have a higher content of aromatic amino acids than soluble proteins,[10] and the side chains of the tryptophan and tyrosine residues tend to be localized at the membrane/water interface, where they form a "collar" at the boundary between the polar and the nonpolar regions of the bilayer.

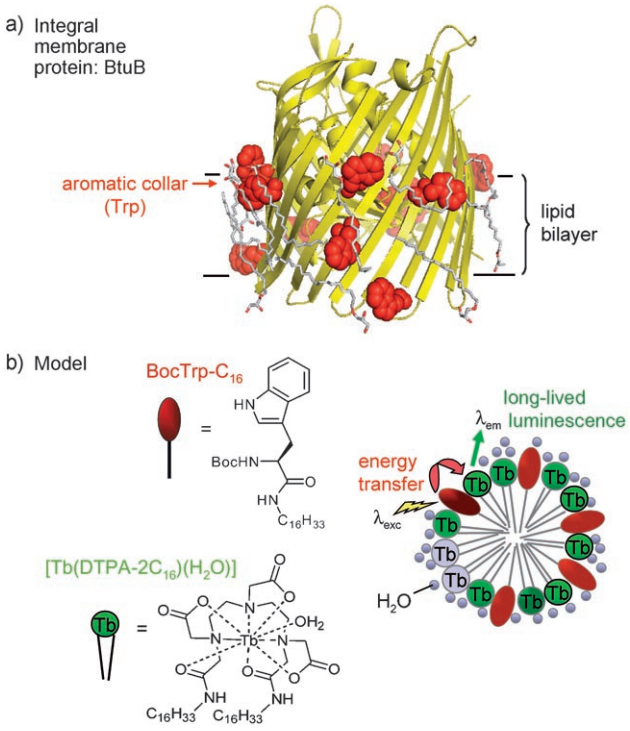

Figure 1. a) Crystal structure of the vitamin B_{12} transporter BtuB (PDB-ID: 2GUF) with tryptophan (space-filling representation, red) and surfactants (stick representation, C gray, O red) highlighted. b) Schematic illustration of the intermolecular sensitization process in a mixed-micelle model system. Boc = *tert*-butoxycarbonyl.

As detailed in the Supporting Information, Tb^{3+} was bound in the head group of H_3-DTPA-2C_{16}.[11,12] The resulting lipid analogue, complex [Tb(DTPA-2C_{16})(H_2O)] (Figure 1 b), was characterized by high-resolution ESI mass spectrometry, infrared spectroscopy, and elemental analysis. Furthermore, fluorescence spectroscopy confirmed that the complex on its own does not show significant terbium-based emission on excitation at 285 nm when solubilized in water (0.21 mM) by

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addition of surfactants such as SDS or *n*-octyl-β-D-glucopyranoside (OG).

To facilitate optimization of the intermolecular sensitization process, the surfactant analogue Boc-Trp-C_{16} (Figure 1b) was synthesized, which models the interface-bound tryptophan residue of an IMP. The indole chromophore of tryptophan is well known to sensitize Tb emission,[13,14] and in SDS micelles the indole group of a similar dodecylamine-modified tryptophan derivative was shown to be located close to the head groups of the surfactant.[15] We found that a slightly longer tail is more suited to our application, and the amine functionality was protected with the Boc group to avoid ionic interactions.

If a mixture of Boc-Trp-C_{16} and the terbium complex in water is excited into the lowest-energy absorption band of the tryptophan at 285 nm, remarkably intense terbium-based emission bands are observed at 494, 550, 590, and 624 nm (Figure 2). Time-delayed detection, which is available in the phosphorescence mode of many spectrofluorimeters, allows any residual emission of tryptophan to be eliminated. A Boc-Trp-C_{16} concentration of 0.07 mM was used to maximize the absorbance while minimizing inner filter effects. At the given concentration of Boc-Trp-C_{16}, the intensity of the sensitized Tb-based emission increases with increasing concentration of [Tb(DTPA-2C_{16})(H_2O)] until it is limited by the solubility of the lipid analogue at a donor to acceptor ratio of 1:3 (relative overall quantum yield $\Phi = 0.1$). The observed Tb-based emission provides evidence for the formation of mixed micelles in which both the donor and acceptor are co-localized at the interface. The formation of micelles under these conditions was confirmed by transmission electron microscopy (Supporting Information, Figure S2).

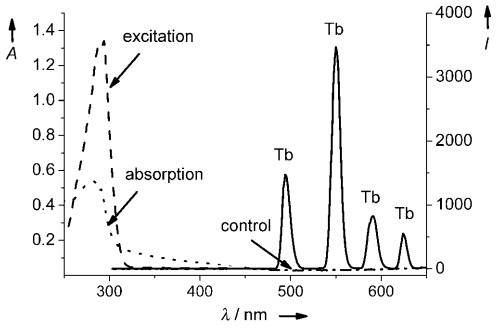

Figure 2. Spectroscopic characteristics of an aqueous micellar system containing [Tb(DTPA-2C_{16})(H_2O)] (0.21 mM) and Boc-Trp-C_{16} (0.07 mM): absorption (dotted line), excitation (dashed line, monitored at 550 nm), and emission spectrum (solid line, $\lambda_{exc} = 285$ nm, phosphorescence scan) in comparison with the emission spectrum (dashed-dotted line, $\lambda_{exc} = 285$ nm, phosphorescence scan) of a solution of [Tb(DTPA-2C_{16})(H_2O)] (0.21 mM) and Trp (0.07 mM) in methanol.

