

# Water-Soluble Monofunctional Perylene and Terrylene Dyes: Powerful Labels for Single-Enzyme Tracking\*\*

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Fluorescence microscopy is the most widely used tool for visualizing subcellular structures and for localizing proteins within cells.<sup>[2]</sup> Single-molecule spectroscopy (SMS) has gone beyond that, and has revealed information about complex biological molecules and processes which are difficult to obtain from ensemble measurements.<sup>[3]</sup> Single proteins, virions, drugs, and other single bioparticles have been labeled and their pathway and interactions followed inside living cells.<sup>[4,5]</sup> One critical issue in observing biological entities at the single-molecule level is the label: It should be water-soluble, highly fluorescent in an aqueous environment, and have a reactive group for attachment to the biomolecule, for example, a protein or enzyme. Moreover, the attachment should not affect the structure or function of the biomolecules, or in the case of an enzyme, its activity. Finally, an exceptional photostability of the label is needed for visualization or tracking over a sufficient period of time. Is there a chromophore that has it all? It appears that rylene dyes fulfil all of these requirements. Rylene chromophores have proven to be a remarkable class of dyes that are characterized by an exceptional thermal and photochemical stability as well as having fluorescence quantum yields close to unity in organic

solvents.<sup>[6,7]</sup> The extreme photostability of several members of the rylene family has been shown in numerous single-molecule experiments.<sup>[8–11]</sup> To covalently attach these outstanding chromophores to proteins, we focused on the synthesis of water-soluble, monofunctional perylene and terrylene chromophores possessing *N*-hydroxysuccinimide ester and maleimide groups. The corresponding rylene-protein conjugates were employed for tracking single phospholipases on their native substrates by real-time wide-field fluorescence microscopy. Enzyme kinetics at the single-molecule level have been previously analyzed by monitoring the conversion of a nonfluorescent substrate into a fluorescent product.<sup>[12–17]</sup> The observed fluctuations in the  $k_{\text{cat}}$  value over time could be correlated with different conformations of the enzyme molecule, with each conformation having its own  $k_{\text{cat}}$  value. Measurement of enzyme activity on native substrates has also been performed at the single-molecule level, namely on DNA-processing enzymes and motor proteins, by using confocal SMS.<sup>[18–20]</sup> However, tracking of single interfacial enzymes such as phospholipases that have complex catalytic pathways and modes of operation has not yet been performed.

