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Novel carbazole-based two-photon photosensitizer for efficient DNA photocleavage in anaerobic condition using near-infrared light

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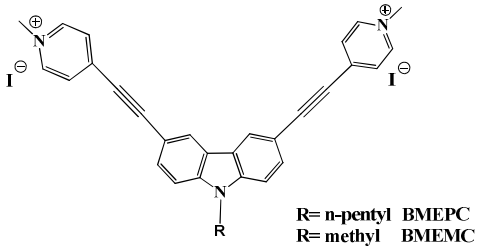
Two novel carbazole derivatives, BMEPC and BMEMC, were designed, synthesized and first reported as two-photon photosensitizer for DNA photodamage, which showed efficient DNA photocleavage ability under near-infrared light exposure *via* two-photon process in anaerobic condition.

Photodynamic therapy (PDT), as one of the minimally invasive radiation therapy technologies for malignant tumors, has attracted great interest due to the potential lower poison, higher targeting by using non-poison photosensitizer.[1] A potentially important mechanism of PDT is that photosensitizers can cause the DNA photodamage in cancer cells when exposed to light with a proper wavelength.[2] Photosensitizers such as metal complexes, porphyrins, anthraquinones, and fullerenes et al. have been confirmed to possess DNA photocleavage activity through type I or type II mechanisms.[3] In contrast to type II mechanism that highly depends on O_2 to generate reactive oxygen species, type I mechanism can still work at low oxygen concentrations through electron transfer or hydrogen abstraction processes, which is of high potential to extend PDT applications into hypoxic cellular areas of solid tumor tissue.[4]

However, most photosensitizers suffer the disadvantage of weak absorbance in the phototherapeutic window of 600-900 nm, limiting their further application in PDT.[5] Thus, photosensitizers with two-photon absorption (TPA) property are very attractive since the photoactivation achieved by two-photon excitation using near-infrared (NIR) light is capable of achieving deep tissue penetration.[6] Moreover, the nonlinear process of TPA on the laser intensity restricts the absorption on the focus of a laser beam, which is beneficial to highly selective targeting damage.[7] Nevertheless, there were relatively few studies on the photosensitizers for PDT or photosensitized DNA damage through TPA process because the conventional photosensitizers usually have a very low TPA cross section (δ_{TPA}), generally in the order of 1-100 GM (1 GM = 10^{-50} cm^4 s $photon^{-1}$ $molecule^{-1}$).[8] Thus, DNA photocleavers with large δ_{TPA} are needed for developing the agents for two-photon excited PDT.

As a known nonlinear materials, carbazole derivatives have been widely used in micro/nanofabrication, optical power limiting and bioanalytical science.[9] In the previous work, we have found carbazole-based cyanines can be employed as two-photon excited fluorescent (TPEF) probes for DNA and cell imaging considering their high binding affinity to DNA, large TPA cross section, and good water solubility.[10] Considering the DNA photodamage ability of some carbazole derivatives by UV-light exposure, we expected that a carbazole-based molecule with large δ_{TPA} and potential interaction with DNA would photosensitize DNA damage under NIR light.[11] Herein, two novel carbazole derivatives 3,6-bis[2-(1-methylpyridinium)ethynyl]-9-pentyl-carbazole diiodide (**BMEPC**) and 3,6-bis[2-(1-methylpyridinium)ethynyl]-9-methyl-carbazole diiodide (**BMEMC**) were designed and synthesized (Scheme 1). The C_{2v} symmetric A-π-D-π-A structure, strong intramolecular change transfer and planar molecular structure with positive charge contribute to the large δ_{TPA}, low fluorescence quantum yield and high binding affinity towards DNA by intercalation mode.[11a, 12] They show efficient DNA photocleavage ability excited not only by visible light but 800 nm NIR light no matter in aerobic or anaerobic condition *via* type I mechanism.



Scheme 1 The chemical structures of **BMEPC** and **BMEMC**.

The synthesis route of these carbazole-based compounds is presented in Scheme S1. Starting from 3,6-dibromo-9H-carbazole, **BMEPC** and **BMEMC** were synthesized *via* Sonogashira reaction to

afford bis-ethynylpyridine carbazole according to our previous work.[13] Subsequent methylation was performed to give the desired product.

