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Tuning the singlet-triplet energy gap: a unique approach to efficient photosensitizers with aggregation-induced emission (AIE) characteristics†

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The efficiency of the intersystem crossing process can be improved by reducing the energy gap between the singlet and triplet excited states (ΔE_{ST}), which offers the opportunity to improve the yield of the triplet excited state. Herein, we demonstrate that modulation of the excited states is also an effective strategy to regulate the singlet oxygen generation of photosensitizers. Based on our previous studies that photosensitizers with aggregation-induced emission characteristics (AIE) showed enhanced fluorescence and efficient singlet oxygen production in the aggregated state, a series of AIE fluorogens such as TPDC, TPPDC and PPDC were synthesized, which showed ΔE_{ST} values of 0.48, 0.35 and 0.27 eV, respectively. A detailed study revealed that PPDC exhibited the highest singlet oxygen efficiency (0.89) as nanoaggregates, while TPDC exhibited the lowest efficiency (0.28), inversely correlated with their ΔE_{ST} values. Due to their similar optical properties, TPDC and PPDC were further encapsulated into nanoparticles (NPs). Subsequent surface modification with cell penetrating peptide (TAT) yielded TAT-TPDC NPs and TAT-PPDC NPs. As a result of the stronger singlet oxygen generation, TAT-PPDC NPs showed enhanced cancer cell ablation as compared to TAT-TPDC NPs. Fine-tuning of the singlet-triplet energy gap is thus proven to be an effective new strategy to generate efficient photosensitizers for photodynamic therapy.

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Introduction

Photosensitized generation of reactive oxygen species (ROS) has attracted great research interest, and has found numerous applications in different fields,^{1,2} including synthetic organic chemistry,³ wastewater treatment,^{5,6} and photodynamic therapy (PDT).⁴ PDT represents a well-consolidated but gradually expanding approach to the treatment of cancer. It involves excitation of a photosensitizer (PS) with specific light wavelengths, which is followed by intersystem crossing (ISC) from its lowest singlet excited state (S_1) to the lowest triplet excited state (T_1); subsequently, energy transfer from the T_1 of the PS to

ground-state oxygen (3O_2) generates ROS, which causes oxidative damage of targets.⁷

The primary cytotoxic agent involved in this photodynamic process is singlet oxygen (1O_2), the efficient generation of which is habitually related to the ISC efficiency of the sensitizer and concentration quenching of the excited state. As such, efficient ISC offers the opportunity to improve the triplet excited state yield, which is highly desirable for efficient 1O_2 generation. In addition, any system with minimized concentration quenching of the excited state is also favorable.

Incorporation of heavy atoms into molecular structures to enhance the spin-orbit perturbations is one of the most widely used approaches to improve the ISC efficiency of PSs.^{8–10} However, the presence of heavy atoms such as selenium, iodine, bromine, and certain lanthanides has often been reported to cause increased “dark toxicity”.¹¹ It is thus important to propose alternative strategies to achieve strong ISC without using heavy atoms, to minimize dark toxicity. Previous studies have shown that the ISC rate constants could be estimated from eqn (1),¹² where H_{SO} is the Hamiltonian for the spin-orbit perturbations (SOP) and $\Delta E_{S_1-T_1}$ (ΔE_{ST}) is the energy gap between the S_1 and T_1 states. ISC can be modeled by mixing of T_1 with S_1 states due to

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SOP. This equation shows the relationship between ISC rate and ΔE_{ST} , which paves the way for improving ISC efficiency, and consequently 1O_2 generation (Scheme 1).

$$k_{ISC} \propto \frac{\langle T_1 | H_{SO} | S_1 \rangle^2}{(\Delta E_{S_1-T_1})} \quad (1)$$

