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Imidazole-modified porphyrin as a pH-responsive sensitizer for cancer photodynamic therapy†

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5,10,15,20-Tetrakis(*N*-(2-(1*H*-imidazol-4-yl)ethyl)benzamide)-porphyrin produced twice as many singlet oxygen ($^1O_2$) molecules at pH 5.0 (quantum yield 0.53 ± 0.01) than at pH 7.4, whereas the $^1O_2$ quenching rate was reduced by a factor of 2.5 for a pH change from 7.4 to 5.0.

Photodynamic therapy (PDT) is based on the administration of a tumor-localizing sensitizer and its activation by light absorption. This technique has been developed for the treatment of various malignancies,[1–4] in which singlet oxygen ($^1O_2$) plays a key role in light-induced cell death. The selectivity of PDT, however, leaves much to be desired because normal cells are also able to accumulate sensitizers, which leads to a prolonged skin photosensitization. One strategy used to solve this problem is to build certain triggers (*e.g.*, pH, bioaffinity) into conventional sensitizers for controllable $^1O_2$ release, which has been the subject of many recent studies.[5–7]

The utility of $^1O_2$ in PDT or organic synthesis is influenced by competitive processes that deactivate $^1O_2$ to ground state oxygen ($^3O_2$) *via* various mechanisms including vibrational energy transfer.[8,9] Quenching of $^1O_2$ can be both good and bad in terms of photosensitizing applications. The vibrational deactivation of $^1O_2$ has been used in stereoselective control of enecarbamate photooxidation.[9] A sensitizer that produces $^1O_2$ at an acidic pH but is deactivated at physiological pH would benefit from the therapeutic selectivity in cancer treatment because the pH in growing malignant tumors tends to be somewhat lower than that in surrounding normal tissue.[10] O'Shea's group prepared a supramolecular agent containing an amine functional group, in which the pH-based reversibility of $^1O_2$ generation was observed.[5] At pH values greater than the pK_b of amines, the intramolecular electron transfer from the adjacent amine quenched the excited sensitizer, hence preventing the energy transfer that led to the production of $^1O_2$. However, upon the protonation of amines, the quenching of the chromophore by electron-transfer was precluded; thus the photosensitized production of $^1O_2$ could ensue. Very recently, Lee and co-workers prepared a polysaccharide/drug conjugate in which glycol chitosan was grafted with 3-diethylaminopropyl isothiocyanate, chlorine e6 and poly(ethylene glycol).[7] At higher pH chlorine e6 is deactivated *via* autoquenching. However, in tumor acidic conditions, a polysaccharide/drug conjugate undergoes conformational change into a uncoiled structure for $^1O_2$ production. In other studies, Ogilby's group reported a DNA sequence-controlled on/off switchable $^1O_2$ sensitizer.[11] In this case, a sensitizer and a quencher were kept in close contact in the "off state" by DNA-programmed assembly but were separated to switch the sensitizer "on" through a process of competitive DNA hybridization. Zheng *et al.* constructed a two-component system consisting of a pyropheophorbide sensitizer tethered with a small peptide sequence to a carotenoid,[12] an effective quencher to both triplet states and $^1O_2$.[13] The tether could be selectively cleaved in the presence of caspase-3 protease to release pyropheophorbide for $^1O_2$ production. The regulation of $^1O_2$ by the interaction of a sensitizer with single-walled carbon nanotubes,[14] TiO_2 nanoparticles[15] and calf thymus DNA[16] was also demonstrated.

Herein, we reported a new system in which a pH-controlled imidazole on/off switch was incorporated into 5,10,15,20-tetrakis(*N*-(2-(1*H*-imidazol-4-yl)ethyl)benzamide) porphyrin (TIEBAP). The four carboxylic acid groups of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (TCPP) were converted into carboxylic amides *via* a typical amide coupling procedure with histamine (see ESI†). Selective control of $^1O_2$ was achieved *via* efficient quenching of triplet states and/or $^1O_2$ at alkaline but not acidic pH. The design and function of TIEBAP are shown in Scheme 1. A special feature in this design is that the imidazole moieties of cationic porphyrin TIEBAP were separated from the porphyrin ring by ethylbenzamide chain spacers, thereby preventing the delocalization of the positive charges onto a porphyrin ring system through direct coupling that could adversely affect the triplet and $^1O_2$ yields.[17–19]

The imidazole heterocyclic ring contained two nitrogen atoms with protonated pK_a values of 7.0 and 14.9 and was functionalized as the proton receptor in TIEBAP. The protonation occurred just below physiological pH. Our results indicate that this pH-responsive sensitizer possesses an ability

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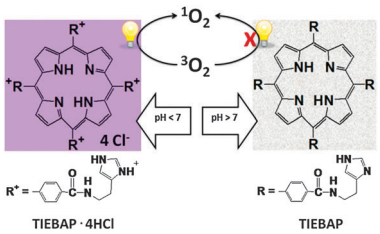
† Electronic supplementary information (ESI) available: Materials and instruments, the synthetic approach of TIEBAP, spectra of ESI-MS, NMR, UV-visible and fluorescence, measurements of Φ_Δ and quenching rate constant of $^1O_2$ by histamine, effect of pH on initial $^1O_2$ intensity, TIEBAP stability tests and cell viability MTT assay. See DOI: 10.1039/c1cc13328d