By monitoring the most intense emission band at 550 nm an excitation spectrum was recorded, which resembles the absorption spectrum of the tryptophan derivative and thereby confirms that the terbium emission is due to intermolecular energy transfer from the tryptophan sensitizer. In addition, the luminescence lifetime of the terbium complex in the optimized micellar system was determined in water (1.96 ms) and D_2O (2.75 ms), in order to estimate the number of Tb-coordinated aqua ligands (Supporting Information).[16] The resulting hydration state of 0.4 points towards an equilibrium between complexes with one or no bound water molecules.

In control experiments in which the Tb complex and tryptophan or Boc-protected tryptophan were dissolved in methanol, no terbium-based emission could be observed under otherwise analogous experimental conditions; that is, sensitization does not occur in solution. We therefore propose that in water Boc-Trp-C_{16} and the terbium complex are likely to be co-micellized as shown in Figure 1b, with the polar head groups close to the micelle/water interface and hydrophobic chains pointing towards the core of the micelle.

To establish whether the aromatic collar of an IMP is able to sensitize terbium in a similar way, [Tb(DTPA-2C_{16})(H_2O)] was added to the OG-solubilized vitamin B_{12} transporter BtuB of *E. coli*, which was purified as previously described.[17] Significant terbium-based emission bands were observed (luminescence lifetime: 1.88 ms), while a control experiment with $[Tb(DTPA)]^{2-}$ [18] gave only very weak terbium emission (Figure 3). The circular dichroism (CD) spectra of OG-solubilized BtuB recorded in the absence and presence of [Tb(DTPA-2C_{16})(H_2O)] are very similar, indicating that the secondary structure of the protein is not changed significantly on addition of the probe (Supporting Information, Figure S5).

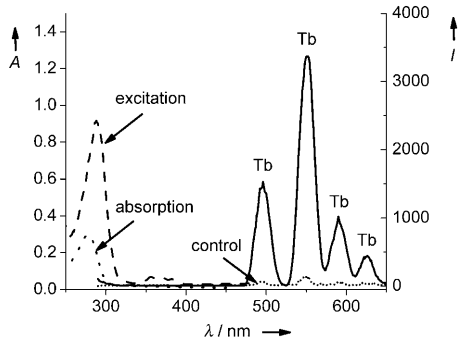

Figure 3. Absorption (dotted line), excitation (dashed line, monitored at 550 nm), and emission spectrum (solid line, $\lambda_{exc} = 285$ nm, phosphorescence mode) of an aqueous system (20 mM Tris buffer pH 7.5) containing BtuB (0.125 mg mL^{-1}, 0.7 μM), OG (1%) and [Tb(DTPA-2C_{16})(H_2O)] (1 mM) in comparison with the emission spectrum (short dotted line, $\lambda_{exc} = 285$ nm, phosphorescence mode) of an analogous system containing BtuB (0.7 μM), OG (1%), and $[Tb(DTPA)]^{2-}$ (1 mM).

A high-resolution crystal structure of BtuB recently confirmed the location of the 13 tryptophan and 41 tyrosine residues within the protein.[19] Interestingly, most of the head groups of the detergent molecules that cocrystallized with the protein are located very close to the tryptophan residues within the aromatic collar, and some are even involved in hydrogen bonding (Figure 1a). To disrupt similar structural arrangements in our system we employed heat denaturation for two minutes at 90 °C in the presence of SDS, which largely unfolds BtuB, as previously reported.[20] As expected, a significant change in the CD spectrum and a drastic decrease

in the intensity of the Tb-based emission of the denatured sample confirmed that the intermolecular sensitization process is inefficient in solution. This was further confirmed by a control experiment with the soluble protein xanthine oxidase as the antenna molecule, in which only very weak emission was observed on addition of the probe. [Tb(DTPA-2C_{16})-(H_2O)] could therefore be used to monitor folding or to assess the reconstitution efficiency of IMPs into amphiphilic environments.

In conclusion, this study confirms that intermolecular sensitization can be achieved in micelles in which the terbium-containing head group of [Tb(DTPA-2C_{16})(H_2O)] is located in proximity to tryptophan-based antenna chromophores. This finding opens up new possibilities for the investigation of IMPs in amphiphilic systems such as vesicles, proteoliposomes, and supported lipid bilayers. Our current work is aimed at improving the quantum yields and solubility of the probes in order to explore these applications in more detail.

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