Supramolecular photodynamic agents for simultaneous oxidation of NADH and generation of superoxide radical

Given that Type-I photosensitizers (PSs) have hypoxia tolerance, developing general approaches to prepare Type-I PSs is of great importance, but remains a challenge. Here, we report a supramolecular strategy for the preparation of Type-I photodynamic agents, which simultaneously generate strong oxidizing cationic radicals and superoxide radicals, by introducing electron acceptors to the existing Type-II PSs. As a proof-of-concept, three electron acceptors were designed and co-assembled with a classical PS to produce quadruple hydrogen-bonded supramolecular photodynamic agents. The photo-induced electron transfer from the PS to the adjacent electron acceptor occurs efficiently, leading to the generation of a strong oxidizing PS•+ and an anionic radical of the acceptor, which further transfers an electron to oxygen to form O2•−. In addition, these photodynamic agents induce direct photocatalytic oxidation of NADH with a turnover frequency as high as 53.7 min−1, which offers an oxygen-independent mechanism to damage tumors.

Photodynamic therapy (PDT) has emerged as a promising treatment for cancer, which operates by administrating photosensitizers (PSs) to produce cytotoxic reactive oxygen species (ROS) under light irradiation1–6. The majority of PSs used in clinical practice or reported in literature produce singlet oxygen (1O2) through energy transfer between the PS in an excited triplet state (3PS*) and molecular oxygen (Type-II mechanism)7–14. However, the yield of 1O2 generated by Type-II PSs is highly dependent on local O2 concentration, meaning the Type-II PSs have a severely lower therapeutic efficacy in treating hypoxic solid tumors, which restricts its application15–19. In contrast, Type-I PSs have been reported to have the benefit of less oxygen content dependency, though this mechanism is not yet well understood20–27. They produce cytotoxic radicals by electron transfer among 3PS*, adjacent substrates, and molecular oxygen28–32.

Unfortunately, Type-I PSs are much less reported, compared to Type-II PSs, due to the lack of general molecular design approaches31–35. This situation is created mainly because the electron transfer reaction is always too inefficient to compete with the energy transfer of the 3PS* to molecular oxygen36–37. It remains a challenge to control the relative contributions of energy transfer and electron transfer by molecular design38,39. In the reported Type-I PDT reactions, the 3PS* first abstracts an electron from nearby substrates, generating an anion radical (PS•−), which further transfers an electron to molecular oxygen to produce a superoxide radical (O2•−)40,41. This is the widely accepted Type-I mechanism, but is normally inefficient because PSs cannot effectively collide with nearby substrates42,43. Theoretically, 3PS* also can transfer an electron to an electron-deficient substrate, and then to O2 to produce O2•−44,45. However, such a Type-I PDT mechanism has never been reported in the literature, presumably due to the lack of suitable electron-deficient substrates in biological systems46. We anticipate that introducing appropriate electron acceptors close to the existing Type-II PS would facilitate the occurrence of an efficient electron transfer between PS and acceptors, leading to the generation of ROS by the Type-I reaction (Fig. 1). This strategy has never been proposed and demonstrated in the literature. It has the following two advantages: (i) it can be employed to convert the existing Type-II photosensitizers to Type-I photosensitizers; (ii) it not only promotes the generation of O2•−, but also yields a strong oxidizing cationic radical of the photosensitizer (PS•+) that directly oxidizes...
biomolecules (e.g., NADH) to cause biological damage in an oxygen-independent manner.