Scheme 1 Design and function of pH-responsive TIEBAP for $^1O_2$ production.

to efficiently kill tumor cells in an acidic environment upon visible irradiation, whereas the production of $^1O_2$ was dramatically reduced at a physiological pH. We attributed this therapeutic selectivity to the improved solubility of TIEBAP and its inertness toward $^1O_2$ in acidic solutions due to the protonation of imidazole ring nitrogen. Sensitizer aggregation occurred upon the deprotonation of imidazole ring nitrogen at slightly alkaline pH, leading to an inefficient formation and potential quenching of the triplet state and/or $^1O_2$.

The positive charges at the imidazole moieties could impact the charge distribution of the porphyrin ring only through inductive effects. An increase in pH promoted the formation of face-to-face H-type aggregation (see ESI†). The change in the UV-visible spectrum of TIEBAP indicated dissociation of the H-type aggregation of this porphyrin to the monomer upon reducing the pH. In addition, the fluorescence spectra were strongly affected by aggregation, showing a weaker two-band emission spectrum at pH 8.2 but a strong one-band emission spectrum at pH 5.3 (see ESI†). The aggregation is known to reduce the quantum yield and lifetime of the excited triplet states of porphyrins, thereby adversely affecting the quantum yield of $^1O_2$ production (Φ_Δ).[20,21]

The effect of pH on $^1O_2$ production was studied by monitoring the $^1O_2$ luminescence at 1270 nm as previously described.[15,22] All of the experiments were performed in D_2O solutions with different pH values, in which $^1O_2$ had a longer lifetime (67 μs)[23] compared to H_2O (4 μs).[24] Keeping the amount of sensitizer constant, the intensity of $^1O_2$ was decreased with an increase in pH (Fig. 1 and ESI†). Compared to the initial value of $^1O_2$ intensity at pH 5.0, the production of $^1O_2$ was reduced by 30% at pH 6.5 and by 50% at pH 7.4, whereas the lifetime of $^1O_2$ in D_2O was increased by a factor of 2.2 at pH 5.0 (71 μs) and 1.5 at pH 6.5 (45 μs) compared to the value of 31 μs at pH 7.4. The first-order solvent deactivation rate constants of $^1O_2$ (k_d) were measured to be 1.3×10^4 s^{-1} at pH 5.0, which was consistent with the literature value of 1.5×10^4 s^{-1} in D_2O.[25] The observed k_d at higher pH values

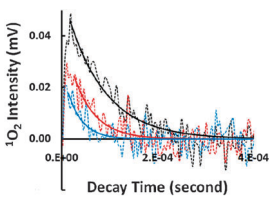


Fig. 1 Kinetics of $^1O_2$ decay after irradiation of air-saturated 7.0×10^{-6} M TIEBAP solutions (with 1% methanol in D_2O) at 532 nm and at pH 5.0 (black), 6.5 (red) and 7.4 (blue); dots: experimental results and solid lines: first-order kinetic simulation

Table 1 Observed first-order solvent deactivation rate constants of $^1O_2$ (k_d) upon irradiation of 7.0×10^{-6} M TIEBAP at 532 nm in air-saturated D_2O solutions of different pH

pH	5.0	6.5	7.4
k_d/s^{-1}	1.3×10^4	2.2×10^4	3.2×10^4

increased as shown in Table 1. Clearly, the quenching of $^1O_2$ in the reaction media containing TIEBAP could be neglected in acidic solutions but not in alkaline solutions. The fast decay of $^1O_2$ in weak alkaline solution ($k_d = 3.2 \times 10^4$ s^{-1} at pH 7.4) could have resulted from the quenching by both aggregates and the imidazole moieties (see quenching results below). Fig. 1 clearly shows how $^1O_2$ generation was virtually switched on in response to decreasing pH.

The quantum yield of $^1O_2$ production (Φ_Δ) for TIEBAP at pH 5.1 D_2O was determined to be 0.53 ± 0.01 by a relative method in comparison to a well-developed reference sensitizer meso-tetrasulfonatophenyl porphyrin (TSPP) with a known Φ_Δ 0.63 in D_2O[26] (see ESI†). This value (0.53) is consistent with the Φ_Δ of TCPP (0.53) in the weak alkaline solutions.[15] To quantify the effect of imidazole protonation on $^1O_2$ quenching, we determined the total quenching rate constant of $^1O_2$ removal (k_T, M^{-1} s^{-1}) by histamine in pH 7.4 and pH 5.1 D_2O solutions by the Stern–Volmer analysis. Measurements were carried out at 532 nm excitation using TSPP as a sensitizer. Our data indicated that the kinetics of $^1O_2$ luminescence decay at 1270 nm followed the Stern-Volmer equation (eqn (1)).