Concentration quenching of the excited state is another common problem that affects the 1O_2 generation efficiency of PSs. This is particularly serious for hydrophobic PSs, which tend to aggregate in aqueous media due to their rigid planar structures, resulting in aggregation-caused quenching (ACQ)¹³ and remarkable reduction in 1O_2 generation efficiency.¹⁴ The quenching is more severe when the PSs, such as porphyrin derivatives, are encapsulated into nanocarriers, which could lead to significantly decreased fluorescence and photodynamic efficiency.¹⁵ Opposite to the ACQ effect, propeller-shaped fluorogens with aggregation-induced emission (AIEgen) characteristics have recently emerged as a powerful and versatile tool for biomedical applications.^{16–25} AIEgens are almost non-emissive in the molecular state but can be induced to emit strong fluorescence in the aggregated state owing to the prohibition of energy dissipation through non-radiative channels.^{26–33} In addition, AIEgen PSs have been found to exhibit enhanced fluorescence and efficient photosensitizing characteristics in the solid state,^{24,34–38} which paves the way for tackling the ACQ problem of PSs. This motivates us to develop more efficient AIEgen PSs for image-guided photodynamic therapy.

To develop more efficient AIEgen PSs, we propose to control the ΔE_{ST} values by incorporation of electron donors and acceptors into π -conjugated systems to manipulate the HOMO–LUMO distribution.^{39–42} By taking advantage of the unique optical properties of AIEgens, in this contribution, a series of AIEgens with methoxy as the electron donor and dicyanovinyl as the acceptor were synthesized, and their ability to generate 1O_2 was studied both in solution and in cells. The work demonstrates for the first time that reducing ΔE_{ST} is an effective strategy for achieving efficient PSs for photodynamic therapy.



Scheme 1 A physical model of 1O_2 generation, depicting the S_1 – T_1 ISC process and the proposed strategy for increasing 1O_2 generation.

Experimental section

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-maleimide-(polyethyleneglycol)-3000] (DSPE-PEG₃₀₀₀-Mal) was ordered from Avanti Polar Lipids. 4-Acetylphenylboronic acid, malononitrile, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and other chemicals were all purchased from Alfa Aesar or Sigma-Aldrich. Cell penetrating peptide HIV-1 TAT was purchased from GL Biochem Ltd. Trypsin–EDTA, Annexin V-FITC, fetal bovine serum (FBS) and Hoechst 33342 were purchased from Life Technologies. TPDC and TPPDC were synthesized according to our previous reports.^{35,37}

Instrumentation

NMR spectra were performed on a Bruker ARX 400 NMR spectrometer. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Proteome X-LTQ. Particle size and size distribution were determined by laser light scattering (LLS) with a particle size analyzer (90 Plus, Brookhaven Instruments Co., United States) at a fixed angle of 90° at room temperature. The zeta potential was determined by a Malvern Zetasizer Nano ZS90 (Worcestershire, UK). TEM images were obtained from a JEOL JEM-2010 transmission electron microscope with an accelerating voltage of 200 KV. UV-vis absorption spectra were taken on a Shimadzu Model UV-1700 spectrometer. Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS 55 spectrofluorometer. All UV and PL spectra were collected at 24 ± 1 °C.

Computational details

All molecules were fully optimized by the hybrid B3LYP, in combination with the 6-31G(d) basis set. The excited-state characteristics were calculated by time-dependent density functional theory (TD-DFT) using optimized ground state geometries. TD-DFT in combination with the B3LYP hybrid functional method and the 6-31G(d) basis set has been shown to provide accurate energies for the excited state of D–A molecular systems with less than 0.1 eV error.^{39–41} The geometry data of TPDC, TPPDC and PPDC are provided in the (ESI).†

Synthesis of PPDC

The synthesis and characterization of the intermediates of DMTPBr and PPac are described in the ESI.† To synthesize PPDC, PPac (100 mg, 0.18 mmol), malononitrile (100 mg, 1.50 mmol) and ammonium acetate (139 mg, 1.80 mmol) were dissolved in a mixture of dichloromethane (20 mL) and methanol (4 mL). Then silica gel (2.4 g) was added to the above mixture, and the solvent was removed under reduced pressure. The resulting mixture was heated at 100 °C for 4 h. The mixture was cooled down and subsequently separated by chromatography (hexane/ethyl acetate = 20/1, v/v) to give the desired product as a yellow powder (74 mg, 73.6% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.65–7.50 (m, 4H), 7.31 (d, J = 8.4 Hz, 2H), 7.11–6.95 (m, 7H), 6.92–6.80 (m, 4H), 6.65–6.49 (m, 4H),



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TPE (1.22 eV). The small ΔE_{ST} suggests that a potentially high ISC rate is possible for efficient 1O_2 generation.

