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# A near infrared heptamethine aminocyanine dye with a long-living excited triplet state for photodynamic therapy

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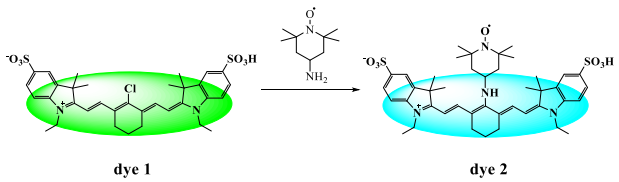
**A water-soluble near-infrared aminocyanine dye have been developed with a long triplet-state lifetime (τ = 9.16 μs in deaerated ethanol). Thereby, extremely high singlet oxygen quantum yield ($\Phi_\Delta$ = 0.20) and low dark cytotoxicity ($IC_{50}$ = 715.4 μM) were achieved. The potential of the dye as a PDT photosensitizer was demonstrated.**

Photodynamic therapy (PDT) has become an effective malignant cancers treatment approach with minimal invasion and high spatiotemporal precision.[1-3] PDT calls for excellent photosensitizers with low dark cytotoxicity and good phototoxicity.[4] Nevertheless, the traditional photosensitizer molecules are mostly hydrophobic molecules with high dark cytotoxicity, which greatly restricts their clinical application and promotion.[5] For example, Photofrin, a porphyrin-based photosensitizer, has complex chemical nature and nonnegligible intracellular accumulation, especially retentions by skin which leads to protracted cutaneous photosensitivity. Therefore, patients were usually required to avoid light exposure for a long time after PDT. Otherwise, it will cause skin allergy and eye damage.[6,7] Besides the toxicity, the photosensitizers are expected to be irradiated by near-infrared (NIR) light. Because working in NIR optical window can provide deeper irradiation depth. In this context, as an ideal and effective photosensitizer for PDT, low dark toxicity but prominent phototoxicity, and strong absorption in NIR region (600-900 nm) are extremely important prerequisites.

Heptamethine cyanine dyes (Cy7) such as indocyanine green (ICG), IR-780, IR-783, IR-808, IR-820, IR-825 have emerged as an excellent NIR imaging agents in fluorescence diagnosis applications due to their low cytotoxicity.[8-12] Some of them are also used as photosensitizers for PDT, like ICG (approved by the FDA in the United States for medical diagnostic application). However, they have low phototoxicity because they are characterized by extremely low singlet oxygen quantum yield, which has limited their practical utility for PDT.[13] Heavy atoms are introduced in the structural skeleton of Cy7 dyes, which is known to efficiently increase ISC process and significantly improve singlet oxygen generation.[14-16] But the resulting modified dyes usually have high dark cytotoxicity. 2,2,6,6-Tetramethylpiperidinyloxy (TEMPO) is a stable radical which is widely used as a nitroxide spin label in obtaining biochemical reaction information in vivo due to its low cytotoxicity.[17,18] Meanwhile, TEMPO is known to enhance inter system crossing (ISC) process for triplet-state photosensitizers.[19-22] So far as we know, introducing TEMPO into Cy7 dye to greatly enhance ISC efficiency for PDT purpose has not been reported yet.

Herein we designed and synthesized a heptamethine aminocyanine dye **2** containing the TEMPO substituent from a hydrophilic known Cy7 dye **1** according to our previous reported method (Scheme 1).[23,24] We aim to provide an efficient NIR PDT photosensitizer with high singlet oxygen quantum yield as well as low dark toxicity for application in oncology.




**Scheme. 1** Synthesis of heptamethine aminocyanine dye **2.**

After the preparation of dye **1** and dye **2** (Scheme S1), their photophysical properties were tested. As we expected, dye **2** possesses a large Stokes shift (~100nm) due to the introduction of 4-amino-TEMPO at the center of the conjugated bridge of dye **1**, Meanwhile, dye **1** has a small Stokes shift (~30nm) (Figure S4). This result of enlarging Stokes shift is consistent with our previous work about heptamethine aminocyanine dyes.[23,24]

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Electronic Supplementary Information (ESI) available: Synthesis and characterization of compounds. HPLC data, Transient absorption measurements, Hela cells culture and staining, Cofocal fluorescence imaging. Cytotoxicity assays. See DOI: 10.1039/x0xx00000x

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Still, the absorption and fluorescence peaks of the dye **2** are in NIR range, which makes it suitable for bio-applications, including PDT for tumors.[8,25,26]

