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Synthesis and in vitro evaluation of a PDT active BODIPY–NLS conjugate

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ABSTRACT

Two new photosensitizers based on the BODIPY scaffold have been synthesized, of which one bears an NLS peptide, which is linked to the BODIPY's core using the copper catalysed azide–alkyne click reaction. The phototoxicities of these BODIPY based photosensitizers have been determined, as well as their dark toxicities. Although the conjugation of a single NLS peptide to the BODIPY did not lead to any observable nuclear localization, the photosensitizer did exhibit a superior photoxicity. Cellular co-localization experiments revealed a localization of both dyes in the lysosomes, as well as a partial localization within the ER (for the peptide-bearing BODIPY).

Photodynamic therapy (PDT) is a minimally invasive cancer treatment depending on three elements: a photosensitizer, light and oxygen.[1] Administration of the photosensitizer (either topically or systemically) is followed by local irradiation with visible light, resulting in the formation of singlet oxygen (^1O) and other reactive oxygen species (ROS).[2–5] The formation of ROS leads to oxidative damage, resulting in cell death either through apoptosis or necrosis.[3,6,7]

Due to the body's therapeutic window (650–900 nm),[8–10] photosensitizers that are systemically administered are generally designed to absorb red light, whereas light with a shorter wavelength can be used when tissue penetration is not necessary (*e.g.* treatment of carcinoma of the bladder wall).[11]

Most of the photosensitizers currently used in clinical environments are tetrapyrrole macrocycles. The drawback of this class of molecules however, is the cumbersome synthesis and purification, as well as the relatively low absorbance of these dyes.[12] The groups of O'Shea,[12] Nagano,[13] Akkaya,[14] Burgess[15] and Monti[16] previously demonstrated that halogenated (aza) BODIPYs (boron dipyrromethenes), utilizing the internal heavy atom effect,[17,18] are viable candidates as photosensitizers, generally exhibiting higher absorbances and excellent singlet oxygen producing abilities. Furthermore the synthesis of these dyes is relatively straightforward and different substitution patterns lead to a very broad range of absorbance maxima of these dyes.[19,20]

Due to short lifetime of ^1O in aqueous media (0.6 μs), resulting in a diffusion distance of 0.1 μm,[21] the localization of the photosensitizers within the cell is of vital importance. It is known that the DNA contained in the nucleus is far more sensitive to the oxidative damage of singlet oxygen than the phospholipids of the plasma membrane.[22,23]

Nuclear localization sequences (NLS) are short peptides governing the active transport of proteins to the nucleus of the cell, of which the NLS of the simian virus (SV40) large T antigen (PKKKRKV) was the first to be discovered.[24–27] In order for the NLS to work, the NLS conjugate must be located in the cytoplasm[28,29] and since most synthetic NLS conjugates undergo endosomal uptake, it is vital for the conjugates to escape from these vesicles.[30,31] Previously a wide range of synthetic molecules, such as peptide-nucleic acids (PNAs),[32–36] gold nanoparticles,[37] metal complexes,[38–40] fullerene[41] and porphyrins (or tetrapyrrole macrocycles)[42–48] have been conjugated to the SV40 large T antigen NLS, with variable success, most likely due to the inability of certain conjugates to escape the endosomes or lysosomes following endocytosis. A hypothesis that was further confirmed by the successful nuclear localization of various conjugates inserted by microinjection[26,49,50] and streptolysin O based reversible membrane permeabilization,[51] thus circumventing the endocytosis pathway. The successful or unsuccessful nuclear delivery of many of the NLS