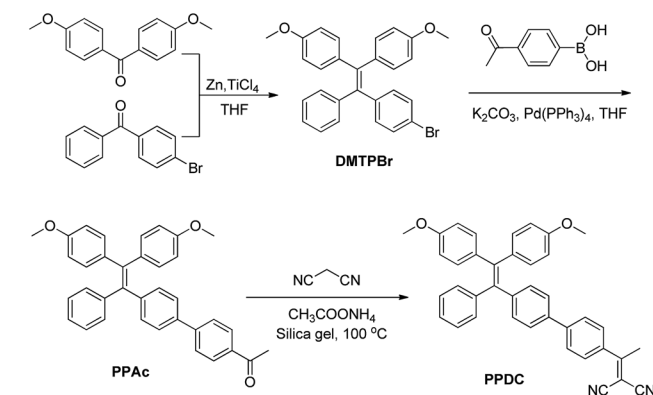
Synthesis

The synthesis of **PPDC** is shown in Scheme 2. **DMTPBr** was synthesized according to the reported procedures.³⁵ **DMTPBr** was first transformed into **PPAc** via Suzuki-coupling with 4-acetylphenylboronic acid. **PPDC** was subsequently obtained in 74.6% yield by the reaction between **PPAc** and malononitrile in the presence of ammonium acetate and pyridine. The final product and intermediates were characterized by NMR and mass spectrometry in Fig. S1–S8,[†] which revealed their correct structures with high purity.

Optical properties and 1O_2 generation of photosensitizers

The optical properties of **TPDC**, **TPDCP** and **PPDC** were investigated first. The AIE characteristics of all the compounds were studied by monitoring their emission properties in DMSO/water mixtures with different volume fractions of water. As shown in Fig. 2, all three compounds are almost non-emissive in pure DMSO and they gradually become emissive after introducing a certain amount of water into the DMSO solution, indicating that they are indeed AIE active. The size distributions of the aggregates formed in DMSO/water (v/v = 1/99) mixtures were evaluated by laser light scattering (LLS), which revealed an average size of ~ 50 nm for each (Fig. S9[†]). Fig. 3A shows the absorption and emission spectra of **TPDC**, **TPDCP** and **PPDC** as nanoaggregates at the same concentration of 10 μ M. Although **TPDC** shows a red-shifted absorption spectrum as compared to **PPDC**, they have similar light absorption in the white light range (>400 nm) by comparing the areas under the absorption spectra.

To assess the capabilities of **PPDC**, **TPDC** and **TPPDC** for 1O_2 generation, a commercial 1O_2 probe, ABDA, was used as an indicator, and RB was used as the standard PS (the 1O_2 quantum



Scheme 2 Synthetic route to **PPDC**.

yield for Φ_{RB} is 0.75 in water).⁴³ As shown in Fig. S10,[†] under white light irradiation, the presence of **PPDC**, **TPDC**, **TPPDC** or RB, in the ABDA solution leads to gradually decreased ABDA absorbance with prolonged irradiation time, indicating degradation of ABDA by the generated 1O_2 in solution. It is important to note that under the same conditions, the white light irradiation on ABDA alone does not lead to an obvious change in absorbance. If we define A_0 and A as the absorbance of ABDA in the presence of these AIEgen PSs at 378 nm before and after irradiation, respectively, the plot of $\ln(A_0/A)$ against time gives straight lines in Fig. 3B. From the slopes, the decomposition rate constants (K_{PS}) of **PPDC**, **TPPDC** and **TPDC** can be calculated as 0.0032, 0.0018 and 0.0013 s^{-1} , respectively. Under the same experimental conditions, the rate constant for RB (K_{RB}) is 0.0055 s^{-1} (Fig. S10[†]). The integrations of the optical absorption in the wavelength range of 400–800 nm (A_{PS}) for RB, **PPDC**, **TPPDC** and **TPDC** are 9.52, 4.68, 7.33 and 5.67, respectively (Fig. S10[†]). Interestingly, **PPDC** shows the second largest K_{PS}



