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A pH-responsive fluorescent probe and photosensitiser based on a self-quenched phthalocyanine dimer†

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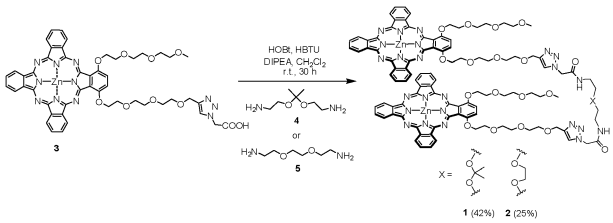
A self-quenched zinc(II) phthalocyanine dimer linked with an acid-sensitive ketal unit has been prepared, which can be activated in an acidic environment (pH = 5.0–6.5) as a result of the cleavage of the ketal linker and separation of the phthalocyanine units, resulting in enhanced fluorescence emission and singlet oxygen production.

Activatable fluorescent probes which can be triggered by tumour-associated stimuli have great potential in molecular imaging of cancer.[1] Generally, these probes are connected to a quencher *via* a short linker, such as peptide[2] or oligonucleotide,[3] which can be selectively cleaved or hybridised upon interaction with a tumour-associated enzyme or nucleic acid. The separation of the fluorophore and the quencher restores the fluorescence emission, thereby allowing specific detection of the target. As an alternative form of these activatable probes, an acid-sensitive unit is linked to the fluorophore by which its fluorescent property can be modulated by changing the pH of the environment.[1b,4] These strategies have been extended to the design of activatable photosensitisers for targeted photodynamic therapy (PDT), which is of much current interest as a promising modality for cancer treatment.[5]

Apart from using a quencher, which deactivates the fluorescence and photosensitisation processes by energy and/or electron transfer, the fluorophore/photosensitiser itself can also be used to induce self-quenching as a result of its intrinsic aggregation tendency. This design has the advantage of amplification of signals due to the presence of more fluorescent and photosensitising units. A significant number of such self-quenched systems immobilised onto polymers, nanoparticles and proteins have been reported, which can be activated by cleavage of the linkers or disintegration of the macro-systems under a specific condition.[1,2,5] However, only a few self-quenched dimeric molecular systems have been reported so far.[6] We report herein a novel pH-responsive fluorescent probe and photosensitiser (compound **1**) in which two phthalocyanine units are linked with an acid-cleavable ketal group. Due to the intrinsic propensity of phthalocyanines to form H-type dimers,[7] compound **1** is in the "OFF" state, resulting in minimal fluorescence emission and singlet oxygen production. However, under an acidic condition (pH < 6.5), the acid-sensitive linker is cleaved and the two phthalocyanine units are separated, thus restoring its fluorescence emission and singlet oxygen generation. On the basis that tumours generally have a lower extracellular pH than normal tissues,[8] this dimer serves as a potential tumour-selective fluorescent probe and photosensitiser for targeted PDT.



Scheme 1 Synthesis of phthalocyanine dimers **1** and **2**.

Scheme 1 shows the synthetic route for **1** and a non-cleavable analogue (compound **2**) which was used as a control. Treatment of the carboxy phthalocyanine **3**[9] with diamino ketal **4**[10] in the presence of 1-hydroxybenzotriazole (HOBt), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIPEA) in CH_2Cl_2 gave the cleavable dimer **1** in 42% yield. Similarly, by treating **3** with the commercially available diamine **5**, the non-cleavable analogue **2** was obtained. Both dimers **1** and **2** could be purified readily by silica gel column chromatography and were fully characterised with various spectroscopic methods and elemental analysis. The assignment of their ^{1}H NMR signals in pyridine-d_5 was confirmed by ^{1}H–^{1}H COSY experiments (ESI†).