$$k_{obs} = k_d + k_T[\text{histamine}] \quad (1)$$

where k_{obs} is the observed 1st-order rate constant of $^1O_2$ decay after laser pulse. k_d is the 1st-order rate constant of $^1O_2$ decay in the absence of histamine. Changes in $^1O_2$ lifetimes were observed by the addition of histamine into the solutions (Fig. 2). Stern–Volmer plots showed a good linear correlation between k_{obs} and quencher histamine concentrations, giving k_T $(5.8 \pm 0.9) \times 10^7$ M^{-1} s^{-1} in pH 7.4 phosphate D_2O buffer and $(5.1 \pm 0.7) \times 10^5$ M^{-1} s^{-1} in pH 5.1 acetic acid/acetate D_2O buffer. Our results are comparable to literature values for histidine, wherein k_T was determined to be 5.0×10^7 M^{-1} s^{-1} at pH > pK_a but less than 10^6 M^{-1} s^{-1} at pH < pK_a.[27] Except for the physical quenching, the large k_T value at higher pH could involve the chemical reactions of $^1O_2$ with the deprotonated imidazole rings *via* a [4 + 2] cycloaddition.[28] Apparently, the efficient quenching of $^1O_2$ by imidazole moieties could result in the limited utility of $^1O_2$ in weak alkaline

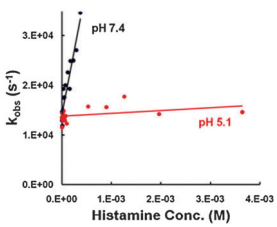


Fig. 2 Stern–Volmer plots for the luminescence quenching of $^1O_2$ by TIEBAP in 0.1 M phosphate D_2O buffer solution of pH 7.4 (black dots) and in 0.1 M acetic acid/acetate D_2O buffer solution of pH 5.1 (red dots). Solid lines are theoretical simulation using a linear least-square fitting method.

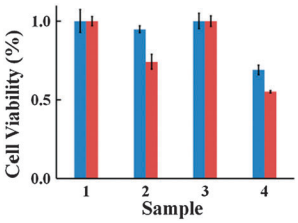


Fig. 3 SK-BR-3 cancer cell viability after 10 minute (samples 2) and 20 minute (samples 4) irradiation (at an average intensity of 7.3 mW cm^{-2}) of 1.0×10^{-5} M TIEBAP-attached 1×10^{6} breast cancer cells at pH 7.4 (blue bars) and pH 6.1 (red bars). Samples 1 and 3 are set to 100% and used as references that represent 10 minute and 20 minute dark control in the absence of TIEBAP, respectively.

solutions or at physiological pH, which is desired in PDT. The rate constants of $^1O_2$ decay in D_2O buffer solutions (k_d) were also extracted from the Stern–Volmer analysis as 1.4×10^4 s^{-1} for both pH 5.1 and 7.4, which was consistent with the literature value of 1.5×10^4 s^{-1} for D_2O.[23]

The human adenocarcinoma breast cell line SK-BR-3 was used to test the photodynamic selectivity at both physiological pH (7.4) and acidic tumor extracellular pH (6.1). The results in Fig. 3 revealed that TIEBAP exhibited a pH-sensitive response to acidic pH. For the same amount of sensitizer, the cytotoxicity was significantly enhanced at pH 6.1 (red bars) when compared to results at pH 7.4 (blue bars). When the pH decreased from 7.4 to 6.1, the cell viability was reduced from 95% to 75% (samples 2 in Fig. 3) and from 70% to 55% (samples 4 in Fig. 3) after visible irradiation of TIEBAP for 10 and 20 minutes, respectively. The cancer cells at pH 6.1 required about half of the irradiation time to obtain the same cytotoxic effectiveness compared to results at pH 7.4. The control experiments performed in darkness at both pH 6.1 and pH 7.4 were used as references for cell viability calculation. Clearly, this imidazole-modified porphyrin showed potential as a selective drug for PDT in cancer treatment.

In conclusion, we propose a new system to improve the selectivity of PDT in cancer treatment, in which imidazole moieties were employed as a pH-sensitive trigger for controllable $^1O_2$ release. The photosensitized production of $^1O_2$ can be switched on in an acidic tumor environment but almost off at physiological pH. A special feature in TIEBAP design is based on the control of photosensitization *via* imidazole moieties that were separated from the porphyrin ring by ethylbenzamide chain spacers to prevent the direct charge distribution onto the porphyrin chromospheres. With an easy synthetic approach, the incorporation of imidazoles into a hydrophobic sensitizer allowed modulation between monomers and aggregates around a neutral pH. The selective control of $^1O_2$ production was achieved by the improved solubility of TIEBAP and its inertness toward $^1O_2$ at slightly acidic pH due to protonation of the imidazoles. The deprotonation resulted in sensitizer aggregation in weak alkaline solutions, hence leading to the inefficient formation and potential quenching of triplet states and/or $^1O_2$. Quenching of $^1O_2$ by the deprotonated imidazole rings in weak alkaline solutions makes imidazole moieties ideal switches to shut down, at least partially, the therapeutic function of a sensitizer in a normal cellular environment. This system can be extended to other sensitizers modified with imidazole groups.

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