Lipid Bioimaging

Synthesis and Self-Assembly of Bay-Substituted Perylene Diimide Gemini-Type Surfactants as Off-On Fluorescent Probes for Lipid Bilayers


Abstract: Interest in bay-substituted perylene-3,4:9,10-tetracarboxylic diimides (PDIs) for solution-based applications is growing due to their improved solubility and altered optical and electronic properties compared to unsubstituted PDIs. Synthetic routes to 1,12-bay-substituted PDIs have been very demanding due to issues with steric hindrance and poor regioselectivity. Here we report a simple one-step regioselective and high yielding synthesis of a 1,12-dihydroxylated PDI derivative that can subsequently be alkylated in a straightforward fashion to produce nonplanar 1,12-dialkoxy PDIs. These PDIs show a large Stokes shift, which is specifically useful for bioimaging applications. A particular cationic PDI Gemini-type surfactant has been developed that forms nonfluorescent self-assembled particles in water (“off state”), which exerts a high fluorescence upon incorporation into lipophilic bilayers (“on state”). Therefore, this probe is appealing as a highly sensitive fluorescent labelling marker with a low background signal for imaging artificial and cellular membranes.

Introduction

Substituted perylene-3,4:9,10-tetracarboxylic diimides (PDIs) have attracted interest for a variety of applications, including organic electronics,[1–3] single molecule spectroscopy[4–6] and bioimaging.[7–9] For bioimaging, fluorescent quenching of PDIs has been used to sense hydrophobicity changes such as in artificial and cellular bilayers.[10–20] However, PDIs as highly sensitive fluorescent probes with a low background signal are still rare. Such off-on fluorescent probes would be appealing for cellular imaging. Also, surprisingly few examples have been reported of water-soluble PDIs not bearing large-MW solubilising substituents (e.g., dendrimer like).[9, 21–23]

Fine tuning of the physical, optical, and electronic properties of PDIs is typically achieved through substitution of the perylene ring system at either the imide-, ortho-, or bay positions, or a combination of the three to customise the optical, electronic and self-assembly properties of the PDIs (Figure 1a).[24, 25] Substituents on the imide nitrogen atoms of PDIs tend to im-
prove solubility\cite{26,27} while retaining the planarity of the perylene aromatic ring system, thus preserving the perylene’s optical properties.\cite{28} N-substitution of PDIs can also influence their self-assembly properties, which has stimulated intensive studies into bulk and solution-based applications of these compounds.\cite{29–32} By contrast, substitution of the perylene ring system at either the ortho- or bay positions can significantly influence the optical and solubility properties of the PDIs, dependent upon the substitution pattern, the steric bulk and electronic properties of the substituent group.\cite{33–36} Bay substitution, for example, increases steric hindrance in this region of the molecule, which is relieved by out-of-plane twisting of the PDIs perylene ring system. This phenomenon causes a disruption to the π–π stacking behaviour of the PDI, which leads to improved solubility and altered physical properties.\cite{33,37} Substitution of PDIs at the bay position is, however, synthetically demanding, involves halogenation reactions, and predominantly four-fold bay-substituted PDIs have been reported.\cite{38,39–43} Partly substituted 1,6- and 1,7-regioisomers, commonly as mixtures,\cite{44–48} have also been synthesised,\cite{44,50} while 1,12-bay-substituted PDIs have been rarely reported. The latter are interesting because the distortion of the perylene ring system by the 1,12-substitution will strongly alter the optical properties of the compound, comparable to their corresponding tetrasubstituted PDIs, while solubility and self-assembly properties will be different.\cite{51–53} Unfortunately, 1,12-bay-substituted PDIs have to be accessed through halogenation/dehalogenation chemistry,\cite{54–57} which is typically inefficient, cumbersome, and low yielding. Controlled and efficient access to 1,12-bay-substituted PDIs is evidently challenging, and yet also potentially highly valuable given their untapped chemical potential and prospective influence on the PDIs optical properties.