The UV-Vis spectra of **1** and **2** were recorded in DMF (Fig. S1, ESI†) and the data are compiled in Table 1, in which the corresponding data for **3** are also included for comparison. Both the spectra of **1** and **2** were typical as those for non-aggregated phthalocyanines, showing a sharp and intense Q band

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† Electronic supplementary information (ESI) available: UV-Vis spectra of **1** and **2** in DMF, comparison of the UV-Vis and fluorescence spectra of **1–3** in DMF, comparison of the rates of photodegradation of DPBF in DMF using **1–3** and ZnPc as the photosensitisers, UV-Vis and fluorescence spectra of **1** and **2** after incubation for 8 h in citrate buffer solutions or phosphate buffered saline with different pH, changes in fluorescence intensity of **1** and **2** in phosphate buffered saline with different pH at different time intervals, comparison of the UV-Vis and fluorescence spectra of **1** and **3** after incubation for 8 h in a citrate buffer solution at pH 5.0, fluorescence images and intensities of HT29 cells after incubation with nigericin and **1** at pH 5.0 or 7.4, comparison of the cytotoxic effects of **1** and **2** on HT29 cells and ^{1}H, ^{13}C{^{1}H} and ^{1}H–^{1}H COSY spectra of **1** and **2** in pyridine-d_5. See DOI: 10.1039/c2cc34327d

Table 1 Electronic absorption and photophysical data for compounds **1–3** in DMF

Compound	λ_{abs}/nm (log ε)	λ_{em}[a]/nm	Φ_F[b]	Φ_Δ[c]
1	342 (5.11), 629 (4.90), 690 (5.49)	703	0.03	0.56
2	341 (5.09), 626 (4.89), 690 (5.51)	703	0.03	0.62
3	336 (4.70), 619 (4.55), 687 (5.30)	698	0.11	0.85

[a] Excited at 610 nm. [b] Relative to ZnPc in DMF as the reference (Φ_F = 0.28). [c] Relative to ZnPc as the reference (Φ_Δ = 0.56 in DMF).

at 690 nm, which strictly followed the Lambert–Beer law. All their B band, vibronic band and Q band were slightly red-shifted (by 3–10 nm) compared with those of **3**, suggesting that there are π–π interactions between the phthalocyanine units in the dimers. Upon excitation at 610 nm, both compounds showed a weak fluorescence emission at 703 nm with a fluorescence quantum yield (Φ_F) of 0.03 relative to the unsubstituted zinc(II) phthalocyanine (ZnPc) (Φ_F = 0.28). Although the Q-band absorptions of **1** and **2** were stronger than that of **3** due to the presence of two phthalocyanine units, their fluorescence emissions were significantly weaker (Fig. S2, ESI†), indicating that a certain degree of self-quenching of the phthalocyanine units existed in these dimers.

To evaluate the photosensitising efficiency of these compounds, their singlet oxygen quantum yields (Φ_Δ) were also determined in DMF by a steady-state method using 1,3-diphenylisobenzofuran (DPBF) as the scavenger. The values were reflected by the rates of decay of DPBF. The results showed that dimers **1** and **2** can generate singlet oxygen but are less efficient than **3** (Fig. S3, ESI† and Table 1). It is believed that dimerisation of the phthalocyanine units in the dimers partially quenches their singlet excited state, reducing the singlet oxygen generation efficiency.

To examine the pH-responsive properties of dimer **1**, its UV-Vis and fluorescence spectra were measured after incubation for 8 h in citrate buffer solutions with different pH. As shown in Fig. S4(a) (ESI†), the Q band becomes more intense and sharper as the pH decreases from 7.4 to 5.0. It is believed that the ketal linker is cleaved under acidic conditions, which separates the two phthalocyanine units and hence reduces their aggregation. By contrast, the Q band of the non-cleavable analogue **2** remains very broad under all these conditions [Fig. S4(b), ESI†]. Upon excitation at 610 nm, the fluorescence intensity of **1** increases significantly at lower pH (Fig. 1), while the fluorescence remains very weak for **2** (Fig. S5, ESI†).

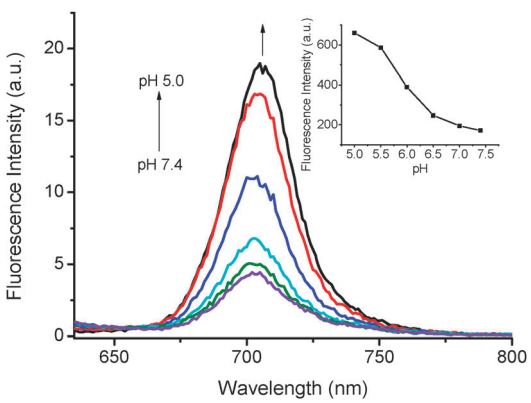

Fig. 1 Fluorescence spectra (λ_{ex} = 610 nm) of **1** (2 μM) after incubation for 8 h in citrate buffer solutions with different pH. The inset plots the variation of the fluorescence intensity with pH.