Fig. 1 Chemical structures and HOMO–LUMO distributions of **TPE**, **TPDC**, **TPPDC** and **PPDC**, optimized structures of the HOMO and LUMO at S_1 were calculated by TD-DFT (Gaussian 09/B3LYP/6-31G(d)).



Fig. 2 Fluorescence spectra of **TPDC** (A), **TPPDC** (B), **PPDC** (C) in DMSO/water mixtures with different volume fractions of water at a concentration of 10 μ M. The excitation wavelengths for (A), (B) and (C) are 400, 390, 420 nm, respectively. The insets show photographs of the compounds in DMSO and DMSO : water (v : v, 1 : 99) under UV light (365 nm) illumination.



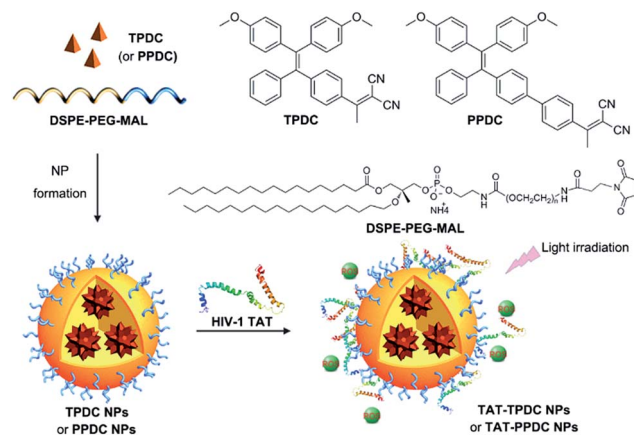
Fig. 3 (A) UV-vis absorption (solid lines) and normalized photoluminescence (PL) spectra (dashed lines) of 10 μM each of PPDC, TPDC and TPPDC in DMSO/water ($v/v = 1/99$). (B) The decomposition rates of ABDA by PPDC, TPDC and TPPDC; A_0 and A are the absorbance of ABDA in the presence of the AIEgen PSs at 378 nm before and after irradiation, respectively.

with the smallest integrated area. The $^1\text{O}_2$ quantum yields of PPDC, TPPDC and TPDC were calculated to be 0.89, 0.32 and 0.28, respectively. These results agreed well with the prediction based on eqn (1). Tuning the ΔE_{ST} is thus an effective strategy to yield PSs with controlled $^1\text{O}_2$ generation efficiency. Due to the similar A_{PS} values for TPDC and PPDC, they were chosen for $^1\text{O}_2$ generation comparison in cells.

Preparation and characterization of TAT-TPDC NPs and TAT-PPDC NPs

The TAT-TPDC NPs and TAT-PPDC NPs were prepared *via* the modified nano-precipitation method (Scheme 3).¹⁹ The maleimide-bearing copolymer DSPE-PEG₃₀₀₀-Mal was used as the matrix to encapsulate TPDC and PPDC to yield maleimide-functionalized AIE NPs, which were subsequently reacted with thiol (-SH) bearing cell-penetrating peptide (RKKRRQRRRC) to yield the TAT-TPDC NPs and TAT-PPDC NPs, respectively.

