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Exploiting the Nitrilotriacetic Acid Moiety for Biolabeling with Ultrastable Perylene Dyes

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Chemical and biological labeling is essential for the exploration of protein function, as fluorescent probes allow detection of molecular interactions, mobilities, and conformational changes. Especially fluorescence resonance energy transfer (FRET) has become an important tool to study conformational distributions and dynamics of biomacromolecules.[1] This, however, requires the ability to introduce fluorescent reporters at specific sites.[1] One of the most frequently used approaches for selective labeling of proteins is to introduce a non-native cysteine at a desired location, while the chromophore possesses a thiol reactive group. This strategy suffers from the disadvantage that the number and position of native cysteines are practically important for the structure and biological function of various proteins such as cysteine-based oxidoreductases, phosphatases, and proteases.[2]

The most widely used genetically encoded tag is the polyhistidine tag, which usually consists of 2–6 consecutive histidine residues.[3,4] It was originally developed for the purification of recombinant proteins by immobilized metal-affinity chromatography. Different modifications of this molecular recognition technique have been exploited, including target protein detection and protein structure studies.[5,6] This strategy has been also utilized to introduce a chromophore containing a nitrilotriacetic acid (NTA) moiety into His-tagged proteins.[7–9] All of the reported chromophores, however, suffered from the same drawback—a severe loss of the fluorescence upon binding of the paramagnetic nickel ion. When Ni^{2+} was complexed with the NTA moiety that was attached to commercial Cy3 and Cy5 dyes, the fluorescence quantum yield dropped by 75%.[9] Although the quenching of the fluorescence is distance dependent, an 80% loss of the fluorescence of Atto-565 was measured, even when a longer spacer between the NTA moiety and the fluorophore was introduced.[8]

Herein, we report a different NTA-modified fluorophore—water-soluble perylene(dicarboximide) dye, connected to a NTA unit, which in contrast to all the other examples reported before contains a much shorter spacer between the dye and the NTA (only two methylene groups). Remarkably, the photophysical properties of the chromophore remain unchanged upon Ni^{2+} binding. The new fluorescent reporter has relatively small molecular weight (1555 g/mol), which makes it a good candidate for labeling proteins at the same time avoiding limitations due to the large size of autofluorescent proteins or quantum dots.

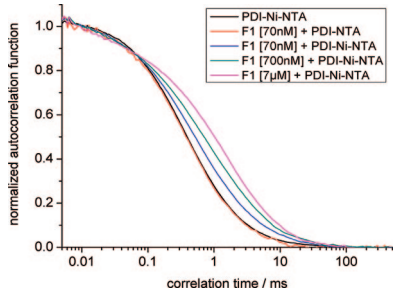

Figure 1. FCS results. Normalized autocorrelation functions of perylene-Ni-NTA and perylene-NTA in the presence of His_6-tagged F_1-ATPase.

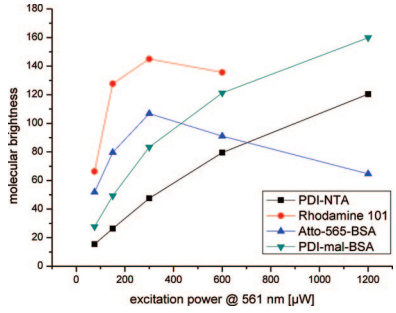

Figure 2. Relative molecular brightness of PDI-NTA, rhodamine 101, and BSA-bound Atto-565.

Protein labeling with the NTA functionalized perylene(dicarboximide) was successfully demonstrated using His-tagged ATP synthase.

The synthesis and application of water-soluble rylene dyes have been reported recently.[10] They exhibited outstanding photophysical and photochemical properties at both ensemble and single molecule level.[11,12] Among these exceptional characteristics are high fluorescence quantum yields, high extinction coefficients, and excellent stability against photooxidation.[10,13,14]

Two synthetic strategies were elaborated to attach the NTA moiety to the perylene chromophore. One is based on solution phase synthesis, while the other relies on a solid phase approach. In the first route, the water-soluble perylene *N*-hydroxysuccinimide ester **1** was reacted with *t*-butyl protected nitrilotriacetic acid (Scheme 1A). After removal of the protective groups using trifluoroacetic acid, the nitrilotriacetic acid functionalized perylene(dicarboximide) (PDI-NTA) **3** was obtained.

