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A disulfide-linked conjugate of ferrocenyl chalcone and silicon(IV) phthalocyanine as an activatable photosensitiser†‡

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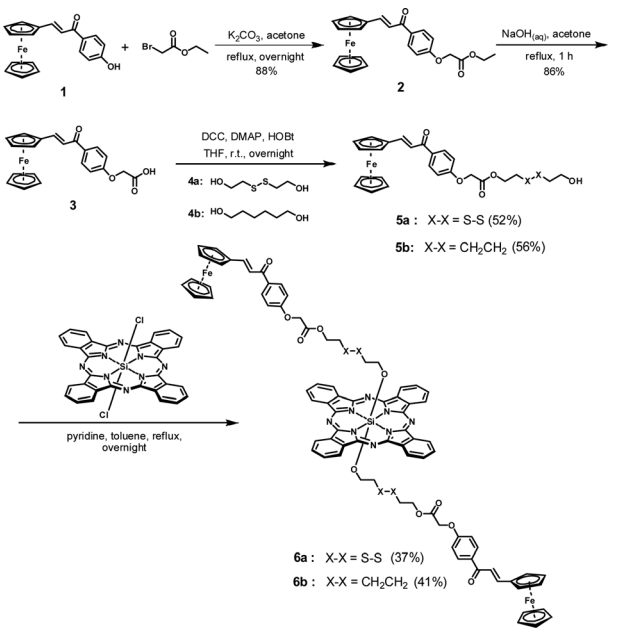
A novel bis(ferrocenyl chalcone) silicon(IV) phthalocyanine has been prepared in which the disulfide linker can be cleaved by dithiothreitol. The separation of the ferrocenyl quencher and the phthalocyanine core greatly enhances the fluorescence emission, singlet oxygen production and *in vitro* photocytotoxicity.

There has been considerable interest in development of efficient photosensitisers for photodynamic therapy (PDT).[1] Apart from their efficiency in generating reactive oxygen species (ROS), their selectivity towards malignant tissues is also a critical factor affecting the therapeutic outcome. To achieve the latter, various strategies have been explored which include conjugation to tumour-specific vehicles such as epidermal growth factor and monoclonal antibody, and encapsulation in nano-systems such as liposomes, polymeric micelles and silica-based nanoparticles.[2] In addition, activatable photosensitisers are also of much current interest.[2a,3] Upon interactions with various tumour-associated stimuli such as the acidic environment of tumours,[4] cancer-related proteases,[5] and mRNAs that have high tumour specificity,[6] the photosensitising ability of these photosensitisers can be triggered, thereby offering control over their tumour selectivity. We have recently reported a ketyl-linked self-quenched phthalocyanine dimer as a rare example of activatable phthalocyanine-based photosensitisers.[7] Under an acidic environment (pH = 5.0–6.5), the ketal linker is cleaved and the disconnection of the phthalocyanine units enhances the photosensitising efficiency. We describe herein another example which has a different mode of activation. It contains a silicon(IV) phthalocyanine core linked to two ferrocenyl chalcone moieties at the axial positions *via* a disulfide linker. The ferrocenyl unit serves as a quencher for the excited phthalocyanine[8] and a potential chemotherapeutic agent,[9] which may promote the anti-tumour effect after cleavage. The incorporation of the disulfide linker, which can be cleaved by biological reducing agents, provides a way for activation. In fact, a substantial number of disulfide-linked systems have been prepared and used for drug delivery and release,[10] and as fluorescent probes for thiols[11] and protein transduction[12] in living cells. The synthesis of this novel compound and the effects of a reducing stimulus, namely dithiothreitol (DTT), on its photophysical properties and *in vitro* photocytotoxicity are described below.

Scheme 1 shows the preparation of this compound and a non-cleavable counterpart. Treatment of 3-ferrocenyl-1-(4′-hydroxyphenyl)-prop-2-en-1-one (**1**)[9a] with ethyl bromoacetate in the presence of K_2CO_3 in acetone afforded compound **2**, which was hydrolysed with NaOH to give compound **3**. This compound was then coupled with 2-hydroxyethyl disulfide (**4a**) or 1,6-hexanediol (**4b**) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP) and 1-hydroxybenzotriazole (HOBt) to give the corresponding alcohols **5a** and **5b**. Further substitution of silicon(IV) phthalocyanine dichloride ($SiPcCl_2$) with these alcohols resulted in the formation of the cleavable phthalocyanine **6a** and the non-cleavable analogue **6b**. In addition, $SiPcCl_2$ was also treated with an excess of alcohol **4a** to give $SiPc(OCH_2CH_2S–SCH_2CH_2OH)_2$ (**7**) as a reference compound, in which the two ferrocenyl chalcone moieties are absent (Scheme S1, ESI‡). The experimental details and the characterisation data are given in ESI.‡



Scheme 1 Synthesis of ferrocenyl phthalocyanines **6a** and **6b**.