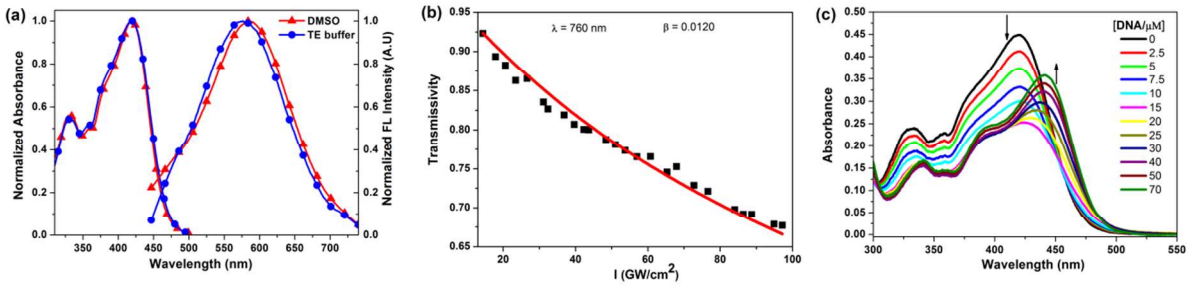


Fig. 1 (a) Normalized absorption and one-photon induced fluorescence spectra of **BMEPC** in DMSO and TE buffer; (b) The plot of transmissivity dependence on light intensity for **BMEPC** at 760 nm. Squares denote the experimental value of transmissivity; solid lines denote the theoretical fitting line; (c) The absorption spectra of **BMEPC** (10 μM) with the addition of CT-DNA (0-70 μM) in TE buffer.

The ionic groups introduced by salification reaction are better electron acceptor groups in the molecule.

The photophysical properties of **BMEPC** and **BMEMC** have been investigated and the data are summarized in Table 1. The normalized absorption and fluorescence spectra of **BMEPC** in dimethyl sulfoxide (DMSO) and Tris-EDTA (TE) buffer are shown in Fig. 1a. The absorption band at 418 nm is the electronic transition from the ground state to the intramolecular charge transfer (ICT) state, and the peak at 331 nm is assigned to the typical π–π* transition corresponding to the locally excited state. Besides the two peaks, there is a weak shoulder peak at 380 nm, which is attributed to the coupling of two ICT branches. The emission peaks locate at ~590 nm with the excitation wavelength of 415 nm. The fluorescence quantum yields of **BMEPC** and **BMEMC** are too low to be calculated accurately either in DMSO or buffer solution. This is most likely induced by the ICT state which mainly deactivates through a nonradiative decay, resulting in more efficient transition from singlet state to triplet state.[14]

Table 1 Optical properties of **BMEPC** and **BMEMC**

	Solvent	λ_{abs}[a] (nm)	$10^4\varepsilon$[b] ($M^{-1}\cdot cm^{-1}$)	λ_{em}[c] (nm)	Φ[d]	δ[e] (GM)
BMEPC	DMSO	419	4.69	591	<0.001	522[f]/401[g]
	TE buffer	418	4.55	584	<0.001	
BMEMC	DMSO	418	4.59	593	<0.001	492[f]/352[g]
	TE buffer	417	4.58	583	<0.001	

[a] the wavelength of absorption maximum; [b] the extinction coefficient; [c] the wavelength of one-photon emission maximum; [d] fluorescence quantum yield; [e] TPA cross section (1 GM = 10^{-50} cm^4 s $photon^{-1}molecule^{-1}$) at [f] 760nm and [g] 800nm.

Both **BMEPC** and **BMEMC** exhibit weak TPEF signals upon excitation at 800 nm femtosecond (fs) laser pulses in DMSO. Since two-photon induced fluorescence method is not suitable for the δ_{TPA} measurement of the two compounds due to their extremely low fluorescence quantum yields, the δ_{TPA} were determined by a non-linear transmission measurement technique using an amplified Ti:sapphire ultrafast laser system (Spitfire ACE, Spectra-Physics) at the wavelength from 750-810 nm.[15] The relationships of transmissivity and light intensity for **BMEPC** and **BMEMC** at 760 nm and 800 nm are shown in Fig. 1b & S2, ESI*. Both of them showed a maximum δ_{TPA} at 760nm (522 GM for **BMEPC** and 492 GM for **BMEMC**) which corresponded with the shoulder peak in one-photon absorption spectra at 380 nm as the coupling of second intramolecular charge transfer state. The large TPA properties imply their potential applications for two-photon photosensitization.