To affirm the PDT potential of dye **2**, we try to arrest its triplet excited state by nanosecond time-resolved transient difference absorption spectra. Because a good PDT photosensitizer normally has a long-living triplet excited state for sensitizing the molecule oxygen in tumor region, which would generate cytotoxic reactive oxygen species (ROS, such as singlet oxygen) to induce cancer cells death. As shown in Figure 1, a salient feature of transient absorption was found between 630 nm and 820 nm after 532 nm laser excitation. Two bleaching peaks are located at ~660 nm and ~790 nm, respectively. The former absorption centred at 660 nm can be assigned to the bleaching of ground state. The later one at 790 nm should be attributed to the transient absorption of the formed long-living triplet excited state ($\tau$ = 9.16 μs under argon, blue kinetic fitting curve in Figure 1b). And this long-living transient was found to be sensitive to oxygen ($\tau$ = 0.38 μs under air, green kinetic fitting curve in Figure 1b), which also confirms that this microsecond scale transient is the formed triplet excited state specie. Under the same experimental conditional of nanosecond time-resolved transient difference absorption spectra, no salient signal was observed for dye **1** (Figure S5). These results indicate that efficient radical-enhanced intersystem crossing occur in dye **2** due to the radical substituent, thereby obtaining a long-living excited triplet state. Such a long-living triplet excited state has not been achieved by the conventional methods relying on heavy atom effect to enhance ISC process of cyanine dyes.[14-16, 27,28] Enhanced ISC by the introduced TEMPO group for dye **2** should be attributed to electron spin polarization (ESP) based on a radical-triplet pair mechanism (RTPM).[29,30,31] As far as we know, such a microsecond scale transient absorption has never been observed in reported cyanine dyes.

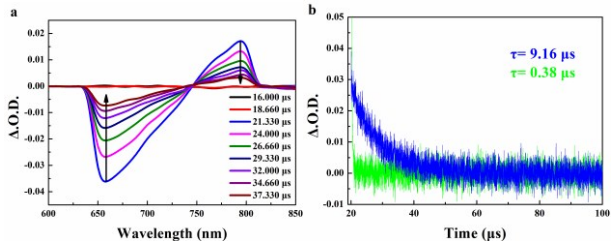


**Fig. 1** (a) Nanosecond time-resolved transient difference absorption spectra of dye **2** (10.0 μM in deaerated ethanol). 532 nm laser pulse, decay times as indicated; (b) Life decay curves of transient species in dye **2** (10.0 μM) under argon (blue line) and air atmosphere (green line). Blue line shows the profile after deoxygenating with argon bubbles ($\tau$ = 9.16 μs, residuals = 0.75) and green line shows the profile after the oxygen-free sample was settled for 24 h under air atmosphere ($\tau$ = 0.38 μs, residuals = 0.82). Excited at 532 nm and monitored at 800 nm. All measurements are performed at room temperature.

We know that production of reactive oxygen species (ROS) is strongly linked to the excited triplet states of the photosensitizer. Therefore, to confirm the ability of dye **2** with a long-living excited triplet state may sensitize more oxygen into singlet oxygen, the singlet oxygen generation abilities of dye **1** and dye **2** were measured through using 1,3-diphenylisobenzofuran (DPBF) as singlet oxygen trapping agent. The absorbance of DPBF at 410 nm decreased remarkably in the presence of dye **2** under irradiation (Figure S6a), while the absorbance decline of DPBF at 410 nm is extremely slow in the presence of dye **1** (Figure S6b). As seen from Figure 2, the $^1O_2$ generation efficiency of both dye **1** and dye **2** shows a nearly linear relationship. But, the singlet oxygen generation ability of dye **2** is extremely prominent when compared to dye **1**. Meanwhile, 4-amino-TEMPO as a control does not cause obvious bleaching of DPBF under LED irradiation for 700 s. These results support our expectation that introducing the radical of nitroxide into heptamethine cyanine dye can enhance the ISC process of cyanine dyes to produce more $^1O_2$. To quantify the singlet oxygen generation abilities of dye **1** and dye **2**, the singlet oxygen quantum yield ($\Phi_\Delta$) of dye **1** and dye **2** were calculated by choosing methylene blue in ethanol with $\Phi_{MB}$ = 0.52 as reference.[32,33] Dye **2** has a considerably higher singlet oxygen quantum yield ($\Phi_\Delta$ = 0.20) than dye **1** ($\Phi_\Delta$ = 0.006) in ethanol. So far as we know, the singlet oxygen quantum yields ($\Phi_\Delta$) of other cyanine dyes like ICG and IR-783 used for PDT are only 0.008 and 0.007, respectively.[26]

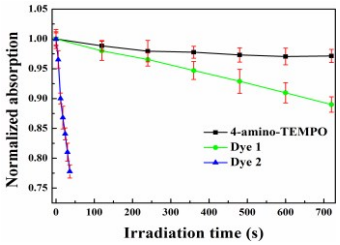


**Fig. 2** The bleaching rate comparison of DPBF at 410 nm. A NIR LED array ($\lambda_{ex}$ = 660 nm; 0.8 mW/cm$^2$) for different irradiation times. Dye **1** and dye **2** were respectively dissolved in absolute ethanol to a final concentration with almost the same absorbance (~0.1) at 660 nm and mixed with DPBF (50 μM). 4-Amino-TEMPO (3 μM) was used with the same concentration as dye **2**. Data are presented as the mean value ± SD (n = 3).