Here we report a one-step, atom-efficient, and regioselective synthesis of a 1,12-dihydroxylated PDI based on violanthrone chemistry (Figure 1a).\cite{58} This bay-substituted PDI acts as an intermediate towards the introduction of structurally diverse polar and apolar side-chains through straightforward O-alkylation chemistry. A particular cationic bay-substituted PDI derivative, resulting from this synthetic approach, can be regarded as a new type of gemini-surfactant\cite{59} bearing two hydrophilic head groups, two hydrophobic groups and a rigid fluorescent core. This amphiphile forms nonfluorescent self-assembled particles in aqueous solution and light upon incorporation in a hydrophobic matrix. This property can be exploited as an off-on fluorescence probe for artificial and cellular lipid bilayers (Figure 1b).

**Results and Discussion**

Starting material PDI 1 was prepared from parent perylene-3,4,9,10-tetracarboxylic diimide and n-amylamine\cite{60} and purified\cite{61} according to reported procedures. Using a procedure derived from violanthrone chemistry,\cite{62} PDI 1 was subsequently oxidised by activated manganese(IV) oxide (MnO₆) in sulfuric acid and then partially reduced using sodium sulfite, introducing two hydroxyl groups at positions 1 and 12, yielding PDI 2 (Scheme 1). The \( ^{1}H \) and \( ^{13}C \) NMR of PDI 2 was found to be remarkably clean. Moreover, a broad vibrational mode associated with OH stretching was observed for PDI 2 in the functional group region of the solid-phase infrared spectrum, providing further evidence for the introduction of the hydroxyl groups (Figure S1). Given its low solubility, PDI 2 was directly used for alkylation without further purification. Substitution of the bay hydroxyls were chosen such that PDI derivatives with varying side-chain polarity were synthesised. This allows for a clear analysis of the influence of side-chain polarity on the photophysical and morphological properties of the products. With 1,12-dihydroxylated PDI 2 in hand, subsequent reactions with 2-ethylhexylbromide, monomethyl triethylene glycol p-toluenesulfonate, 1,6-dibromohexane and n-octylbromide yielded PDI 3–5 and 7, respectively (Scheme 1). Notably, all introduced linear and branched bay substituents ensured excellent solubility of PDI 3–5 and 7 in organic solvents as compared with the solubilities of their parent PDIs 1 and 2. After separation of PDI 5 from its cyclised side product by silica gel chromatography, substitution of the bromides with trimethylamine yielded doubly charged cationic species PDI 6, which was conveniently purified by precipitation from acetone. As discussed previously, substitution at bay area positions, such as the 1,12-positions, can be particularly challenging because of steric restrictions. In contrast, this synthetic route has allowed for satisfactory overall yields of 43, 60, 43 and 13 % for PDI 3–5 and 7, respectively.

Examination of PDI 2 revealed no evidence for the formation of mono-, tri- or tetra-bay hydroxylated derivatives by mass spectrometry, \(^{1}H \) and \(^{13}C \) NMR analyses. Considering the much improved solubility of the substituted derivative of PDI 2, a detailed 2D-NMR study was performed on PDI 3 (Figures S2–S5 in the Supporting Information). The perylene-bound protons of PDI 3 were assigned by the presence of \(^{3}J_{CH} \) correlations between protons H₆ (\( \delta = 8.55 \) ppm) and carbonyl carbons C₅ (\( \delta = 163.9 \) ppm) and between H₄ (\( \delta = 8.43 \) ppm) and carbonyl car-
bons $C_7$ ($\delta = 163.8$ ppm) and by the absence of this correlation for $H_a$ ($\delta = 8.48$ ppm) (Figure 2a). The presence of additional $\gamma_{CH}$ correlations between protons $H_a$ ($\delta = 8.55$ ppm) and $H_d$ ($\delta = 8.43$ ppm) with carbon $C_6$ ($\delta = 123.6$ ppm) and the absence of a $\gamma_{CH}$ correlation between one single carbon both with $H_a$ ($\delta = 8.48$ ppm) and $H_d$ ($\delta = 8.43$ ppm) simultaneously, is consistent with either a 1,7- or a 1,12-substituted $^{[56]}$ isomer. Protons $H_g$ were observed as two signals at $\delta = 4.28$ and 4.02 ppm, both correlating with a single carbon signal $C_5$ at $\delta = 72.8$ ppm in a HSQC experiment (Figure S3). Cross peaks between these proton signals were observed in a COSY experiment (Figure S4), indicating that each signal arises from two protons $H_g$ each bound to a different $C_g$ carbon, and that protons $H_g$ are thus diastereotopic. In a NOESY experiment, these protons show a through-space dipolar interaction with protons $H_g$ corroborating the 1,12-substitution topology. Moreover, cross peaks between protons $H_g$ were herein also observed, which is expected for nonequivalent protons bound to the same carbon (Figure S5). However, these nonexchangeable protons show the same phase as the diagonal, indicating conformational exchange, classified as restricted rotation, at these positions.$^{[63]}$ These conformational exchange peaks lead to the assumption that the 1,12-substitution pattern distorts the aromatic ring system of the perylene, hence altering the physical properties as noted before herein.