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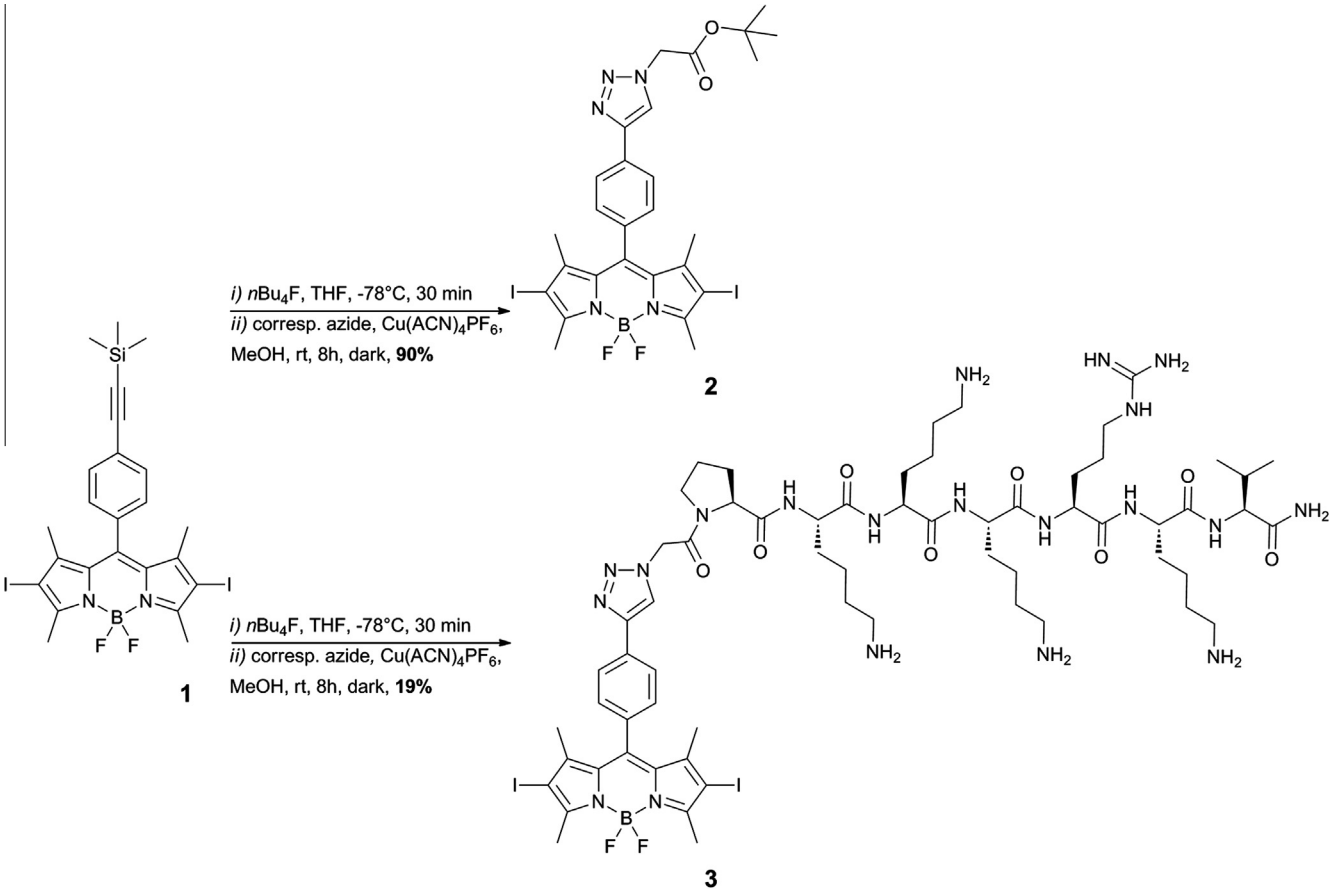
conjugates above was established by covalent attachment of fluorescein[38–41] or rhodamine[36] to the conjugates. Remarkably, conjugates simply consisting of fluorescein and an NLS did not exhibit nuclear localization (and did not escape the endosomes or lysosomes),[38] whereas the structurally related rhodamine dye of a rhodamine–NLS conjugate did exhibit nuclear localization,[36] even in the case where the NLS peptide was replaced by a hybrid PNA–peptide mimetic. Also noteworthy is that, although the NLS-conjugated porphyrins did not manage to enter the nucleus, the presence of an NLS peptide did result in increased phototoxicities.[47,48]

Here, we report the synthesis of a dihalogenated BODIPY, conjugated with an NLS peptide utilizing the chemical orthogonality of the copper catalysed azide–alkyne cycloaddition,[52,53] as well as a reference dye, also containing a triazole. We study the phototoxicity and subcellular localization behavior of these BODIPY dyes after endocytosis to assess the ability of these BODIPY dyes to undergo nuclear localization.

The synthesis of the NLS containing BODIPY (**3**) and the reference molecule (**2**) was initiated by the deprotection of a TMS (trimethylsilyl) protected acetylene containing diiodoBODIPY (**1**)[54] by tetra-*n*-butyl ammonium fluoride in THF (tetrahydrofuran) at −78 °C. The deprotected BODIPY subsequently underwent a copper (I) catalyzed azide–alkyne cycloaddition (also known as the click reaction),[52,53] using tetrakis(acetonitrile)copper(I) hexafluorophosphate as a copper source. The reaction was carried out under an inert atmosphere and protected from the light, to eliminate the risk of decomposition due to singlet oxygen formation. The reaction proceeded in an excellent yield in the case of **2**. By contrast, the reaction yielding **3** only resulted in a 19% yield, which may be explained by π–π stacking of the unreacted BODIPY (or its decomposition products) with the target molecule, preventing full recovery of this molecule after the filtration of the reaction mixture (Scheme 1).