In the second approach, we took advantage of a straightforward solid phase synthesis of a nitrilotriacetic acid moiety that was developed by Meredith et al.[15] The NTA unit was

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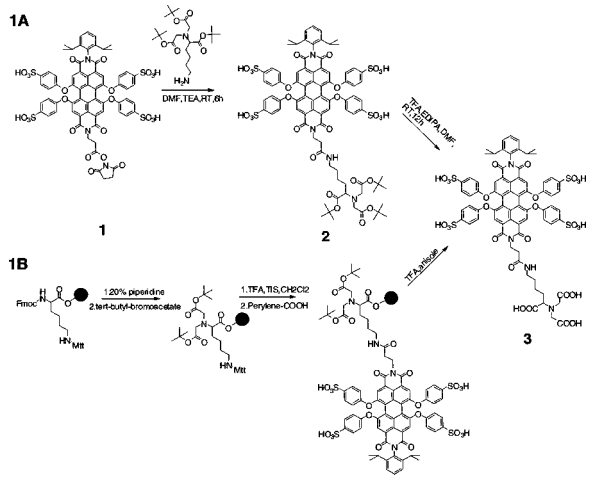
Scheme 1. Synthesis of the NTA Perylene Conjugate: (A) Solution Phase Synthesis and (B) Solid Phase Synthesis



conveniently coupled to a carboxyl functionalized perylene derivative by amide bond formation (Scheme 1B). For both routes, the water-soluble perylene NTA derivative **3** was purified by size exclusion chromatography and obtained in 70 and 15% yield for the solution and solid phase syntheses, respectively. The new chromophore has an absorption maximum at 562 nm ($\epsilon = 35\,000\ M^{-1}\ cm^{-1}$). The emission maximum is located at 615 nm. A fluorescence quantum yield of 15% for **3** was determined by using Cresyl Violet in methanol as reference.[16] When the same quantum yield measurement was carried out with the Ni^{2+} complexes of **3**, no decrease in the fluorescence was observed. In addition, the absorption and emission maxima remained unchanged.

To prove the suitability of **3** for protein labeling, the Ni complex of the chromophore was incubated with His_6-tagged F_1 complex of F_0F_1-ATP synthase from *Escherichia coli*. The F_1 complex with subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$ contained three His_6 tags, one at the N-terminus of each of the β subunits. These His_6 tags had been used to attach the F_1-ATPase to a glass surface and to demonstrate the ATP-driven rotation of the γ subunit.[17] In order to detect the binding of the perylene dye to this rotary motor protein in solution, fluorescence correlation spectroscopy (FCS) experiments were carried out. Confocal laser excitation at 561 nm was used to match the absorbance maximum of **3**. Different concentrations of F_1-ATPase were premixed with 1 nM PDI-NTA (**3**) complexed with Ni^{2+} (PDI-NTA-Ni^{2+}). As a control, the dye was mixed with F_1-ATPase in the absence of Ni^{2+}. Increasing amounts of F_1-ATPase resulted in increasing diffusion times of the PDI-NTA-Ni^{2+} due to conjugate formation with the His-tagged protein (Figure 1). Using a two-component model for bound and unbound dye to fit the autocorrelation functions of the FCS experiments, a dissociation constant $K_D = 3 \pm 1\ \mu M$ was obtained for the PDI-NTA-Ni^{2+}, which is in good agreement with binding constants reported for other Ni-NTA labels.[18–20] In the absence of Ni^{2+}, no evidence for binding of the PDI-NTA dye was found by FCS measurements (Figure 1).

FCS was also used to determine the mean photon count rate or "molecular brightness" of a single PDI-NTA in the absence of Ni^{2+} (Figure 2).[21] Rhodamine 101 and Atto-565 were used as the references. For rhodamine 101, a maximum fluorescence of about 145 000 counts/s/molecule saturated with an excitation power of 300 μW on the back aperture of the microscope objective was detected. At higher excitation power, photobleaching and increased population of the nonfluorescent triplet state reduced the brightness per molecule. Similar saturation behavior was observed for Atto-565, which was bound to bovine serum albumin (BSA). In contrast, the molecular brightness of the PDI-NTA steadily increased up to an excitation power of 1.2 mW, reaching 120 kHz and supporting the high photostability of the perylene chromophore. At this high laser power, the triplett yield was only 11% compared to about 45% for Atto-565 bound to BSA. In the presence of Ni^{2+}, the relative brightness of the perylene derivative was reduced to about 77%. Binding of the PDI-NTA-Ni^{2+} to the His tags of F_1-ATPase did not reduce the molecular brightness further but rather increased slightly.

In summary, we have presented the synthesis of a new water-soluble perylene(dicarboximide) dye functionalized with a nitrilotriacetic acid moiety. This chromophore combines the exceptional photophysical properties of the rylene(dicarboximide) dyes and a recognition unit for site-specific labeling of proteins. An important feature of the label is the unchanged emission of the dye upon complexation with nickel ions. Moreover, the suitability of the fluorophore for labeling His-tagged proteins was demonstrated by FCS. Due to its small size, its proven exceptional photostability and the possibility for site-specific attachment, the PDI-NTA offers great potential for the characterization of protein functions and interactions.

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Supporting Information Available: Detailed description of the synthesis, purification, and spectroscopic characterization of the compounds, and a description of the FCS experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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