The absorption and emission spectra of TAT-TPDC NPs and TAT-PPDC NPs are shown in Fig. S11.† When the same concentrations of TAT and NPs were used, the TAT conjugation efficiencies were calculated to be $62 \pm 5\%$ and $58 \pm 4\%$ for TAT-TPDC NPs and TAT-PPDC NPs, respectively. The size distributions of both NPs were evaluated by laser light scattering (LLS), which revealed an average size of ~ 50 nm for each (Fig. 4). Likewise, TAT-TPDC NPs and TAT-PPDC NPs exhibit similar morphology according to the TEM images shown in the insets of Fig. 4. In addition, the zeta potentials for TAT-TPDC NPs and TAT-PPDC NPs are 23.7 mV and 23.4 mV, respectively. In view of the similar TAT conjugation efficiency, particle size, and surface charge of TAT-TPDC NPs and TAT-PPDC NPs, it is reasonable



Scheme 3 Schematic illustration of AIE NP formation and surface modification with TAT.

to expect that the two NPs would show similar uptake efficiency by the same cancer cells.

Imaging of live cancer cells

TAT-TPDC NPs and TAT-PPDC NPs were incubated with human cervix carcinoma HeLa cells for 4 h and their confocal fluorescence images are shown in Fig. 5. Red fluorescence either from TAT-TPDC NPs or TAT-PPDC NPs in the cell cytoplasm and blue fluorescence from Hoechst in the cell nuclei are simultaneously observed. Further quantitative fluorescence intensity analysis of both samples of NPs was performed by comparing the fluorescence intensity ratio (TPDC/PPDC) of the NPs retained in the medium before and after cell incubation and cell removal. The same intensity ratio indicates that the HeLa cell uptake of both types of NPs is similar.

Photodynamic therapy

Low cytotoxicity in dark conditions but high toxicity upon exposure to light irradiation is essential for phototherapy. Quantitative evaluation of the therapeutic effect of TAT-TPDC NPs and TAT-PPDC NPs on HeLa cells was studied by standard MTT assay. The cytotoxicity of HeLa cells upon incubation with TAT-TPDC NPs and TAT-PPDC NPs in dark conditions was first evaluated. As shown in Fig. 6A, after 24 h incubation, no significant cytotoxicity is observed in the dark. However, after exposure to light irradiation, a dose-dependent cytotoxicity is

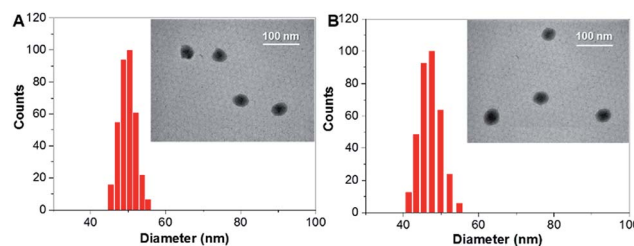


Fig. 4 Size distribution and TEM images (inset) of TAT-TPDC NPs (A) and TAT-PPDC NPs (B).

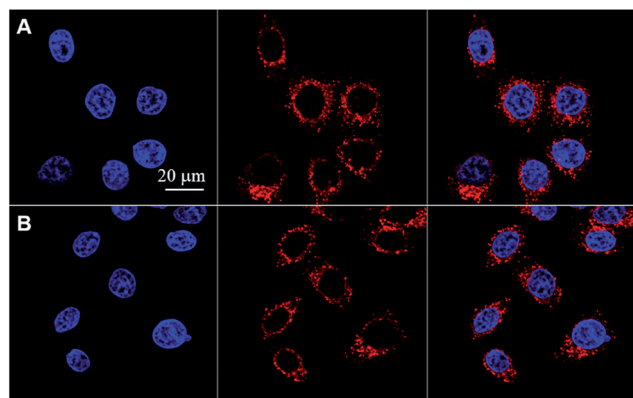


Fig. 5 Confocal images of HeLa cells upon incubation with TAT-TPDC NPs (A) and TAT-PPDC NPs (B) for 4 h. The red fluorescence is from NPs and the blue fluorescence is from Hoechst 33342-stained cell nuclei. All the images share the same scale bar of 20 μm .