To be used in bio-applications, the chemical durability of dye **2** should be evaluated. Because the *meso* position substitution of the Cy7 fluorophores has been doubted to be labile when such a Cy7 dye is circulating throughout the blood stream. Besides, reducing substances in the blood may cause dye **2** to be reduced into dye **2-OH** (Scheme S1). We found the reduction product dye **2-OH** shows better ionization capacity than dye **2** under high resolution mass spectra (HRMS) condition (Figure S1, S2). Therefore, HRMS was employed to evaluate the chemical stability of dye **2**. Dye **2** was treated with 100% ICR mice serum for 4 h and 24 h before evaluation. For comparison, a control sample by mixing dye **2-OH** and dye **2** (mole ratio of dye **2-OH** : dye **2** is 1:9) was also checked by HRMS. As shown in Figure S7, the results of HRMS data indicate that the content of possible reduction product was far below 10% even after 24 h treatment with serum. It means that dye **2** has a robust

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chemical stability and can be used for PDT applications in biological environment.

Based on excellent chemical durability of dye **2**, we subsequently try to verify dye **2** a better photosensitizer for PDT in living cells. It is desirable to develop photosensitizers with low dark cytotoxicity. The amphiphilic dye molecules ICG with low dark cytotoxicity as a photosensitizer for PDT has been studied.[26-28,32-34] However, its low singlet oxygen quantum yield ($\Phi_\Delta$ = 0.008) limits its practical application. Protoporphyrin IX (PpIX), a known PDT photosensitizer, has an excellent photocytotoxicity. But its high dark cytotoxicity is the mainly side effect for PDT. Here, we evaluated both phototoxicity and dark cytotoxicity of dye **1**, dye **2** and PpIX towards HeLa cells by MTT assays. As shown in Figure 3a, dye **1** did not show significant photocytotoxicity to HeLa cells with $IC_{50}$ value at 429.6 μM. The dose-dependent phototoxicity of dye **2** was recorded with $IC_{50}$ value at 14.2 μM, which tells that dye **2** has a moderate phototoxicity and can induce a time-dependent process of cell death (Figure S8). Although PpIX showed a much higher phototoxicity with $IC_{50}$ value at 5.9 μM than dye **2**. But, the dark cytotoxicity of PpIX is much higher than that of dye **1**, dye **2** (Figure 3b, $IC_{50}$ of dye **1** and **2** were 659.7 and 715.4 μM respectively, while that of PpIX was 76.3 μM). The dark cytotoxicity of dye 2 was similar with that of dye 1 after the introduction of 4-amino-TEMPO. The phototoxicity index (PI) (the ratio of $IC_{50}$ of dark cytotoxicity to $IC_{50}$ of phototoxicity) are calculated for PpIX (PI = 12.9), dye **1** (PI = 1.5) and dye **2** (PI = 50.4) to evaluate their potentials as PDT photosensitizers. Although PpIX have a high phototoxicity, a high dark toxicity makes its PI value lower than that of dye **2**. This result suggests that dye **2** should be a better photosensitizer for clinical PDT.

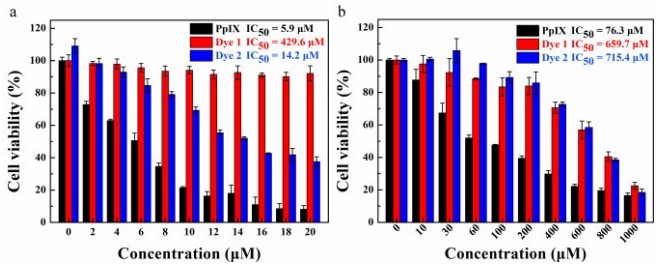


**Fig. 3** (a) Phototoxicity evaluation of dye **1**, dye **2** and PpIX on HeLa cells for 20 minutes irradiation ($\lambda_{ex}$ = 660 nm, 50 mW/cm$^2$), MTT array was measured after another 6 h incubation. (b) Dark cytotoxicity evaluation of dye **1**, dye **2** and PpIX on HeLa cells. Data are presented as the mean value ± SD (n = 6).