The absorption and emission spectra of the halogenated BODIPY **2** were determined in methanol (Fig. S1, see supplementary material). The absorption spectrum reveals a maximum at 531 nm and the emission spectrum (upon excitation at 531 nm) presents a maximum at 549 nm, resulting in a Stokes shift of 18 nm. The quantum yield of fluorescence of **2** in methanol was determined relative to rhodamine 6G in ethanol and was found to be 2%, which is in good accordance with the reported quantum yield of fluorescence of a previously reported diiodinated BODIPY.[13] A virtually identical absorbance and emission profile was found for **3** (Fig. S2, see supplementary material). In order to assess ^1O production efficiency of dyes **2** and **3**, the ^1O luminescence emission of both dyes was determined relative to Rose Bengal (RB) in dimethylformamide (DMF) (Table 1). Judging from these corrected relative emission intensities, both BODIPY dyes show a similar ^1O generation capacity. The relative emission intensities are in good accordance with the proportion of the ^1O luminescence emission of the diiodinated BODIPY core versus RB in MeOH.[13]

The in vitro phototoxic activity of **2** and **3** against T24, a human urinary bladder carcinoma cell line,[55] was determined after an irradiation with 4 J/cm^2 of a broad spectrum light using an MTT (methylthiazolyl-diphenyltetrazolium bromide) assay.[15,56] A second assay in which the cells were not irradiated was also carried out to determine the dark toxicity. The IC_{50} (the concentration of sensitizer that inhibits the proliferation rate by 50% compared to cells



Scheme 1. Synthesis of BODIPYs **2** and **3**.

Table 1
The relative ^1O emission intensities vs RB in DMF upon excitation at 532 nm; and the in vitro phototoxicity of **1** and **2**, at a dose of 4 J/cm^2, as well as the dark toxicity

BODIPY	Relative ^1O luminescence emission vs. RB	IC_{50} (μM)[a]	
		0 J/cm^2	4 J/cm^2
2	1.76	>10	0.042 ± 0.004
3	1.61	>10	0.016 ± 0.003

[a] The values represent the mean ± the standard deviation of 3 measurements.

that were not exposed to the sensitizer) of cells treated with **3** was 2.6 times lower than the IC_{50} of cells exposed to the reference molecule without the peptide (**2**) (Table 1 and Fig. S3, see supplementary material). Both compounds showed no signs of dark toxicity at a concentration of 10 μM. These data show an increase in toxicity of at least 240–625 times upon irradiation with 4 J/cm^2 (for **2** and **3**, respectively).

In order to get a better understanding of the underlying cause of the observed toxicity difference between the BODIPY substituted with the NLS peptide and the one without, confocal laser scanning microscopy was performed on T24 cells incubated with either **2** or **3**. Co-staining images of the dyes and ER Tracker Blue-White DPX or LysoTracker Blue DND-22 revealed that neither **2** or **3** showed any signs of nuclear localization (judged from the absence of fluorescence originating from the cell's nucleus). The BODIPY **2** showed a clear co-localization with the lysosomes (Fig. S4, see supplementary material), as well as a weak overlap with the ER (endoplasmic reticulum). As was the case with **2**, **3** showed a localization with the lysosomes. In contrast with **2**, **3** also showed a better partial overlap with the ER (Fig. 1).

In view of the similar ^1O generating proprieties of **2** and **3**, the difference in cellular localization seems to be the major factor determining the phototoxicity of these BODIPY photosensitizers. The presence of **3** in the ER points to the successful liberation of (a fraction of) this peptide-bearing dye into the cytosol, in contrast to **2**. However, this did not lead to any significant nuclear uptake. A concentration dependent increase in nuclear uptake could be imagined, as was previously demonstrated for a ruthenium complex bearing cationic peptide, showing nuclear uptake in concentrations higher than 10 μM.[40] Yet, in the present case these experiments would not show any representative cellular distribution, in view of the IC_{50} being lower by at least three orders of magnitude. It has been previously observed that targeting photosensitizers to lysosomes leads to the permeabilization of these organelles, releasing the photosensitizer as well as hydrolytic enzymes into the cytosol (which in turn might lead to the photosensitization of tubulin)[3,57]; photosensitization of the more sensitive ER has been reported to directly lead to apoptosis via activation of the caspase cascade.[58–60] Therefore, it would not be unlikely that the latter pathway is the cause of the observed