The interaction of **BMEPC** and **BMEMC** with DNA was investigated by absorption titration. The absorption spectra of **BMEPC** and **BMEMC** upon addition of calf thymus DNA (CT-DNA) at different concentrations in TE buffer are very similar (Fig. 1c and S3, ESI*). Obvious decrease of the absorption intensity (42% and 36%, respectively) accompanied with negligible bathochromic shift under low concentration of DNA and then hyperchromism with a bathochromic shift (~20 nm) at high concentration were observed. The spectra changes as a function of the concentration of DNA indicate that the interaction of **BMEPC** and **BMEMC** to DNA is a complex process, including at least two binding modes. The compounds may aggregate on the surface of CT-DNA helix at low DNA concentration by the electrostatic interactions and then intercalate into CT-DNA base pairs at high DNA/compounds ratios leading to the increased absorbance and large red-shift of λ_{max}, which is basically in line with the result as reported in literature.[16]

The binding capabilities of **BMEPC** and **BMEMC** towards CT-DNA were investigated by fluorescence titration (Fig. S4, ESI*). The compounds showed obvious fluorescence enhancement upon the addition of CT-DNA, which is attributed to the reduction of the nonradiative decay caused by the restricted intramolecular rotation after interacting with DNA. The binding constants (K_b) of **BMEPC** and **BMEMC** with CT-DNA estimated by the nonlinear curve fitting analysis are 2.9 × 10^5 M^{-1} and 3.3 × 10^5 M^{-1}, respectively, which are comparable to some other DNA-binding molecules mentioned in literatures.[17] The result indicates that the compounds possess high binding affinity to DNA due to their symmetric bis-cationic and planar structures, which implies the potential of them as DNA photocleavers.

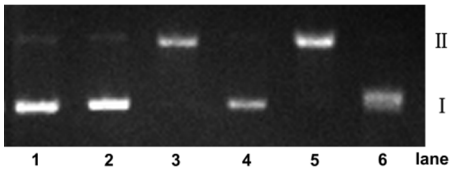


Fig. 2 Agarose gel electrophoresis patterns of the photocleaved supercoiled pBR322 DNA (31 μM in base pair) by **BMEPC** and **BMEMC** (20 μM) upon visible-light irradiation (> 400 nm) for 25 min in air-saturated Tris/CH_3COOH/EDTA buffer (pH = 7.4). Lane 1, DNA alone (in dark); lane 2, DNA + irradiation; lane 3,

DNA + **BMEPC** + irradiation; lane 4, DNA + **BMEPC** (in dark); lane 5, DNA + **BMEMC** + irradiation; lane 6, DNA + **BMEMC** (in dark). Form I and II denote supercoiled circular and nicked circular forms, respectively.

BMEPC and **BMEMC** were applied to photocleavage supercoiled pBR322 DNA upon light irradiation in air-saturated buffer. As shown in Fig. 2, the plasmid is in the supercoiled form (Form I) with a small amount of nicked circular form (Form II) in the absence of compounds (lanes 1 and 2). DNA was not cleaved in the presence of the compounds (20 μM) in the dark (lanes 4 and 6). Under light irradiation (λ > 400 nm, 25 min), **BMEPC** and **BMEMC** can lead to single-strand DNA cleavage, as evidenced by the transformation from Form I to Form II (lanes 3 and 5). The results show the light radiation is necessary for DNA cleavage by **BMEPC** and **BMEMC**.

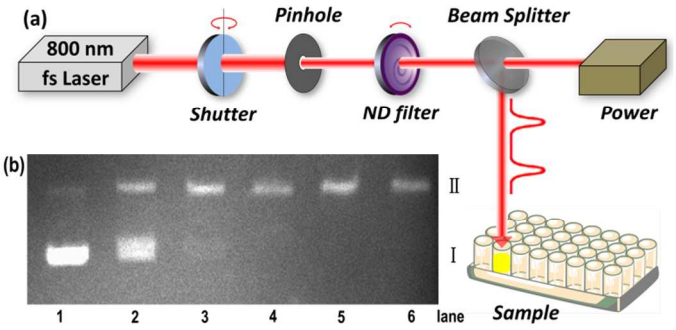


Fig. 3 (a) Experimental setup for two-photon DNA photocleave; (b) Agarose gel electrophoresis patterns of the photocleaved supercoiled pBR322 DNA (31 μM in base pair) by **BMEPC** upon 800 nm femtosecond (fs) laser (0.3 W/cm^2) irradiation for 35 min in air-saturated Tris/CH_3COOH/EDTA buffer (pH = 7.4). Lane 1, DNA alone; lane 2, DNA + **BMEPC** (10 μM); lane 3, DNA + **BMEPC** (20 μM); lane 4, DNA + **BMEPC** (30 μM); lane 5, DNA + **BMEPC** (40 μM); lane 6, DNA + **BMEPC** (50 μM). Form I and II denote supercoiled circular and nicked circular forms, respectively.