Here, we show a powerful strategy for enhancing the Type-I PDT reactions by introducing electron acceptors to the existing Type-II PS through the supramolecular assembly. As a proof-of-concept, three different types of electron acceptors are designed and co-assembled with a conventional Type-II PS to form quadruple hydrogen-bonded supramolecular photodynamic agents (Fig. 2). Proximity of the PS to electron acceptors with matched redox potential promotes, upon light irradiation, the electron transfer from the PS to the electron acceptor, then to O$_2$, which leads to an efficient generation of O$_2$•−. In addition, this process is accompanied by the generation of a strong oxidizing cationic radical of the PS (PS+$^+$), which effectively oxidizes 1,4-dihydronicotinamide adenine dinucleotide (NADH), an important coenzyme in living cells, aggravating biological damage of tumor cells in an oxygen-independent manner. Strikingly, these supramolecular PSs show the highest NADH oxidation turnover frequency (TOF) of $53.7 \text{min}^{-1}$, which is more than 1–3 orders of magnitude higher than those of conventional transition-metal catalysts. As a result, the supramolecular PS exhibited superior PDT performance in treating tumor cells even under hypoxic environments (2% O$_2$). Distinct solid tumor ablation in vivo was also achieved in the tumor treatment of mouse models.

**Results and discussion**

A classical Type-II PS, iodide-BODIPY (D)$^{47,48}$, and three different types of electron-deficient acceptors (A$_1$, A$_2$, A$_3$) were modified with quadruple hydrogen-bonding units 2-ureido-4[1H]-pyrimidinone (Upy)$^{49}$, which is one of the most commonly used building blocks in supramolecular assembly (Fig. 2b)$^{50,51}$. The synthetic routes are illustrated in the Supplementary Information. All products were characterized by $^1$H NMR, $^{13}$C NMR, and HR-MS.

The electron acceptor of A$_1$ was first chosen to assemble with D to form photodynamic agent DA$_1$ (Supplementary Fig. 1). The formation of the supramolecular polymer was confirmed by diffusion-ordered NMR spectroscopy (DOSY). The diffusion coefficient decreased from $6.31 \times 10^{-10}$ to $1.32 \times 10^{-10}$ m$^2$ s$^{-1}$ as the mixture of D and A$_1$ (molar ratio 4:6) increased from 10 to 50 mM, suggesting the formation of supramolecular polymers (Supplementary Fig. 2). Scanning electron microscopy (SEM) image revealed that DA$_1$ were uniformly distributed in a spherical shape and the average hydrodynamic diameter of DA$_1$ estimated by dynamic light scattering was $69.2 \pm 6.4$ nm (Fig. 3a and Supplementary Fig. 3). The quadruple hydrogen-bonds are crucial in assembly and formation of nanoparticles with well-defined size and morphology (Supplementary Fig. 4). The aqueous dispersion of DA$_1$ show the highest NADH oxidation turnover frequency (TOF) of $53.7 \text{min}^{-1}$, which is more than 1–3 orders of magnitude higher than those of conventional transition-metal catalysts. As a result, the supramolecular PS exhibited superior PDT performance in treating tumor cells even under hypoxic environments (2% O$_2$). Distinct solid tumor ablation in vivo was also achieved in the tumor treatment of mouse models.

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exhibits excellent photostability and thermal stability in PBS and complete medium, which is beneficial to the biological application (Supplementary Fig. 5). The aqueous dispersion of DA1 absorbs at 665 nm and emits weak fluorescence at 730 nm (Supplementary Fig. 6).

The enhancement of the Type-I PDT reactions for DA1 was initially verified by evaluating the generation of O$_2^\cdot$ with the specific O$_2^\cdot$ indicator dihydroethidium (DHE). Upon light irradiation, the fluorescence of the DHE solution in the presence of DA1 increases significantly, indicating that DA1 generates O$_2^\cdot$ efficiently (Fig. 3b). The increase of fluorescence intensity for D is negligible, suggesting that no O$_2^\cdot$ is generated without electron acceptor (Supplementary Fig. 7).

As shown in Fig. 3c, the rate of O$_2^\cdot$ generated by the irradiation of DA1 is 1.7-fold that of methylene blue (MB) under the identical condition. Then, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) was employed as a1O$_2$ indicator to evaluate the1O$_2$ generation of DA1. According to the above mechanism, the photo-oxidation of NADH by DA1 was evaluated. NADH, as an important coenzyme in many oxidoreductases, participates in the maintenance of the intracellular redox balance 2–14. The oxidation of NADH in cancer cells would disturb the intracellular redox balance, thus leading to cell death 15–17. When NADH is oxidized to NAD$^+$, the absorption at 339 nm is reduced and the absorption at 260 nm is enhanced (Fig. 4a).