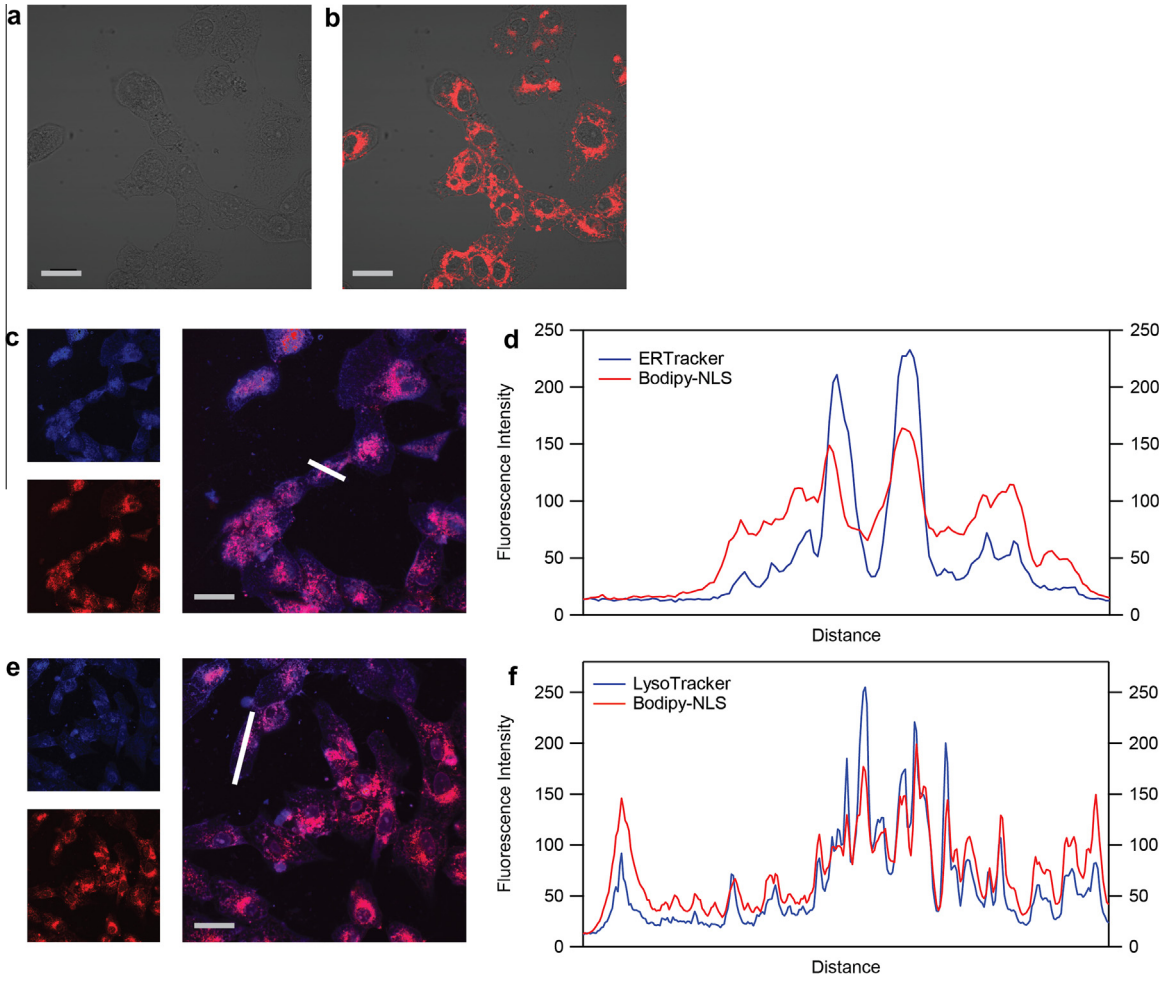


Fig. 1. Subcellular localization of **3** in T24 cells at 100 nM for 30 min. Confocal fluorescence images (c and e) and fluorescence topographic profiles (d and f) of T24 cells costained with 100 nM **3** and respective organelle tracers. (a) Transmission image of T24 cells using DIC. (b) Subcellular localization of **3**. (c and d) Endoplasmic reticulum was labelled with 100 nM ERTracker. (e and f) Lysosomes were labelled with 100 nM LysoTracker. White lines indicate the longitudinal transcellular axis analyzed to generate the fluorescence topographic profiles. A scalebar of 30 μm is indicated in lightgrey.

differential phototoxicity, albeit that it is not possible to rule out any other mechanism of cell death that could be triggered outside of the nucleus.

In conclusion, we described the synthesis of two BODIPY based photosensitizers, **2** and **3**. The applied modular pathway, using the click reaction, can be easily adapted to allow the synthesis of a number of BODIPY based photosensitizers, decorated with different peptides or other targeting molecules. The phototoxicity of dihalogenated BODIPY photosensitizers can be significantly improved by conjugating the dye with a NLS peptide, without significantly improving the dark toxicity of the drug. Although the conjugation with a single NLS did not result in (any observable) nuclear localization, it however did result in an improved toxicity due to a differential cellular localization, of which the exact processes involved are yet unknown.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03. 128.

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