We further examined the DNA photocleavage activity of **BMEPC** through two-photon absorption process by 800 nm laser pulse with a pulse width of 120 fs and a repetition rate of 1 kHz. As shown in Fig. 3a, supercoiled pBR322 DNA within different concentrate of **BMEPC** was irradiated for 35 min under defocused laser beam with an average power of 0.3 W/cm^2. Control experiment proves that the presence of **BMEPC** is necessary for DNA cleavage (Fig. 3b). Less DNA cleavage was observed for 10 μM **BMEPC** upon irradiation, while DNA converted from supercoiled circular form to nicked circular form completely at the concentration of 30 μM (lanes 2 and 4). These experiment results indicate that the high DNA photocleavage activities of **BMEPC** and **BMEMC** can be induced by not only one-photon absorption process, but two-photon absorption process, which enable them to be excited with a NIR light source.

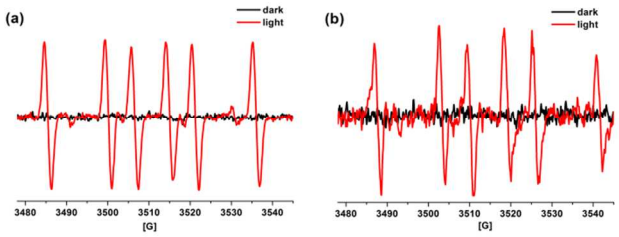


Fig. 4 EPR spectra of DMPO spin adducts: (a) N_2-saturated DMSO solutions of 1 mM **BMEPC** with mercury lamp, the splitting parameters are g=2.0046, $a_{N\alpha}$=14.5 G and $a_{H\beta}$=21.1 G; (b) N_2-saturated PBS (pH 7.4) solutions of 50 μM **BMEPC** and pBR322 DNA (31 μM in base pair) with mercury lamp, the splitting parameters are g=2.0063, $a_{N\alpha}$=15.6 G and $a_{H\beta}$=22.3 G. Dark control means the sample without light irradiation.

To investigate the possible mechanism of the photosensitized DNA damage by **BMEPC**, a control experiment performed in the N_2 atmosphere showed that there was no significant difference for the DNA photocleavage results between aerobic and anaerobic conditions (Fig. S5, ESI*). The results suggest that the photocleavage mainly results from type I mechanism as oxygen is not the essential cofactor for DNA photocleavage which is also in agreement with the very low efficiency in singlet oxygen production of **BMEPC** (data not shown).[18] Furthermore, negligible inhibiting effect for DNA cleavage was observed in the presence of superoxide dismutase (SOD) and mannitol, indicating that superoxide anion (O_2^{-}·) and hydroxyl radicals (·OH) were not involved (Fig. S5, ESI*). Electron transfer from guanine to the photoexcited BMEPC can be excluded on the account of the relatively short excited state lifetime of **BMEPC** (< ns level) and it is also impossible in the terms of energy according to the estimated Gibbs free energy (+0.15 eV) of the process (Fig. S6, ESI*).[19] A possible explanation for the photocleavage activity of **BMEPC** is the reactive intermediates generated by photochemical effects. The planar carbazole molecule with pyridinium cations as strong acceptor group can form N-centered radical cations through electron transfer.[20] As shown in Fig. 4a, the EPR signals of **BMEPC** used 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as spin trapping agent can be assigned to aminyl radicals, which shows good accordance to results reported in the literature.[20b, 21] The similar signals were also obtained in the PBS solution in the presence of pBR322 DNA (Fig. 4b). The amine radical cations generated by photoirradiation may abstract hydrogen from the adjacent deoxyribose, leading to the DNA cleavage.[21-22] In addition, the inhibiting effect for DNA cleavage in the presence of NaN_3 was likely caused by the quenching of the radical species, which is reasonable that NaN_3 is a singlet oxygen scavenger but not strictly specific.[23]

In summary, we have successfully designed and synthesized two novel carbazole derivatives **BMEPC** and **BMEMC** as photosensitizers for DNA photocleavage. The molecular structure characteristics contribute to the large TPA cross section and the high binding affinity towards DNA. DNA photocleavage can be achieved efficiently in the presence of **BMEPC** and **BMEMC** excited not only by visible light but 800 nm NIR light through TPA process. The experimental evidence supports the fact that **BMEPC** and **BMEMC** photocleave DNA mainly via hydrogen abstraction by N-centered radicals (Type I mechanism), contributing to the DNA photocleavage ability in anaerobic conditions. Such carbazole-based photocleavers are valuable for the development of new two-photon excited PDT agents. Further studies are underway to investigate the the detailed mechanism, cytotoxicity and PDT experiment *in vivo*.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Fig. S1-S6, experimental details, compound characterization, fluorescence titration, electrochemical properties. See DOI: 10.1039/c000000x/

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