Water-Soluble Dyes

Ionic Perylenetetracarboxdiimides: Highly Fluorescent and Water-Soluble Dyes for Biolabeling**

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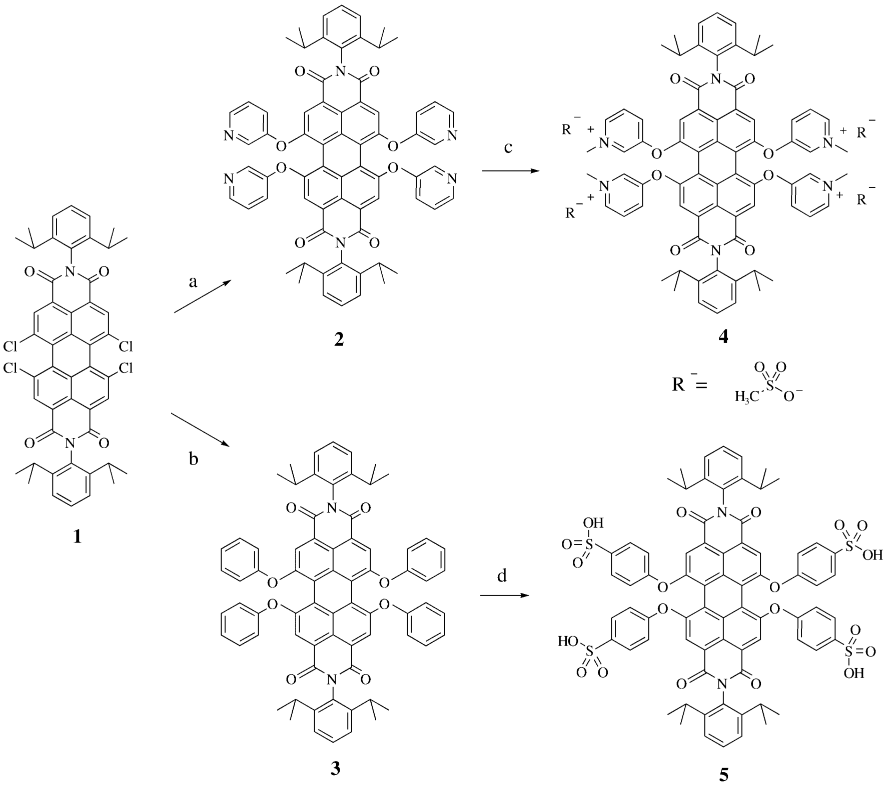
The use of water-soluble fluorescent labels for cells, antibodies, and DNA has rapidly expanded in recent years and has gained additional momentum owing to single-molecule spectroscopy.[1–3] Although there are a large variety of water-soluble chromophores commercially available today, most of them exhibit relatively low fluorescence quantum yields and/or photochemical stabilities. Cyanine dyes, for example, are highly instable towards oxygen and light, while xanthene dyes tend to aggregate in aqueous medium.[1,4] The ideal dye label should have the following characteristics: 1) good water solubility, 2) high fluorescence quantum yield, 3) high chemical and photostability, 4) nontoxicity, 5) good biocompatibility, 6) availability of monofunctional derivatives as an extrinsic covalently bound probe, as well as 7) possible commercial viability and scalable production. The highly fluorescent perylene-3,4,9,10-tetracarboxdiimide (PDI) chromophore is widely used as a commercial dye and pigment due to its outstanding chemical, thermal, and photochemical stability.[5] Furthermore, because of its brilliant color, strong absorption, and fluorescence, it has been extensively investigated as an active compound in reprographic processes,[6] photovoltaic cells,[7] light-emitting diodes,[8] other photonic devices,[9] and light-harvesting complexes.[10] Owing to the unique properties of PDIs, they should also be excellent biological probes; however, the retention of high fluorescence in aqueous medium is a critical issue. Up to now, only a few water-soluble PDI derivatives with functionalities in their imide moieties have been reported. They exhibit poor water solubility and/or very weak fluorescence in water with moderate fluorescence quantum yields of around 10% because of aggregation of the perylene chromophores.[11,12]

It is well-known that the solubility of PDI derivatives in organic solvents dramatically increases when substituents are attached in the bay region, resulting in a twist within the perylene core,[13] and that water solubility can be achieved by introducing charged groups on the hydrophobic dyes or pigments.[14] By successfully combining both structural features, we can produce highly fluorescent, water-soluble, and photostable PDIs for the first time. Furthermore, PDIs that contain multiple charged groups have been prepared as a monocarboxylic acid derivative for covalently attached labels in the field of biology. Initial cell-visualization experiments by using the novel labels are described below.