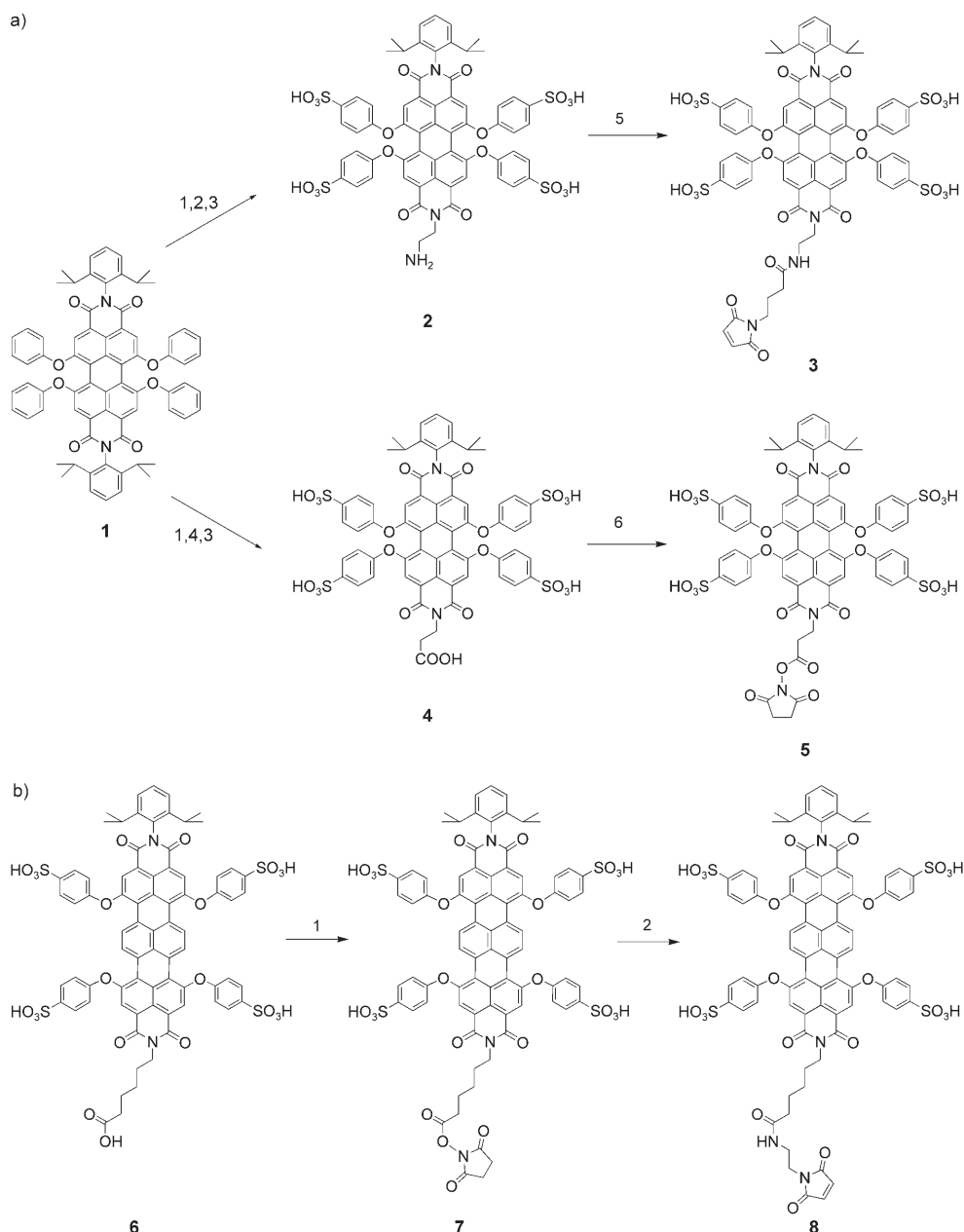
The synthesis of the herein described perylene derivatives starts from the symmetrical perylene diimide (PDI) **1**. One of the imide groups was saponified under strong basic conditions and then further treated with 1,2-ethylenediamine or 3-aminopropanoic acid to give the corresponding monofunctionalized perylenes. Water solubility was achieved by introducing four sulfonyl substituents at the phenoxy groups in the bay regions.<sup>[21]</sup> Compound **2** was treated with 4-maleimido-butyric acid *N*-succinimidyl ester (GMBS) in dry DMF in the presence of triethylamine to give **3** in high yield (Scheme 1a). *N*-hydroxysuccinimide ester **5** was obtained from **4** by using *N,N,N',N'*-tetramethyl(succinimido)uronium tetrafluoroborate (TSTU) and diisopropylethylamine (DIPEA) at RT (Scheme 1a).<sup>[22–24]</sup> Compounds **3** and **5** are highly soluble in water and have high fluorescence quantum yields ( $\Phi_f$ ) in aqueous media (Table 1).<sup>[25,26]</sup> The higher homologue terrylenediimide (TDI) was synthesized by formally introducing one additional naphthalene unit into the perylene scaffold. The water-soluble terrylene dye **6** absorbs above 600 nm, and thus is ideal for single-molecule and live-cell-imaging experiments because autofluorescence of cellular components at that wavelength region is minimal. The synthesis of the monocarboxylic acid functionalized TDI was recently reported,<sup>[9]</sup> and its transformation into the activated ester **7** was accomplished by its treatment with TSTU and DIPEA, as described for **5** (Scheme 1b). Compound **7** was employed as the starting material for the maleimide-functionalized TDI

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[\*\*] Support from the FWO (grant G.0366.06), the KULeuven Research Fund (GOA) 2006/2, Centre of Excellence INPAC, Centre of Excellence in Catalysis (CECAT), the Flemish Ministry of Education (ZWAP 04/007), the Portuguese Foundation for Science and Technology (FCT), the Federal Science Policy of Belgium (IAP VI), and the EU Sixth Framework Programme (STREP Bioscope) is acknowledged.



Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



**Scheme 1.** Synthesis of perylene and terrylene labels: a) Reagents, conditions and yields: 1) KOH, isopropanol, 140 °C, 12 h, 24%; 2) ethylenediamine, toluene, 60 °C, 3 h, 60%; 3) H<sub>2</sub>SO<sub>4</sub>, RT, 16 h, 97%; 4) 3-amino-propanoic acid, propionic acid, 140 °C, 2 h, 65%; 5) GMBS, triethylamine (TEA), DMF, 5 h, 95%; 6) TSTU, DIPEA, DMF, water, 1,4-dioxane, 1 h, 85%. b) Reagents, conditions, and yields: 1) TSTU, DIPEA, DMF, water, 1,4-dioxane, 1 h, 80%; 2) 1-(2-aminoethyl)pyrrol-2,5-dione, DMF, TEA, 5 h, 90%.

derivative **8**. The synthesis of **8** was realized by treating **7** with 1-(2-aminoethyl)pyrrole-2,5-dione in DMF.