observed in HeLa cells for both types of NPs. The half-maximal inhibitory concentrations (IC_{50}) of TAT-TPDC NPs and TAT-PPDC NPs for HeLa cells are 3.44 and 1.28 $\mu\text{g mL}^{-1}$, respectively. The lower IC_{50} of TAT-PPDC NPs relative to that for TAT-TPDC NPs should be attributed to the higher $^1\text{O}_2$ quantum yield and more efficient light-induced $^1\text{O}_2$ generation upon light irradiation for the former. The 2.6-fold lower IC_{50} of TAT-TPDC NPs is reckoned significant in cancer cell inhibition. Furthermore, to validate the exposure time and light power-dependent PDT, both TAT-TPDC NP- and TAT-PPDC NP-incubated HeLa cells were irradiated with light for different time durations or at different power densities. For example, when the cells were incubated with 1.0 $\mu\text{g mL}^{-1}$ each of the NPs, followed by white light irradiation at a power density of 100 mW cm^{-2} , the time required to reach 50% cell viability was 73 s for TAT-PPDC NPs and 113 s for TAT-TPDC NPs. By keeping the NP concentration the same at 1.0 $\mu\text{g mL}^{-1}$, and the irradiation time as 60 s, varying the light power densities also results in different cell viabilities (Fig. 6C). Under all the tested conditions, TAT-PPDC NPs showed enhanced inhibition of cell viability as compared to TAT-TPDC NPs, which clearly demonstrates the importance of PS design in photodynamic therapy. These results also show that therapeutic efficiency can be regulated by controlling the laser irradiation time or the light power density.

Fluorescein isothiocyanate (FITC)-tagged Annexin V, a cell apoptosis indicator, was subsequently used to study whether cell apoptosis occurred in the PDT process. FITC-tagged Annexin V is commonly used to distinguish viable cells from apoptotic ones, as Annexin V can selectively bind to the exposed phosphatidylserines on the outer cytoplasmic membrane of apoptotic cells. As shown in Fig. S12,[†] after incubation of HeLa cells with TAT-TPDC NPs or TAT-PPDC NPs followed by light irradiation and FITC-tagged Annexin V staining, strong green fluorescence attributed to FITC is clearly observed on the cell membranes, indicating that the cells undergo the apoptosis process. On the other hand, no green fluorescence signal is observed in HeLa cells under dark conditions, indicating that



Fig. 6 (A) The viability of HeLa cells upon treatment with TAT-TPDC NPs and TAT-PPDC NPs with or without light irradiation and further incubation for 24 h in fresh medium. The viability of HeLa cells incubated with TAT-TPDC NPs and TAT-PPDC NPs for different durations of light irradiation (B) or at different power densities (C), followed by further incubation for 24 h in fresh medium. Data represent mean values \pm standard deviation, $n = 3$.

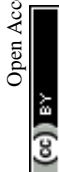
TAT-TPDC NPs and TAT-PPDC NPs show low dark toxicity. The results indicate that the $^1\text{O}_2$ clearly causes cell death.

Conclusions

In this contribution, three AIEgen PSs of TPDC, TPPDC and PPDC were designed and synthesized with fine-tuned ΔE_{ST} via HOMO-LUMO engineering. Among the three AIEgens, PPDC shows the highest $^1\text{O}_2$ quantum yield of 0.89, owing to its lowest ΔE_{ST} value (0.27 eV). This led to higher HeLa cell inhibition for TAT-PPDC NPs relative to TAT-TPDC NPs upon white light illumination. These observations validate our hypothesis that reducing the ΔE_{ST} values could yield higher $^1\text{O}_2$ generation and more efficient PDT. Distinguished from traditional PSs that show ACQ characteristics with reduced $^1\text{O}_2$ generation in aggregates (e.g. NPs), the AIEgen PSs developed in this work show strong fluorescence emission and efficient $^1\text{O}_2$ generation in NPs, which make them highly favorable for image-guided cancer therapy. When the fine-tuning of the ΔE_{ST} approach is further extended to a large variety of fluorophores, such as BODIPY, rhodamine, and phthalocyanine, etc., one would expect to develop new PSs with enhanced $^1\text{O}_2$ generation for more efficient PDT.

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