Scheme 1. a) 3-Pyridinol, *N*-methylpyrrolidone (NMP), K_2CO_3, 80 °C, 15 h, 85 %; b) phenol, NMP, K_2CO_3, 80 °C, 15 h, 89 %; c) 1) methyl iodide, methanol, reflux, 24 h, 95 %; 2) silver methanesulfonate, methanol, room temperature, 5 h, 92 %, d) conc. sulfuric acid, room temperature, 15 h, 93 %.

The synthesis of the positively charged PDI **4** involves a phenoxylation with 3-pyridinol in 1-methyl-2-pyrrolidone followed by a quaternization, beginning with the tetrachloroperylenetetracarboxdiimide **1** (Scheme 1).[15] Because of the good solubility of PDI **2** in alcohols, the quaternization is performed with an excess of methyl iodide in methanol followed by a quantitative exchange of iodide with methanesulfonate counterions to give the desired water-soluble PDI **4**. A PDI with sulfonyl substituents **5** is obtained after phenox-

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ylation of **1** with phenol (by using a similar procedure as for **2**), and the subsequent sulfonation with concentrated sulfuric acid at room temperature.

The asymmetric, chloro substituted PDI **7** is obtained by imidization by using two different arylamines with and without the carboxylic function. Isolation of the monoacids **7**, **8**, and **9** is easily performed by column chromatography, the eluent being a mixture of dichloromethane/acetone (10:1 v/v). The monofunctional water-soluble PDIs **10** and **11** are prepared in high yields by using the same reaction conditions as for **4** and **5** (Scheme 2). The structures of all compounds are confirmed by means of field desorption (FD) mass spectra, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry and/or NMR spectroscopy.[16] These new chromophores have good thermal stability, with decomposition temperatures above 300 °C, and high water solubility. The saturation concentration of PDI **4** in water is above 1.0×10^{-2} M and 8.0×10^{-2} M for **5**. Monofunctional ionic PDIs **10** and **11** show similar water solubility in comparison to the symmetric ionic PDIs. Molecular-modeling studies[17] of PDIs **4** and **5** reveal a twist of the two naphthalene rings of about 28°[18] and a polar shell embedding the PDI fluorophore (not show here).

The UV/Vis absorption and emission spectra of **4**, **5**, **10**, and **11** are measured in water (Figure 1) and their fluorescence quantum yields are determined. An overview of the optical properties of all chromophores is given in Table 1. PDIs **4** and **5** have high extinction coefficients in water in comparison to tetraphenoxyperylenetetracarboxdiimide **3** in organic solvents (≈45 000 $\text{M}^{-1}\text{cm}^{-1}$). Furthermore, the absorption spectra of all ionic PDIs in water in a concentration rage of 10 mg mL^{-1}–1.0×10^{-2} mg mL^{-1} exhibited the same vibrational fine structure of PDI, which suggest that ionic PDIs still present as monomers in high concentrations. A PDI that has positive charges, **4**, shows a blue shift

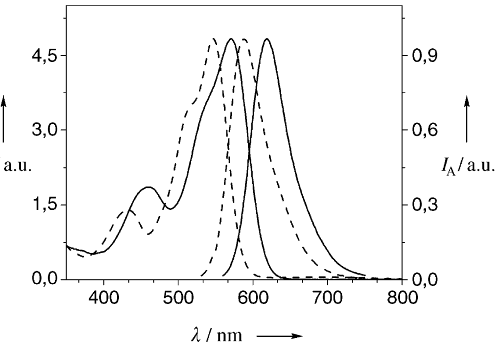
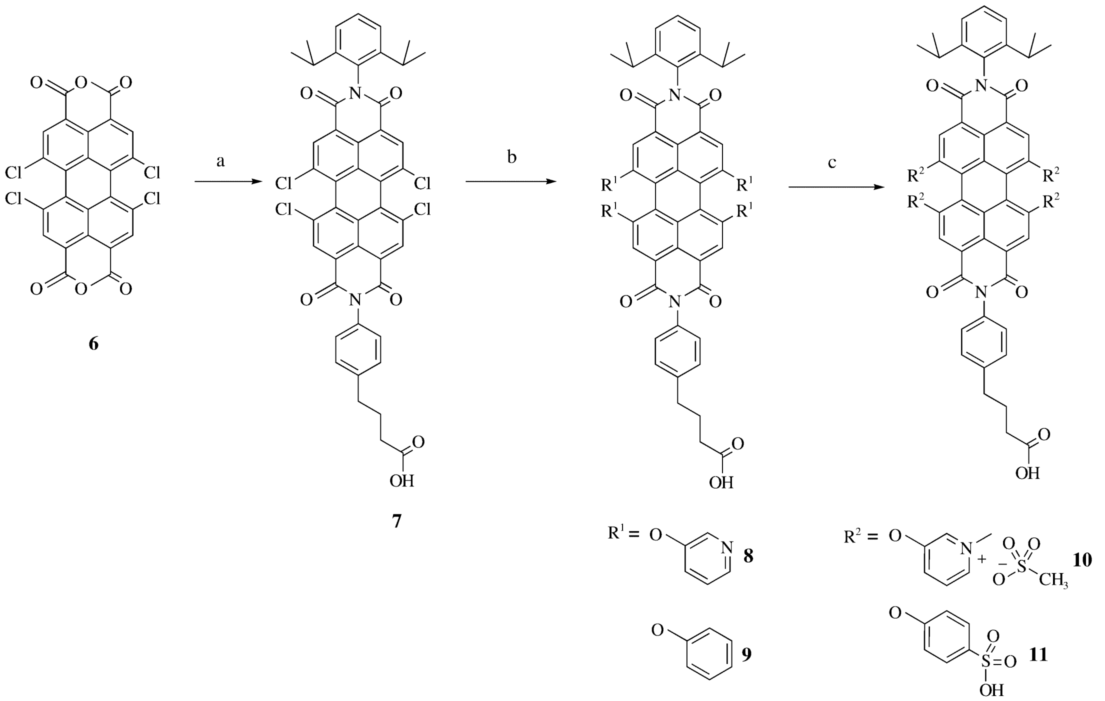