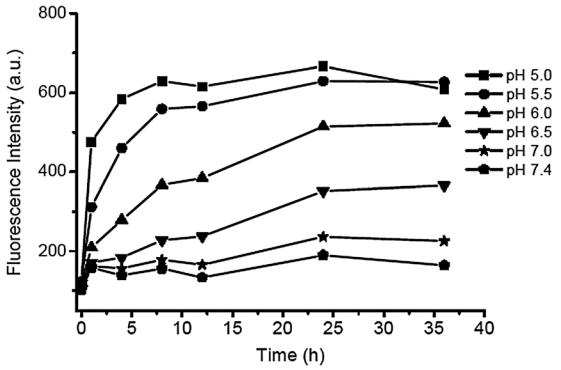

Fig. 2 Changes in fluorescence intensity of **1** (2 μM) in citrate buffer solutions with different pH at different time intervals.

Fig. 2 shows the changes in fluorescence intensity of **1** in citrate buffer solutions with different pH at different time intervals. It can be seen that the fluorescence intensity increases by more than 4-fold in 1 h at pH 5.0. This indicates that a significant portion of the ketal linker is cleaved within an hour and the linked phthalocyanines are separated. The fluorescence intensity is almost restored after incubation for 8 h in the buffer solution with pH 5.0 as shown by the almost identical UV-Vis and fluorescence spectra with those of **3** (Fig. S6, ESI†). As expected, the rate of cleavage of the ketal linker, as reflected by the rate of fluorescence increase, decreases as the pH increases. The fluorescence intensity remains almost unchanged at pH 7.0 and 7.4 over 36 h. For the non-cleavable analogue **2**, there is no significant change in the fluorescence intensity at different pH over 36 h. The UV-Vis and fluorescence spectra of **1** and **2** were also recorded in phosphate buffered saline at different pH (5.0, 6.0 and 7.4) at different time intervals (up to 24 h) (Fig. S7–S9, ESI†). The results were very similar to those recorded in citrate buffer solutions.

In addition, the effect of pH on the singlet oxygen generation efficiency of these compounds was also examined. Fig. 3 compares the rates of decay of DPBF induced by dimers **1** and **2** after incubation for 10 h in the buffer solutions at pH 5.5 and 7.4. It can be seen that dimer **1** shows the highest efficiency in generating singlet oxygen at pH 5.5 and the efficiency is

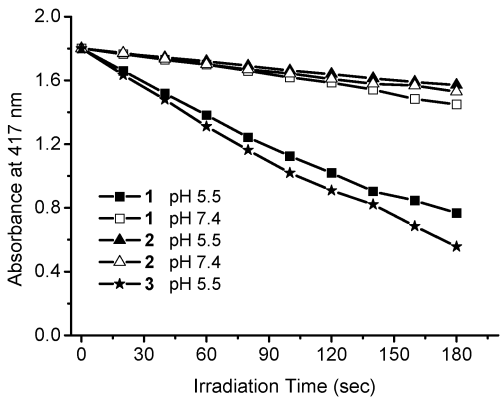

Fig. 3 Comparison of the rates of photodegradation of DPBF with irradiation time. The mixtures contained freshly prepared DPBF (80 μM) and **1** (2 μM), **2** (2 μM) or **3** (4 μM), which were incubated previously in a citrate buffer solution at pH 5.5 (closed symbols) or pH 7.4 (open symbols) for 10 h.