Fig. 3c. Upon light irradiation the solution of NADH in the presence of DA1 was measured by laser flash photolysis. As shown in Fig. 4b, c, upon light irradiation the solution of NADH in the presence of DA1, the absorption of NADH at 339 nm decreases significantly, whereas it keeps stable when only NADH is present. This result indicates that NADH is photo-oxidized and catalyzed by DA1. Excitingly, the NADH oxidation TOF was calculated to be 24.8 min$^{-1}$, which is much more efficient than widely explored transition-metal-containing photocatalysts 15–17. The decrease of NADH absorption is negligible in the presence of D after irradiation for 4 min, suggesting that it cannot be efficiently oxidized by 1O$_2$ (Supplementary Fig. 9).

To further confirm the photo-oxidation of NADH in the presence of DA1, 1H NMR spectroscopy was employed to monitor the conversion from NADH to NAD$^+$ (Fig. 5a). Upon irradiation of DA1 in aqueous solution with BMPO as a spin-trap agent for O$_2^\cdot$, a characteristic signal of the adduct of O$_2^\cdot$ with BMPO was observed, further confirming the generation of O$_2^\cdot$. The above results demonstrate that introducing an electron acceptor to the Type-II PS is an effective strategy for converting the Type-II process to Type-I.

In order to understand the formation of O$_2^\cdot$ and the oxidation of NADH, the electron transfer from exited D to A1 was studied. The Stern–Volmer quenching of the emission of D with A1 was initially performed. As shown in Fig. 5a, the emission of D is gradually quenched with the addition of A1. The Stern–Volmer plots show a linear correlation between the concentration of A1 and the (I$_0$/I) ratio, suggesting that A1 quenches the excited state of D by electron transfer (Fig. 5b).

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acceptor of A1 decreases from 5.28 to 1.11 µs, indicating that A1 quenched the excited triplet state of D by electron transfer. In order to further study the intermolecular electron transfer, cyclic voltammetry was conducted to determine the oxidation potential (Eox) of D1 and the reduction potential (Ered) of A1. As shown in Fig. 5e, the Eox of D is +0.440 V (VS Fc/Fc+) and the Ered of A1 is −1.183 V (VS Fc/Fc−). According to the Rehm–Weller equation, the Gibbs free energy (ΔG) of electron transfer between D and A1 was estimated as −22.2 kJ mol−1, confirming the thermodynamic feasibility of intermolecular electron transfer (Fig. 5f and Supplementary Fig. 12).

To verify the universality of this strategy, we further evaluated the photoinduced O2− generation and NADH oxidation of D2A2 and D3A3. As shown in Supplementary Fig. 13, the rate of O2− generated by the irradiation of D2A2 and D3A3 is 2.0- and 1.6-fold that of MB under the identical condition and the total ROS generation efficiencies of supramolecular photodynamic agents are enhanced compared to their monomers (Supplementary Fig. 14). The ESR spectroscopy was further employed to confirm the O2− generation by sensitization of D2A2 and D3A3 (Supplementary Fig. 15). Gratifyingly, D2A2 and D3A3 also efficiently oxidize NADH with TOF of 53.7 and 24.3 min−1, respectively (Supplementary Fig. 16). Then, the Stern–Volmer quenching titration of the emission of D was employed with A2 and A3, and the Stern–Volmer quenching constants, Ksv, are 7.7 and 4.9 M−1, respectively (Supplementary Fig. 17). In addition, the calculated ΔG of electron transfer between D and A2 or A3 are both negative and the lifetime of a triplet of D is also decreased in the presence of A2 or A3 (Table 1 and Supplementary Figs. 18, 19). All the above results show that the introduction of an electron acceptor enhances the electron transfer from the PS to the electron acceptor, thereby generating O2− and oxidizing NADH.