The above detailed 2D NMR study unequivocally proves that the applied oxidative chemistry to acquire dihydroxylated PDI 2, indeed results in 1,12-dialkoxy PDI regioisomers after alkylation. Note that this 1,12-assignment is in contradiction with reported 1,7-assignments (that were based solely on $^1H$ NMR spectra) for PDIs that have been prepared using a procedure similar to that delineated here.$^{[63,64]}$ but that the 1,12-assignment is in line with regioisomer assignments as reported for hydroxylated violanthrones.$^{[58]}$ A comparative synthesis of a 1,7-substituted PDI and its 1,12-isomer (see the Supporting Information) provides further evidence for the assignment of the 1,12-substitution topology for PDIs prepared by our procedure.

Figure 2b shows the stacked $^1H$ NMR spectra of the 1,7- and 1,12-substituted regioisomers of di-$n$-octyl-substituted PDIs 11 and 7, respectively. The resonance peaks corresponding to protons $H_g$ in the aromatic region of PDI 11 shift significantly downfield through intramolecular hydrogen-bonding of $H_g$ with the adjacent oxygen atom in the 1,7-substitution topology. In contrast, the corresponding $H_g$ proton signals in PDI 7 are shifted more upfield, presumably because no intramolecular hydrogen bonding can take place in this 1,12-substituted PDI. Moreover, PDI 11 shows an expected triplet for protons $H_g$ at circa 4.3 ppm, whereas the restricted rotation of the alkoxy substituents in PDI 7 results in a broad peak for these protons (Figure S6). This loss of clear multiplicity for protons $H_g$ is a feature of all 1,12-substituted PDIs studied in the paper.

The bay-substituted PDIs 3, 4 and 6 were studied by UV/Vis and fluorescence spectroscopy in their molecularly dissolved state in anhydrous dimethyl sulfoxide (DMSO). This solvent was chosen to ensure complete solubility for the doubly charged PDI 7. Non-bay-substituted PDI 1 is notably less soluble than PDIs 3–7 and tends to aggregate at higher concentrations in DMSO. Gratifyingly, PDI 1 was sufficiently soluble in anhydrous tetrahydrofuran (THF) to enable optical measurements at the desired concentration. Table 1 shows the maximum absorption and emission wavelengths, including the extinction coefficient and the quantum yield, of non-bay- and 1,12-bay-substituted PDIs 1, 3, 4 and 6 as derived from the UV/Vis and fluorescence spectroscopy studies. The absorption spectra of PDIs bearing a 1,12-substitution topology clearly show a bathochromic shift, broadening of the lowest energy absorption band and thus a loss of sharp vibronic progressions as compared with parent PDI 1 (Figure S7). The latter effect is generally attributed to an out-of-plane distortion of the perylene ring system because of the sterically encumbered substituent groups.$^{[65,66]}$ The overall bathochromic shift of the maximum absorption wavelength is due to the $\pi$-donating effect of the oxygen substituents on the perylene ring system.$^{[52]}$ The fluorescence spectra of 1,12-bay-substituted PDIs are a mirror image of their corresponding absorption spectra (Figure 3a),
including the loss of vibronic fine structure accompanied by a relatively large Stokes shift as compared with PDI 1. The distorted planarity of the perylene ring system additionally contributes to a large bithropic shift of ca. 100 nm. Quantum yields of ca. 70–80% were measured for all PDI derivatives, independent of the substituents and spectral shapes (Table 1).