Figure 1. The normalized absorption spectra and fluorescence spectra of **4** (dash) and **5** (solid) in water.

Table 1: Absorption ($\lambda_{\text{max,abs}}$) and fluorescence maxima ($\lambda_{\text{max,flu}}$), and fluorescence quantum yields (Φ_f)[a] in water for **4**, **5**, **10**, **11**, and **12**.

Compound	$\lambda_{\text{max,abs}}$ [nm⁻] (ε [$\text{M}^{-1}\text{cm}^{-1}$])	$\lambda_{\text{max,flu}}$ [nm⁻]	Φ_f
4	434 (9766), 516 (24 628), 547 (33 751)	588	0.66
5	461 (10 708), 541 (21 026), 571 (27 800)	619	0.58
10	432 (9456), 516 (21 818), 547 (29 974)	592	0.58
11	459 (9540), 541 (19 261), 566 (23 160)	618	0.49

[a] Φ_f was measured at room temperature using Cresyl Violet in methanol (the standard value is 0.54) as reference.[19]



Scheme 2. a) 2,6-Diisopropylaniline, 4-(4-aminophenyl)butyric acid, propionic acid, 140 °C, 5 h, 30%; b) **8**: 3-pyridinol, NMP, K_2CO_3, 80 °C, 15 h, 81%; **9**: phenol, NMP, K_2CO_3, 80 °C, 15 h, 80%; c) **10:** 1. methyl iodide, methanol, reflux, 24 h, 94%; 2. silver methanesulfonate, methanol, RT, 5 h, 90%; **11**: conc. sulfuric acid, RT 15 h, 92%.

(≈20 nm) in comparison to the negatively charged PDI **5** due to the stronger electron withdrawing effect of pyridinium. All synthesized PDIs can be excited with 488 and 514 nm (Ar), 532 nm (Nd:YAG) laser lines, and with a 546 nm mercury arc line, owing to the presence of a broad and strong absorption band, to yield intense fluorescence. The dyes have high fluorescence quantum yields (Φ_f), which are measured by using Cresyl Violet in methanol as a reference.[19] PDIs with positive charges have similar Φ_f values to those that have negative charges (0.66 for **4**, 0.58 for **5**). The monofunctional dyes **10** and **11** show very similar optical properties to the analogous symmetric PDIs **4** and **5**. However, the fluorescence quantum yields of the monofunctional dyes are slightly lower (0.58 for **10**, 0.49 for **11**). Clearly the imide structure of the ionic PDIs influences the fluorescence in water.[20] These results validate our concept of introducing water solubility and high fluorescence quantum yield of PDIs in water by introducing substituents in the bay region and also explain the failure of approaches found in the early literature that were based upon functionalization of the imide units.[11,12] The ionic PDIs in water appear to retain photostability in comparison to PDIs in organic solvents or in the solid state.[21] The UV/Vis absorption and the fluorescence spectra of the water solution of PDIs **4** and **5** remain almost unchanged (their intensities decrease by about 6% for **4** and 1% for **5**) in sun light or after radiation with under UV light (365 nm) for one week.