The perylene derivative **5** was used for the labeling of phospholipase (PLA1). As the seven solvent-exposed lysine residues of the enzyme were targeted, **5** was applied in 20-fold excess to the enzyme. The purification of the conjugates was accomplished by coupling the unreacted dye to a solid support (see the Supporting Information). The resin consisted of a low-cross-linked polystyrene matrix onto which polyethylene glycol containing a free terminal amino group was grafted;

such resins are usually employed for solid-phase organic synthesis. Here the unreacted dye was covalently captured after addition of the support to the labeling solution, and the labeled enzyme was isolated by filtration. Gel electrophoretic analysis confirmed that the unreacted label was removed very efficiently (see Figure S2 in the Supporting Information).

Further evidence for the effective removal of unreacted dye was obtained by performing fluorescence correlation spectroscopy measurements (see the Supporting Information). A diffusion coefficient of  $2.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  was measured for the free dye, whereas a value of  $1 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  was found for the labeled protein. The values are in agreement with calculations based on the molecular weight of the protein (see the Supporting Information). Labeling of the enzyme with TDI derivative **7** was achieved in a similar way. This novel strategy allows the convenient and fast separation of labeled enzymes without the need to perform time-consuming chromatographic or electrophoretic purification steps.

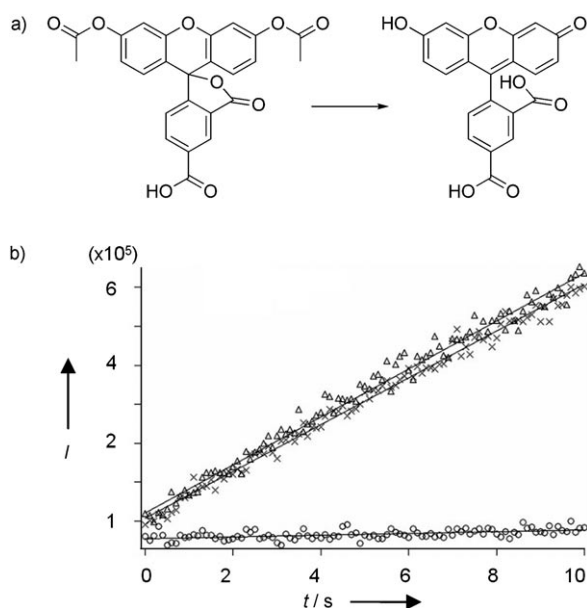
The bulk activity of the labeled enzyme was tested. PLA1 catalyzes the saponification of the acetate groups of the fluorescein derivative, which is nonfluorescent (Figure 1a). The hydrolysis of the substrate can be followed

by the increase in the fluorescence intensity arising from the formation of the fluorescent product by the enzymatic reaction. Figure 1b shows the changes in the fluorescence intensity of a similar solution of pro-fluorescent substrate as a function of time without the enzyme, after adding non-labeled enzyme, and after adding the labeled enzyme. The rate constants of the hydrolysis were estimated to be  $(3001.3 \pm 29.1) \text{ s}^{-1}$  and  $(3083.1 \pm 38.1) \text{ s}^{-1}$  for the labeled and the nonlabeled enzyme, respectively. The autohydrolysis of 5-CFDA was measured as  $(110 \pm 17.9) \text{ s}^{-1}$ . The observed

**Table 1:** Absorption ( $\lambda_{\text{max,abs}}$ ) and fluorescence maxima ( $\lambda_{\text{max,flu}}$ ), as well as fluorescence quantum yields ( $\Phi_f$ )<sup>[a]</sup> in water for **2–5**, **7**, and **8**.