Nano-sized assemblies of the PDIs were prepared through reprecipitation in aqueous media. Rapid injection of a DMSO stock solution, containing molecularly dissolved PDI, into filter-sterilised demi water resulted in self-assembled architectures for all PDIs bearing a bay substitution topology. PDI 1 did not form stable self-assembled architectures, but instead formed clearly visible and much larger aggregates (i.e., precipitates) in aqueous solution, also at low concentrations, preventing determination of its optical properties in water. The hypsochromic shift of the absorption maxima of 1,12-bay-substituted PDIs through reversal of the vibronic transitions revealed the formation of H-type π–π stacks, which is accompanied by a distinct overall drop in absorption intensity (Figure 3b). Whereas vibronic structure blurring was already observed in organic solvent because of core planarity distortion, a significant drop in overall fluorescence intensity and quantum yield is typically attributed to intermolecular π–π interactions. Even though PDIs are known for their nonradiative relaxation from their excited-state upon polarity induced face-to-face interactions, these bay-substituted PDI derivatives show a large Stokes shift of approximately 100 nm and a bathochromically shifted fluorescence spectrum.

The morphological characteristics of the formed architectures were determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS analysis showed a strong scattering correlation of PDI 3 and 4 (Figure S8). Fits of the scattering intensity distribution revealed hydrodynamic radii of 83 and 48 nm, respectively (Figure 3c), which are consistent with the observations in TEM images (Figure 3d). Intuitively, the larger particle size of PDI 3 could be explained by the hydrophobicity of the side-chains. Moreover, the drop-casted particles were spherical with a fairly monodisperse distribution. In contrast to PDIs 3 and 4, neither scattering correlation nor electron microscopic visualisation of cationic PDI 6 could be achieved, suggesting the absence of large aggregates. Whereas the optical data of PDI 6 shows signs of self-assembly, we envision the formation of only small aggregates. The deviating behaviour for PDI 6 is likely due to charge repulsive forces, as has been observed for other polar PDI derivatives. In summary, these novel PDI derivatives show an electronic S_0–S_1 transition resulting in strong absorption in the visible region between 500 and 650 nm in both monomeric and self-assembled state, which makes them suitable for fluorescence microscopy. Moreover, they display bathochromically shifted fluorescence spectra, as compared with non-bay-substituted perylenes.

A challenge in bioimaging is to date is to find nontoxic fluorophores of small size and high brightness that operate beyond the optical spectral window of biomatter. From this perspective, all three 1,12-bay-substituted perylene derivatives exhibit interesting physical properties, with PDI 6 being most interesting for bioimaging applications because of its cationic amphiphilic nature, which potentially favours cellular uptake. The potential of PDI 6 as bioimaging probe was investigated by first studying its interaction with artificial lipid bilayers. Therefore, a 5 μM aqueous solution of PDI 6 was incubated in the presence of giant unilamellar vesicles (GUVs) prepared from dipalmitoylphosphatidylcholine (DPPC) by using a standard reverse-phase evaporation method. The resulting fluorescence emission spectrum showed a maximum emission wavelength at 616 nm similar to the maximum observed for this perylene derivative in DMSO, and hypsochromically shifted as compared with the fluorescence in aqueous solution (Figure 3b). This effect indicates that, in the presence of GUVs, PDI 6 is surrounded by a

### Table 1. Optical characteristics of PDI derivatives 1, 3, 4 and 6 in THF (for 1) and DMSO (for 3, 4 and 6) and in H₂O.