Taking D1A1 as an example, the PDT activity was evaluated in vitro. D1A1 is internalized by HeLa cells and reaches the maximum at about 6 h after incubation (Supplementary Fig. 20). ROS indicator 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and a specific O2− probe DHE, were used to detect the cellular ROS generation during PDT in normoxic (21% O2) and hypoxic (2% O2) environment. As shown in Fig. 6a, after illumination, HeLa cells treated with D1A1 and DCFH-DA or DHE manifest obvious fluorescence signals, indicating that D1A1 produces ROS efficiently under either normoxic or hypoxic conditions. Then we investigated NADH depletion in HeLa cells with NAD/NADH assay kit with WST-8. As shown in Fig. 6b, the intracellular NADH levels reduced with the increment of D1A1 concentration upon light irradiation, while in the absence of light irradiation, the NADH levels were unaffected. The PDT effect of D1A1 on HeLa cells was further evaluated by cell counting kit-8 (CCK-8) assays. As shown in Supplementary Fig. 21, there is no toxicity of D1A1 to HeLa cells without irradiation in both normoxic and hypoxic environments. After irradiation with 660-nm light (20 mW cm−2) for 10 min, D1A1 showed obvious cytotoxicity to HeLa cells with a half-maximal inhibitory concentration (IC50) of 0.88 µM under hypoxia and 1.15 µM under hypoxia (Fig. 6c). Then, Calcein-AM and propidium iodide (PI) assays were performed to further investigate the apoptosis process of HeLa cells during PDT, in which viable cells were stained by calcein-AM to emit fluorescence in the green channel and apoptotic cells were stained by PI to emit fluorescence in the red channel. As shown in Fig. 6d, HeLa cells treated with D1A1 and illumination emit fluorescence in both green and red channels, indicating partial cell death. The proportion of red fluorescence increased with the increase of D1A1 concentration, suggesting the inhibition of HeLa cells by D1A1 under irradiation. By contrast, HeLa cells without illumination merely exhibited fluorescence in the green channel under identical conditions, indicating no cell death.

Motivated by the excellent experiment results in vitro, the PDT efficiency of D1A1 was further investigated in immunodeficient mouse models by using a subcutaneous tumor model of HeLa in the BALB/c mice. The fluorescence imaging in mice indicated D1A1 was enriched in tumor position due to the enhanced permeability and retention (EPR) effect (Supplementary Fig. 22). Then we proceeded to study the antitumor efficiency of D1A1 in mice bearing HeLa tumor (primary tumor volume: ~100 mm3). D1A1 (130 µM, 60 µL) was subjected to the mice by tail vein injection, followed by illumination treatment (660 nm, 120 mW cm−2) on tumor sites at 9 h post intravenous injection. The body weights (Fig. 6e) and tumor volumes (Fig. 6f) were recorded during the subsequent 14 days. The weight of the mice increased slightly, indicating that the systemic cytotoxicity of D1A1 is negligible. For the group treated with D1A1 and light, the growth of
tumor was inhibited compared with the control group, suggesting that DA1 effectively suppressed the tumor in vivo. And the anti-tumor effect of D without an electron acceptor under identical condition was obviously inferior to that of DA1, suggesting that the introduction of electron acceptors can significantly improve the efficacy of PDT. The mice were sacrificed on the 14th day and all tumor tissues were peeled and weighed. As shown in Fig. 6g, h, the volume and weight of tumor of the treatment group were significantly smaller than that of the control groups, indicating that DA1 well inhibits tumors in vivo.

In conclusion, we report the preparation of Type-I photodynamic agents by introducing electron acceptors to existing Type-II PSs through the quadruple hydrogen-bonded supramolecular assembly. The photoinduced electron transfer from the PS to the electron acceptor is realized by the shortened distance between the PS and the electron acceptor with matched redox potential. Such a process enhances the formation of radical ion pairs. On the one hand, the anionic radical of the acceptor transfers an electron to molecular oxygen to produce $\text{O}_2^{-}$; on the other hand, the cationic radical of the photosensitizer efficiently oxidizes NADH to cause biological damage with an oxygen-independent mechanism, enhancing the PDT efficacy. Experiments show that the prepared photodynamic agents efficiently produce $\text{O}_2^{-}$ and oxidize NADH with a TOF as high as $53.7 \text{ min}^{-1}$ upon electron acceptor and d DA1 dispersed in water (20 μM); the excitation wavelength was 660 nm and the monitoring wavelength was 550 nm. Cyclic voltammogram of e D and A1 in DCM with 0.1 M (n-Bu)₄NPF₆ as a supporting electrolyte, Ag/Ag⁺ as a reference electrode, glassy-carbon electrode as a working electrode, and Pt wire as a counter electrode; scan rate, 100 mV s⁻¹; Fc/Fc⁺ was used as an external reference.