Compound	$\lambda_{\text{max,abs}}$ [nm] ( $\epsilon$ [M <sup>-1</sup> cm <sup>-1</sup> ])	$\lambda_{\text{max,flu}}$ [nm]	$\Phi_f$
<b>2</b>	450 (10850), 534 (21825), 562 (22243)	620	0.39
<b>3</b>	450 (8367), 534 (16199), 566 (16569)	620	0.40
<b>4</b>	450 (11454), 534 (22510), 564 (25059)	620	0.66
<b>5</b>	450 (9951), 534 (18867), 566 (21071)	622	0.58
<b>7</b> <sup>[b]</sup>	426 (4569), 638 (24468)	—	—
<b>8</b> <sup>[b]</sup>	426 (2782), 638 (16633)	—	—

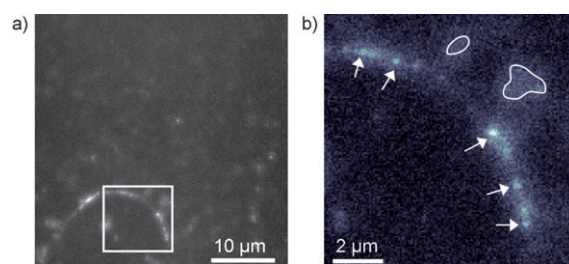
[a]  $\Phi_f$  was measured at room temperature using cresyl violet in methanol ( $\Phi_f = 0.54$ ) as a reference.<sup>[25]</sup> [b] The water-soluble terylene derivatives form nonfluorescing H aggregates in water under the conditions employed here. However, when highly diluted they are well suited for single-molecule studies.<sup>[9]</sup>



**Figure 1.** Relative enzymatic activity in the bulk phase. By using a pro-fluorescent substrate it is possible to follow the reaction kinetics through the increase in the fluorescent intensity. a) Hydrolysis of nonfluorescent 5-carboxyfluorescein diacetate (5-CDFA) to the fluorescent product 5-carboxyfluorescein (5-FAM). b) Fluorescence intensity of 5-FAM as a function of time. For the activation of the surface enzyme in solution (for example, to open the lid protecting the active site), tritonX-100 was added to the solution; 0.7 mM 5-CDFA, 0.5 mM tritonX-100. x: nonlabeled PLA1 (3E-8M), (3001.3 ± 29.1) s<sup>-1</sup>; Δ: PLA1 labeled with *N*-hydroxysuccinimideperylene derivative **5** (3E-8M), (3083.1 ± 38.1) s<sup>-1</sup>; ○: 5-CFDA autohydrolysis (110.54 ± 17.9) s<sup>-1</sup>. The labeling of the enzyme has no effect on its activity towards 5-CFDA.

rate constants clearly show that no activity was lost by the labeling.

In the next step, single-enzyme tracking was performed by using wide-field fluorescence microscopy. For this, the labeled PLA1 was added on to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) supported bilayers and a snapshot was taken every 50 ms. Figure 2a shows the fluorescence image obtained with the enzyme labeled with PDI **5**. The individual labeled enzymes can be clearly observed as bright spots (Figure 2). Furthermore, it is possible to distinguish



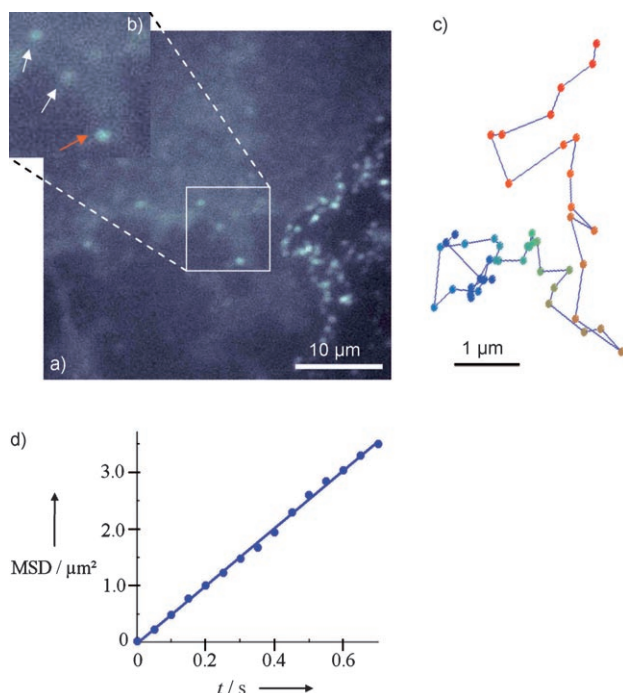
**Figure 2.** Fluorescence image of individual labeled enzymes on POPC layers. The phospholipid bilayers were prepared by the rehydration method using mica as a support<sup>[1]</sup> a) PLA1 labeled with PDI **5** on a nonlabeled POPC layer; b) magnification of the area in image (a) indicated by the white square. The arrows indicate clearly distinguishable individual enzymes located on the edge of the layer. Note that the signal-to-noise is much higher than for the non-adsorbed molecules diffusing in solution (outlined). Exposure time for each frame 50 ms; the excitation wavelength for the dye was 532 nm.

between PLA1 interacting with the edge of the POPC layers and ones diffusing on the top of the layer (Figure 2b). Individual enzymes interacting with the POPC layers edge show up as diffraction-limited spots in the image (Figure 2b), whereas freely diffusing enzymes appear as blurry spots (Figure 2b). Similar results were obtained using phospholipase labeled with terylene derivative **7** (data not shown).