<table>
<thead>
<tr>
<th></th>
<th>UV/Vis absorption λ [nm] (ε [10⁸ M⁻¹ cm⁻¹])</th>
<th>Fluorescence λₘₑₓ [nm] (φₘ [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>THF or DMSO</td>
<td>H₂O</td>
</tr>
<tr>
<td>1</td>
<td>430 (9.1); 455 (20.1); 485 (46.8); 520 (74.0)</td>
<td>_IN</td>
</tr>
<tr>
<td>3</td>
<td>400 (9.5); 417 (11.1); 547 (27.2); 582 (36.2)</td>
<td>418 (4.6); 547 (11.9); 585 (9.8)</td>
</tr>
<tr>
<td>4</td>
<td>396 (7.1); 416 (9.2); 546 (26.4); 583 (37.1)</td>
<td>423 (4.4); 540 (13.7); 606 (5.5)</td>
</tr>
<tr>
<td>6</td>
<td>396 (6.6); 417 (8.5); 546 (24.8); 585 (36.8)</td>
<td>424 (3.5); 556 (11.3); 607 (6.1)</td>
</tr>
</tbody>
</table>

[a] N,N'-Bis(pentylhexyl)perylen-3,4:9,10-tetracarboxylic diimide (φ₀ = 0.99) in methylene dichloride was used as a reference. [b] PDI 1 is insoluble in H₂O and hence precipitated at 1.5 x 10⁻³ M.
less polar environment than pure water, and is presumably incorpo-
rated in the lipid bilayer (Figure 4a). Moreover, the in-
creased overall perylene fluorescence of the GUVs led us to hy-
pothesise that the molecules are incorporated into the lipid bi-
layer in monomeric form. Results from temperature-dependent
fluorescence studies strengthen this hypothesis as the perylene
fluorescence was observed to increase at temperatures above
the DPPC transition temperature of 41 °C (Figure 4a). Dye in-
corporation most likely occurs through insertion of the apolar
perylene moiety into the lipid bilayer, whereas the polar heads
are exposed to the outer environment along with the polar
lipid headgroups.[70] Fluorescence microscopy images show
highly fluorescent GUVs after excitation of PDI 6, and confocal
laser scanning microscopy (CLSM) images strongly suggest
that the fluorescence originates from the lipid bilayer, corrobor-
ating the hypothesis of incorporation of PDI 6 into the lipid
bilayer (Figure 4b–d).

Next, the optical properties as well as the aggregation state
of PDI 6 were studied in phosphate buffered saline (PBS),
having a higher ionic strength than pure water. The absorption
spectra of PDI 6 in water and PBS were similar (Figure S9 a),
indicating an aggregated state of PDI 6 in both solutions. Interest-
ingly, a slight drop in intensity of the electronic S0→S1 transition
can be observed in PBS, which is nevertheless indicative of dif-
fences. Figures S9b and S9c show the scattering corre-
lation and the fitted hydrodynamic diameter, of around
275 nm, of PDI 6 in PBS. This DLS analysis shows that the
doubly charged perylene derivative is assembled in larger
nanostructures in PBS, in contrast to water, most likely because
of the ionic strength of the buffer.

Cellular uptake studies were performed to study the poten-
tial of PDI 6 as an off-on fluorescent lipid probe or organelle
stain for bioimaging applications.[71,72] For these applications,
the toxicity of a probe needs to be kept to a minimum; thus,
the cellular toxicity of PDI 6 was measured with a MTT-cell ac-
tivity assay. PDI 6 was observed to be nontoxic at probe con-
centrations up to the highest test concentration (100 μM) after
incubation for two hours (Figure S10). Based on these findings,
a 5 μM concentration was chosen for all cellular incubation
studies to ensure good fluorescence signal while minimising
toxicity.

Hela cells were incubated with PDI 6 for two hours and ana-
ysed by CLSM after washing. Cellular uptake of this cationic
probe was clearly observed under the aforementioned condi-
tions (Figure 5) compared with the negative control in which

![Figure 5. CLSM images of Hela cells after two hours incubation with a 5 μM solution of PDI 6 (nuclei are stained with Hoechst dye, scale bar: 50 μm).](image)

no probe was administered (Figure S11). Subsequently, the cell-
ular uptake process was monitored by incubating Hela cells
with the probe under the same conditions, followed by time-
course analysis by CLSM (Figure S12). Interestingly, immediately
upon administration, the probe was observed to accumulate
at the plasma membrane, which reached saturation within
5 minutes incubation time. The first signs of cellular uptake
were then seen after 15 minutes incubation in the form of
small vesicular structures at the inside of the plasma mem-
brane. These small vesicular structures then appeared to trans-
locate further into the cell, apparently towards the Golgi appa-
ratus as they eventually surround the nucleus after about
30 minutes, post-administration. This distribution pattern indi-
cates cellular uptake of the probe through an endocytic path-
way. It is important to note here that the cells were not
washed prior to imaging and that the PDI nanostructures were
not visible in the surrounding media, thus only become highly
fluorescent upon interaction with the cellular membrane.