After high phototoxicity and low dark toxicity has been confirmed, dye **2** was investigated to clarify the mode of action for its PDT effect. According to our design, the low dark toxicity should be due to its good water solubility. It is curious to know whether the dye **2** can enter the living cells, and how its high phototoxicity is achieved. HeLa cells were chosen again to be co-stained with dye **2** and DAPI, a nuclear fluorescence dye. As shown in the confocal fluorescence images of Figure S9, the dye **2** was internalized and uniformly stained in the region of cytoplasm. Then, we carried out the PDT to check whether intracellular reactive oxygen species (ROS) was generated by using Dihydroethidium (DHE) staining. DHE is a ROS fluorescent probe. After DHE being oxidized by ROS, the cells' nucleus should exhibit bright fluorescent signal. As shown in Figure S10, the oxidized DHE migrated from the cytoplasm (green arrow) to the nucleus (red arrow) and produced a bright red fluorescence during the process of PDT. Meanwhile, HeLa cell morphology changed significantly after light exposure, accompanying by nuclear condensation. These results indicate that dye **2** can enter the cells and produce ROS during irradiation, although it has water solubility.

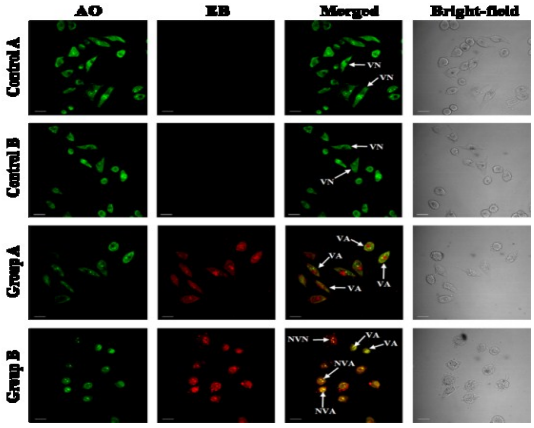


**Fig. 4** Morphology of HeLa cells stained by acridine orange/ethidium bromide (AO/EB) after PDT treatment under confocal fluorescence imaging. Dye **2** (20 μM) was co-cultivated with Hela cells for 12 h, then HeLa cells were irradiated for 20 min before stained with AO/EB. LED array irradiation ($\lambda_{ex}$ = 660 nm, power density of 50 mW/cm$^2$). Control A-HeLa cells were irradiated for 20 min without dye **2** co-cultivation; Control B-HeLa cells were co-cultivated with dye **2** for 12 h without irradiation; Group A-HeLa cells were immediately stained by AO/EB after irradiation; Group B-HeLa cells were stained by AO/EB after continuing incubation for 6 hours after irradiation. VN-viable cells; VA-early apoptotic cells; NVA-late apoptotic cells; NVN-necrotic cells. Scale bar = 20 μm.

At last, we tried to demonstrate the practical feasibility of dye **2** as a PDT photosensitizer in a visual mode by using fluorescence confocal imaging experiments. Acridine orange (AO) stains the DNA and RNA of living cells and gives out green fluorescence. Ethidium bromide (EB) stains DNA and RNA of dead cells and gives out red fluorescence. Through the cells' AO/EB staining, the quantization differences of living cells (VN), necrotic cells (NVN), early apoptotic cells (VA) and the late stage apoptotic cells (NVA) can be visually distinguished by confocal fluorescence microscopy (Figure 4). As shown in Figure 4 (control A), HeLa cells appeared as irregular shuttle with uniformly green fluorescence of AO after light irradiation, while the characteristic red fluorescence of EB was not detected. These results indicate light irradiation from the LED light source used in our experiments cannot cause phototoxicity. In the samples of control B, HeLa cells were stained with dye **2** (20 μM) for 12 h without light irradiation. No obvious cytotoxicity was observed either, which supports that dye **2** at the used

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concentration do not induce cells death. Meanwhile, after PDT under the same light irradiation and at the same concentration of dye **2**, early apoptotic cells (VA) are dominated right after irradiation (Group A), and obvious increased late stage apoptotic cells (NVA) and some necrotic cells (NVN) can be observed for cells with another 6 h incubation after irradiation (Group B). Taken together, these results suggest that cells death caused by PDT of dye **2** is predominantly via apoptosis but not necrosis.[35] And more importantly, these results support dye **2** has a low dark cytotoxicity and a good practical application potential in PDT.

In summary, we have developed a near infrared aminocyanine dye **2** with a long-living excited triplet state (τ = 9.16 μs) by introduction of a radical into water-soluble cyanine dye structure. Its potential in PDT as a photosensitizer has been evaluated. In the one hand, dye **2** exhibits significantly high singlet oxygen quantum yield ($\Phi_{\Delta}$ = 0.20) and a superior PDT effect in the living cells level. In the other hand, dye **2** has a very low dark cytotoxicity and a robust chemical stability. After PDT, dye **2** can induce cell death predominantly via apoptosis. To fulfil its potential in PDT, an improvement with target function based on dye **2** is being done. We firmly believe that new compounds based on dye **2** have a bright prospect for biomedical and clinical applications in the future.

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## Conflicts of interest

There are no conflicts to declare.

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