To correlate the diffusion of the labeled enzyme with hydrolysis of the POPC layers, 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) was incorporated into the phospholipid layers to render them fluorescent, albeit at a different emission wavelength than the label. The use of labeled phospholipid layers allows a clear visualization of the edge between two consecutive layers or between a bilayer and the support (Figure 3a). Furthermore, hydrolysis of the layers can also be followed by the drop in the quantum yield of DiO as a result of the increase in *cis-trans* isomerization when it is freely diffusing in aqueous solution. Even in these difficult conditions for SMS (introduction of a fluorescent background), the new labels allow us to obtain a sufficient signal-to-noise ratio to discriminate and track individual enzymes on the labeled POPC layers (Figure 3a,b).

Figure 3c shows the trajectory of an enzyme diffusing along the edge of the layer (see the movie in the Supporting Information). The diffusion coefficient can be quantified by calculating the mean-square displacement from these trajectories. The diffusion constant of the phospholipase molecule shown by the trajectory in Figure 3 is (1.276 ± 0.014) μm<sup>2</sup>s<sup>-1</sup>. The interaction between enzymes and the substrate layers results in the value of the diffusion constant being lower than the usual values obtained for the free diffusion of proteins in solution (ca. 10<sup>2</sup> μm<sup>2</sup>s<sup>-1</sup>).

Most of the enzymes in the recorded movies stayed on the layers for less than 0.3 s. It has been shown that the water-soluble PDI label **5** has a survival time of 120 s when immobilized in poly(vinyl alcohol).<sup>[27]</sup> Therefore, the disappearance of the labeled enzymes after 0.3 s is probably associated with enzyme activity as well as its mode of action (nonprocessive hydrolysis of the layers).



**Figure 3.** Fluorescence image of individual enzymes labeled with PDI 5 on POPC layers labeled with DiO. The PDI label allows sufficient photons to be obtained to discriminate individual enzymes even with a fluorescence background from the layers. a) Labeling of the layers allows visualization of steps in the layers as well as the preferential adsorption of the enzyme molecules at the steps. The image was obtained after accumulating 8 frames (400 ms) b) Magnification of the area indicated by the white square in image (a). Single enzyme molecules are indicated by arrows. The exposure time for each frame was 50 ms; the excitation wavelengths for PDI and DiO were 532 and 488 nm, respectively. The fluorescence from both dyes is detected through the same filters, see the Supporting Information. c) Trajectory described by the enzyme indicated by the orange arrow in (b) which is on the edge of a POPC layer labeled with DiO. d) Plot of the mean-square displacement (MSD) of the enzyme for the first points of the track. From these data it is possible to calculate the diffusion constant of a single active enzyme as  $(1.28 \pm 0.02) \mu\text{m}^2\text{s}^{-1}$ .

In summary, new water-soluble, fluorescent, monofunctional perylene- and terrylenediimide derivatives have been introduced. These dyes can be attached to a variety of proteins through their reactive functional groups. In regard to protein labeling, a convenient procedure for the removal of unreacted dye from labeled enzymes has been developed which involves capturing excess dye with a solid support.

The performance of the new fluorescent probes was assessed by single-particle tracking. The measurements revealed that single enzymes could even be visualized on a fluorescently labeled substrate. The outstanding photostability of the dyes and their extended survival times under strong illumination conditions allow the actions of enzymes to be characterized on their natural substrates, in this case, phospholipase acting on phospholipid-supported layers. By using this approach, enzyme mobilities could be correlated with the catalytic activity. Furthermore, this assay allows the validation

of the influence of the layer composition, fluidity, etc. on both parameters (enzyme mobility and activity).

Received: November 26, 2007

Revised: February 11, 2008

Published online: March 26, 2008

**Keywords:** dyes/pigments · enzymes · fluorescent probes · kinetics · single-molecule studies

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