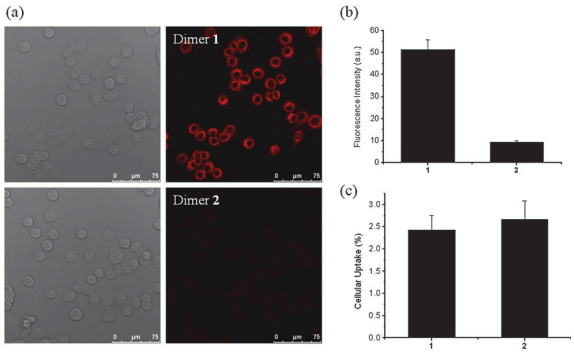

Fig. 4 (a) The bright field (left column) and intracellular fluorescence (right column) images of HT29 cells after incubation with **1** or **2** (5 μM) for 2 h. (b) Comparison of the intracellular fluorescence intensities of **1** and **2**. Data are expressed as the mean ± standard deviation (number of cells = 50). (c) Percentage cellular uptake of **1** and **2** determined by an extraction method. Data are expressed as the mean ± standard error of the mean of three independent experiments.

comparable to that of **3** under the same conditions. By contrast, dimer **1** (at pH 7.4) and dimer **2** (at pH 5.5 and 7.4) are not efficient singlet oxygen generators.

To study the activation of **1** at the cellular level, human colon adenocarcinoma HT29 cells were incubated with **1** and **2** (as a control) for 2 h. The bright field and fluorescence images of the cells were then captured, and the intracellular fluorescence intensities were determined. As shown in Fig. 4(a), dimer **1** can be activated inside the cells causing strong intracellular fluorescence throughout the cytoplasm. By contrast, the fluorescence was very weak for **2**. The intracellular fluorescence intensity of **1** is about 5-fold higher than that of **2** [Fig. 4(b)]. To take into account that these two compounds may have different efficiency in emitting fluorescence inside the cells, we also employed an extraction method to quantify the cellular uptake. After incubation with **1** or **2** for 2 h, DMF was used to lyse the cells and extract the dyes. The dye concentrations inside the cells were quantified by measuring their Q-band absorbance. It was found that the cellular uptakes of **1** and **2** are actually similar [Fig. 4(c)]. The results suggest that the stronger intracellular fluorescence of **1** is due to the cleavage of the ketal linker in the acidic compartments of the cells.

As a further study, the pH-dependent fluorescence emission of **1** in HT29 cells was also investigated. The cells were first incubated with the ionophore nigericin used to equilibrate the intra- and extra-cellular pH[11] at pH 5.0 and 7.4, which were used to roughly mimic the environments around tumours and normal tissues respectively. The cells were then further incubated with **1**. As shown in Fig. S10 (ESI†), the intracellular fluorescence intensity of **1** at pH 5.0 is about 4-fold stronger than that at pH 7.4. The results further demonstrate that **1** is preferentially activated at lower pH at the cellular level, which is a desirable characteristic for tumour-selective imaging probes.

Finally, the photocytotoxicity of **1** and **2** was also briefly examined against HT29 cells. Both of them were essentially non-cytotoxic in the dark, but exhibited substantial cytotoxicity upon illumination with red light ($\lambda > 610$ nm) (Fig. S11, ESI†). The IC_{50} value of **1** (0.35 μM) was significantly lower than that of **2** (0.59 μM). Interestingly, the non-cleavable dimer **2** was still photocytotoxic despite its weak intracellular fluorescence. It is likely that the quenching of singlet oxygen production is less effective than the quenching of fluorescence emission inside the cells. The singlet oxygen produced, even in a small amount, can effectively trigger the oxidative damage of the cells. In fact, the percentages of fluorescence and singlet oxygen quenching were found to be 84% and 66%, respectively, for **2** in the buffer at pH 7.4. These values are similar to those observed for a self-quenched pyropheophorbide *a* dimer reported earlier (80% and 60% respectively).[6a]

In summary, we have developed a novel pH-responsive fluorescent probe and photosensitiser based on the self-quenching mechanism of phthalocyanine units. This dimer shows enhanced fluorescence emission and singlet oxygen generation in acidic environments due to cleavage of the linker. By changing the nature of the linker, the quenching efficiency can be optimised and the activation mechanism can be extended to other tumour-specific stimuli. This provides a useful strategy to enhance the efficacy of this class of therapeutic agents.

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