Co-localisation studies on perylene probe PDI 6—two stain-
ing compartments involved in endocytic routes and the third
stains parts of the cytoskeleton (Figure 6)—revealed that the
perylene probe did not interact with actin in the cytoskeleton
nor did they localise in the endoplasmatic reticulum (ER). The
lysosomal tracker shows a high correlation and, thus, the
nature of the observed vesicular structures can be assigned as
lysosomes. The lack of strong co-localisation with the ER as
well as high lysosomal co-localisation are not uncommon for
endocytic pathways. Nanostructures that are endocytically in-
ternalised are commonly transported to lysosomes.[73] However,
whereas nanosized architectures are usually internalised as a
whole inside the lysosome where the digestive processes occur, it is hypothesised that PDI 6 stains hydrophobic compartments by merging with the membrane. Indications for the insertion of probe into the fluid lipid membrane were found by performing full wavelength fluorescence measurements on the cells. Figure 7 shows the emission spectrum of cells incubated with 5 \( \mu \text{M} \) of PDI 6 for two hours as well as the spectra of the probe in DMSO and water at similar concentrations as measured by CLSM, respectively.

![Figure 6](image1.png)

**Figure 6.** CLSM images of Hela cells after two hours incubation with a 5 \( \mu \text{M} \) solution of PDI 6 co-stained with different organelle markers, showing the lack of co-localisation with actin and ER markers while strong co-localization with a lysosomal marker is shown (nuclei are stained with DAPI in fixed cells and with Hoechst in live cells, scale bar: 50 \( \mu \text{m} \)).

![Figure 7](image2.png)

**Figure 7.** Normalised fluorescence spectra of 5 \( \mu \text{M} \) solution of PDI 6 in DMSO and water and Hela cells incubated with 5 \( \mu \text{M} \) solution of PDI 6 as measured by CLSM, respectively.

Conclusions

We have shown an efficient and facile synthetic entry into exclusively 1,12-bay-substituted perylene diimides (PDIs) through a direct hydroxylation reaction. Accordingly, this substitution pattern is accessed without the need for sequential halogenation and dehalogenation reactions. Further derivatisation of the two hydroxyl groups allows a wide range of substituents to be to potentially introduced through straightforward O-alkylation and leads to PDIs that display an increased solubility in organic solvents. The 1,12-substitution of the PDIs results in a bathochromic shift of the optical properties of the compounds, which is favourable for imaging applications. Upon introducing hydrophilic groups, the PDIs can be regarded as a new class of gemini surfactants having unprecedented optical and self-assembly properties. For example, GUVs could be subjected to nonfluorescence aggregates of a cationic PDI derivative bearing two quaternary ammonium moieties and the lipophilic bilayer of the GUVs turned highly fluorescent immediately after administration. Fluorescence measurements showed the incorporation of the PDI derivatives in the lipophilic bilayer. Subsequent cellular studies showed similar results, as PDI fluorescence was strongly observed in cellular membranes and cellular uptake was observed only 15 minutes after administration. Co-localization studies and full wavelength cell fluorescence measurements revealed that the quenched aggregates merge with cellular membranes. Given the concomitant monomerisation and, hence, fluorescence recovery of the perylene ring system, this synthetically easily accessible PDI amphiphile is functioning as an off-on fluorescent probe for imaging lipid bilayers. Moreover, this probe can also be envisioned to function as a potential trackable platform to anchor bio-active compounds into membrane-containing cellular compartments.

Experimental Section

General remarks

Details on used chemicals and equipment can be found in the Supporting Information.

Dynamic light scattering

DLS experiments were performed with a Malvern Instruments Limited Zetasizer \( \mu \text{V} \) (model: ZMV2000). The incident beam was produced with a HeNe laser operating at 632 nm. Samples were measured unfiltered at 1.5 \( \times 10^{-3} \) M.