f Gibbs free energy of electron transfer between D and A1.

Table 1 | The relevant data on photoinduced electron transfer between D and A

| Electron acceptor | $K_{sv}$ (M⁻¹) | $E^{\text{red}}$ (V) | $\Delta G$ (KJ mol⁻¹) | $\tau_T$ (μs) |
|-------------------|----------------|---------------------|----------------------|--------------|
| A1                | 16.0           | 1.183               | -22.2                | 1.11         |
| A2                | 7.7            | 1.106               | -29.9                | 0.96         |
| A3                | 4.9            | 1.175               | -23.2                | 2.17         |

*The Stern–Volmer quenching constants ($K_{sv}$) were measured by titration.

*Reduction potentials were measured by cyclic voltammetry with ferrocene as the standard.

*The Gibbs free energy ($\Delta G$) was calculated according to the Rehm–Weller equation.

*The lifetime of the triplet state ($\tau_T$) of D in the supramolecular agent was measured by laser flash photolysis.
light irradiation, and they exhibit excellent PDT performance, even under a hypoxic environment. We envision that this work will broaden the insight into Type-I PDT and inspire researchers to develop effective photodynamic agents for cancer treatment.

**Methods**

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Cell culture media, PBS, fetal calf serum (FBS), and CCK-8 (cell counting kit-8) were purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate), DHE (Dihydroethidium), WST-8 (NAD'/NADH Assay Kit), and Calcein-AM/propidium iodide (PI) detection Kit were purchased from Beyotime Biotechnology (Shanghai) Co., Ltd. All chemical synthesis reagents are purchased from Energy Chemical (Shanghai) Co., Ltd. and TCI Chemical Co., Ltd. Female BALB/c mice (6–8 weeks) were used as an animal model in this study. All mouse models are provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were housed in individually ventilated cage (IVC) systems (ambient temperature: 23 ± 3 °C; relative humidity: 40–70%). The instruments and equipment involved in the article are listed in Supplementary Information.

**Data analysis**

All plotted and calculated statistical analyses were performed on Origin 8.5 and SPSS.

**Ethical statement**

All animal experiments were performed following the protocols evaluated and approved by the Animal Ethics Committee of Capital Medical University (Ethics Approval Number: AEEI-2018-097).
irradiation (660 nm LED light, 20 mW cm\(^{-2}\)), the absorption intensity of ABDA at 378 nm was recorded by the UV-Vis absorption spectrometer.

In vitro \(\text{O}_2^*\) detection by ESR spectroscopy

BMPO (5-tert-butoxy carbonyl 5-methyl-1-pyrroline N-oxide) was employed as a spin-trap agent for \(\text{O}_2^*\) in vitro ESR spectroscopy was employed to detect the ESR signals of the following five groups of samples: (i) 100 \(\mu\)M of DA1 was dispersed in water containing 25 mM of BMPO and illuminated with a xenon lamp (500–1200 nm); (ii) 100 \(\mu\)M of DA1 was dispersed in water containing 25 mM of BMPO without light; (iii) 100 \(\mu\)M of D was dissolved in DMSO containing 25 mM of BMPO and illuminated with a xenon lamp; (iv) 25 mM of BMPO was dissolved in DMSO and illuminated with a xenon lamp; (v) 25 mM of BMPO was dispersed in water without light.