The biological applicability of PDI **4** was demonstrated with a cardiac cell culture.[22] When they were subjected to a dye concentration of 3.0 mm, these cells became intensely fluorescent, which was most pronounced in the perinuclear region (Figure 2). This observation indicates an intracellular deposition of the dye, although the mechanism for entering the cells is not yet clear. After they were stained, the cells appeared healthy, as judged from their morphology, and were devoid of vacuoles and any other sign of structural degradation. In addition, the combination of the PDI treatment with nuclear staining by the blue fluorescent dye 4,6-diamidino-2-phenylindole (DAPI)[1] revealed the presence of double nuclei in some cells (indicated by arrows in Figure 2). Thus, the cells continued to proliferate in the presence of the PDI dye, which underlines the nontoxicity of the labels. Photostability and fluorescence intensity remained for a long period of time after contact with the cell culture, thus indicating that the dye is not metabolized by the cells. In addition, the fixation of the cells after staining does not degrade the fluorescence properties of the water-soluble PDI. These primary results are encouraging in terms of the applicability of commercially available dyes/pigments (PDIs) as biological fluorescent probes.

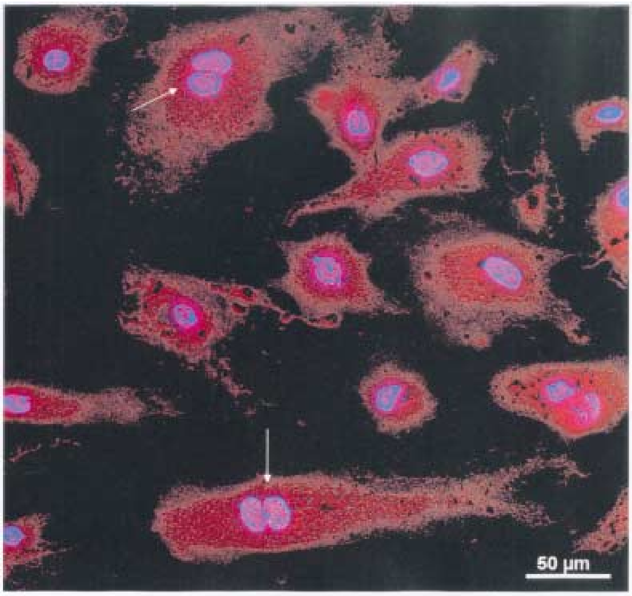


Figure 2. Staining of cardiac fibroblast and muscle cells by PDI **4** (red). The cells' nuclei (blue) were labeled by DAPI. Double nuclei (arrows) indicate ongoing cell division. Micrographs of both staining conditions were separately taken and subsequently overlaid (scale bar 50 µm).

In summary, novel ionic PDI dyes and monofunctionalized ionic PDIs with positively or negatively charged substituents in the bay regions have been developed with simple procedures in high yields. This is the first time that water-soluble PDIs with high fluorescence quantum yields, high extinction coefficients, as well as significant photostability are reported. Due to their broad absorption bands, they can be excited with different excitation sources. The results with ionic PDI on living cells show that the water-soluble dyes are nontoxic and maintaining high fluorescence intensity and photostability. Our future research will concentrate on the mechanism of water-soluble PDIs entering the cells, the role of PDI dyes in specific cell organelles, and the study of DNA and proteins labeled with monofunctional PDIs through covalent bonds, thereby, also using methods of single molecule spectroscopy.[23,24]

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[17] The energy-minimized structures of ionic PDI have been obtained by applying the MM+ force field and the Conjugate Gradient algorithm implemented in the HyperChem 6.0 program from Hypercube Inc. The 3D structure of the PDI-core molecule was first calculated separately by using the semi-empirical PM3 method, and a global minimum was evaluated. Further, the four functional groups were attached to the core, and the geometry of the complete molecule was calculated with the MM+ force field and the Fletcher Reeves algorithm.

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[22] Cardiac cells were obtained from embryonic rat hearts (CD rats; Charles River) and cultivated on fibronectin (Roche Diagnostics, Mannheim, Germany) coated cover-slips for 4 days. Cultures were subjected to PDI dye **4** (0.3 mM in water) for 30 min at 37 °C, rinsed in nutrient mixture F-10 (Invitrogen Corp., Karlsruhe, Germany), and then fixed with 2% paraformaldehyde for 15 min at 4 °C. Subsequently, nuclear staining was achieved by incubation with 0.25 $\mu g\, mL^{-1}$ 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, Taufkirchen, Germany) in PBS pH 7.4 for 4 min at room temperature. Cultures were mounted on microscopic slides and analyzed by using an Olympus IX70 microscope with a U-MNG (PDI dye) and U-MNU (DAPI) fluorescence filter set. Digital micrographs were taken by a cooled CCD device (Photometrics CoolSNAP fx; Roper Scientific, Germany) controlled by the imaging system MetaMorph (Universal Imaging Corp., Downingtown, PA, USA).

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