Transmission electron microscopy

Visualisation by TEM was performed with a Technai G2 Sphera by FEI operating at an acceleration voltage of 80 kV. Samples were prepared by drop-casting a 1.5 \( \times 10^{-3} \) M aqueous PDI solution on a carbon film on a 400 square mesh copper grid and dried for one minute.

Particle formation

Nanosized architectures were prepared from unimolecular building blocks by means of reprecipitation. This includes rapid injection of...
15 μL of a $1 \times 10^{-3}$ M PDI stock solution dissolved in anhydrous dimethyl sulfoxide (≥ 99.9%) into 1 mL filter sterilised demineralised water followed by manual stirring, yielding a $1.5 \times 10^{-3}$ M nanoparticle solution.

**Optical measurements**

UV/Vis absorption spectra were measured with a Jasco V-650 spectrophotometer equipped with a PerkinElmer PTP-1 Peltier temperature control system. The spectra were measured in quartz cuvettes and extinction coefficients were calculated from Beer-Lambert’s law. Fluorescence spectra were recorded with a Varian Cary Eclipse fluorescence spectrophotometer. Fluorescence quantum yields ($\phi$) were calculated from the integrated intensity under the emission band (I) using Equation (1), where OD is the optical density of the solution at the excitation wavelength and $n$ is the refractive index; N,N-bis(pentadecyl)pentacyclo-3,4,9,10-tetraarbicyclooctadecane ($\phi = 0.99$) in methylene dichloride was used as reference.

$$\phi = \frac{I \cdot OD \cdot n^2}{R \cdot OD \cdot n^2}$$  

(1)

**Liposomes**

Dipalmitoylphosphatidylcholine lipids (DPPC, 10 mg) were dissolved in a solution of chloroform (5 mL) containing 5% methanol. The aqueous phase (5 mL of water) was carefully added along the flask walls. The organic solvent was removed in a rotary evaporator at 40 °C at 40 rpm. After drying, an opalescent fluid was obtained with a volume of approximately 4.5 mL containing a mixture of heterogeneous DPPC liposomes. Liposomes were characterised by brightfield and fluorescence microscopy with a Zeiss Axio Observer D1 equipped with an LD Plan-Neofluar 40×/0.6 Korr Ph 2 lens. A HE DsRed reflector (Exc: 538–562; Em: 570–640) was used to image PDI 6. CLSM measurements were performed in a similar fashion as for the cells.

**Confocal microscopy**

HeLa cells were seeded in eight-well uncoated glass-bottom plates (30,000 cells per well) and cultured for 24 h at 37 °C/5% CO₂ in Dulbeco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen/Strep). Prior to imaging, the medium was removed and replaced by a 5 μM PDI 6 solution in cell culture medium without phenol red. After two hours incubation, the solution was removed, the cells were washed with PBS and fresh DMEM was added. The cells were imaged with a Leica TCS SPS AOBS equipped with an HCX PL APO CS 63/1.2 NA water-immersion lens and a temperature-controlled incubation chamber maintained at 37 °C. PDI 6 was excited with a white-light laser at 530 nm with the pinhole set to one airy unit. The detection range was set to 550–750 nm. The organellar markers (Alexa Fluor 680 Phaloidin, ER-tracker Green and Lyso-Tracker Green DND-26) were used according to the manufacturer’s protocol in the co-localisation studies. For the actin co-stained samples, the cells were fixed with paraformaldehyde in PBS for 10 min at room temperature. The microscopy images were analysed using Leica Application Suite X.

**Supporting Information**

Details on used chemicals and equipment, synthetic procedures and characterisation, DLS, UV/Vis absorption and emission spectra, MTT-cell activity assay and CLSM supporting figures can be found in the Supporting Information.

**Acknowledgements**

The research was supported by funding from HTSM through STW grant 12859-FuNanoPart and the Netherlands Organization for Scientific Research (NWO) via Gravity program 024.001.035, VICI grant 016.150.366 and ECHO grant 711.013.015.

**Conflict of interest**

The authors declare no conflict of interest.

**Keywords:** bioimaging · dyes/pigments · nanoparticles · surfactants · supramolecular chemistry