Calculation of turnover frequency (TOF)

The TOF of photocatalytic oxidation of NADH was calculated from the difference in NADH concentration after 1 min irradiation divided by the concentration of photosensitizer. The concentration of NADH was calculated using the extinction coefficient \(\varepsilon_{339} = 6220\ \text{M}^{-1}\text{cm}^{-1}\) in PBS buffer.

Cyclic voltammetry measurement

We employed a three-electrode system to conduct the cyclic voltammograms experiment. Specifically, a platinum-carbon compound electrode was used as the working electrode, the Pt wire electrode was used as the auxiliary electrode, and the Ag/Ag\(^+\) electrode was used as the reference electrode. The measurement was conducted in dichloromethane and 0.1 M tetrabutylammonium hexafluorophosphate was added as a supporting electrolyte. The scan rate was 100 mV/s. Fc/Fc\(^+\) was used as an external reference.

ROS detection in living cells

DCFH-DA was used as a ROS indicator to detect intracellular ROS generation. Human cervical cancer (HeLa) cells were planted onto 35-mm confocal dishes at a density of 1 \(\times\) 10\(^5\) cells. After incubation for 24 h under normoxic (21% O\(_2\)) or hypoxic (2% O\(_2\)) conditions, the medium was removed and washed with PBS three times. Fresh medium containing 0.5 \(\mu\)M DA1 was added and incubated for 0.5 h. After that, the medium was removed and washed with PBS three times. After irradiation with a 660 nm LED light (40 mW cm\(^{-2}\)) for 5 min, fluorescence images were promptly captured by confocal laser scanning microscopy (CLSM).

In vitro \(\text{O}_2^*\) detection in living cells

DHE was used as an \(\text{O}_2^*\) indicator to detect intracellular \(\text{O}_2^*\) generation. HeLa cells were planted onto 35-mm confocal dishes at a density of 1 \(\times\) 10\(^5\) cells. After incubation for 24 h under normoxic (21% O\(_2\)) or hypoxic (2% O\(_2\)) conditions, the medium was removed and washed with PBS three times. Fresh medium containing 0.5 \(\mu\)M DA1 was added and incubated for another 6 h. Then, the medium was replaced with fresh medium containing 2 \(\mu\)M DCFH-DA and incubated for 0.5 h. After that, the medium was removed and washed with PBS three times. After irradiation with a 660 nm LED light (40 mW cm\(^{-2}\)) for 5 min, fluorescence images were promptly captured by confocal laser scanning microscopy (CLSM).

In vivo imaging

Tumor imaging in vivo were studied with a subcutaneous HeLa tumor model in the immunocompetent BALB/c mice. DA1 (400 \(\mu\)g mL\(^{-1}\), 60 \(\mu\)L) were subjected to the mice by tail vein injection, followed by fluorescence imaging recorded at 1, 3, 6, and 12 h by an animal imaging system.

Ex vivo biodistribution of DA1

After 12 h of tail vein injection, mice were sacrificed and their major organs, including heart, liver, spleen, lung, kidney, pancreas, and tumor were carefully removed for visualization under the imaging system.

In vivo PDT experiment

The HeLa tumor-bearing mice were divided into five groups (each group contains five mice): Group I: PBS injection; Group II: D injection without irradiation; Group III: DA1 injection without irradiation; Group IV: D injection with irradiation; Group V: DA1 injection with irradiation. After 9 h of tail vein injection, a 660 nm LED light (120 mW cm\(^{-2}\)) treatment was performed on Group IV and Group V by irradiating the tumor region for 20 min. The effect of the different treatment groups was monitored by measuring tumor size (tumor size = width \(\times\) length/2) and mice body weight for 14 days after PDT treatment. After 14 days, the tumors of the mice were dissected and weighed.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data generated in this study are available within the article, Supplementary Information, and Source Data. Source data are provided with this paper.

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**Author contributions**

Q.-Z.Y. proposed the concept, Q.-Z.Y., L.-Y.N., N.X., and K.-X.T. designed the experiments. Q.-Z.Y., L.-Y.N., and N.X. supervised the project. K.-X.T. synthesized compounds and performed the experiments. Q.-Z.Y., L.-Y.N., N.X., and K.-X.T. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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