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The electronic absorption spectra of **6a**, **6b** and **7** in DMF (Fig. S1a–c, ESI‡) were typical for non-aggregated phthalocyanines. They showed a B-band at 353–355 nm, an intense and sharp Q-band at 674–676 nm together with two vibronic bands at 608 and 642–646 nm (Table S1, ESI‡). The spectra of **6a** and **6b** showed two additional bands at 330–332 and 494–498 nm, which can be attributed to the absorptions of the ferrocenyl chalcone moieties. It was supported by the absorption spectrum of **5a**, which displayed two absorption bands at 311 and 489 nm in DMF (Fig. S1d, ESI‡). Upon excitation at 610 nm, these phthalocyanines showed a fluorescence emission at 684–688 nm. The fluorescence quantum yields of **6a** and **6b** (Φ_F = 0.04–0.05) were greatly reduced compared with that of **7** (Φ_F = 0.28) (Table S1, ESI‡). This can be attributed to the presence of ferrocenyl moieties, which effectively quench the singlet excited state of the phthalocyanines by photoinduced electron transfer.[8]

The singlet oxygen quantum yields (Φ_Δ) of these phthalocyanines in DMF were also determined by a steady-state method using 1,3-diphenylisobenzofuran (DPBF) as the scavenger. Fig. S2 (ESI‡) compares the rate of photodegradation of DPBF sensitised by these compounds and the unsubstituted zinc(II) phthalocyanine (ZnPc) used as a reference. The values of Φ_Δ are also listed in Table S1 (ESI‡). Similarly, the values of **6a** (Φ_Δ = 0.17) and **6b** (Φ_Δ = 0.13) are significantly lower than that of **7** (Φ_Δ = 0.34), which again can be explained by the reductive quenching by the ferrocenyl moieties, which disfavours the intersystem crossing and eventually the formation of singlet oxygen.

To demonstrate that **6a** is responsive towards reducing stimuli, we recorded the fluorescence spectra of **6a** in phosphate buffered saline (PBS) in the presence of 0.5% Cremophor EL and different concentrations of DTT (0–40 mM) over 24 h (Fig. S3a, ESI‡). As shown in Fig. 1, the fluorescence intensity increases with time and the concentration of DTT. In the absence or presence of 2 μM of DTT, which mimics the extracellular reducing environment, the fluorescence intensity only increases slightly. However, upon addition of 10 or 40 mM of DTT, which mimics the intracellular reducing environment, particularly in tumour, the fluorescence intensity increases substantially within the first 6 h. This can be attributed to the cleavage of the disulfide linker by DTT preventing quenching by the ferrocenyl moieties. For the non-cleavable

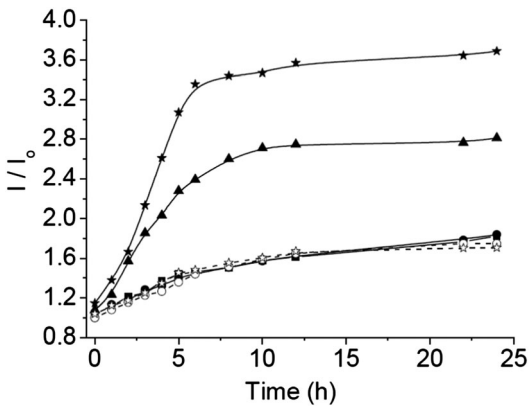

Fig. 1 Changes in fluorescence intensity of **6a** (closed symbols) and **6b** (open symbols) upon exposure to 0 μM (circles), 2 μM (squares), 10 mM (triangles) and 40 mM (stars) of DTT in PBS with 0.5% Cremophor EL.

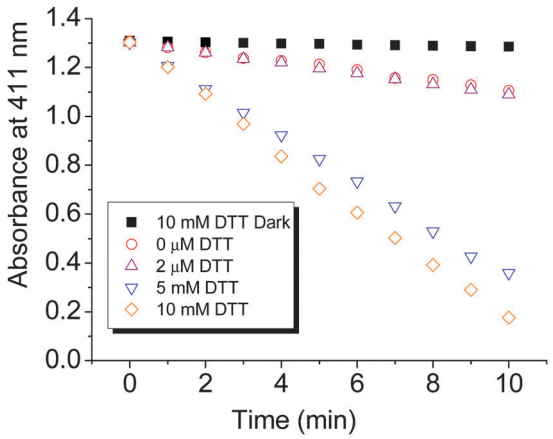

Fig. 2 Comparison of the rate of photodegradation of DPBF (initial concentration = 70 μM) sensitised by **6a** (4 μM) upon exposure to different concentrations of DTT for 24 h in PBS with 0.5% Cremophor EL. The dark control is shown as closed symbols.

analogue **6b**, the fluorescence intensity was only increased slightly even in the presence of 40 mM of DTT (Fig. S3b, ESI‡ and Fig. 1). The absorption spectra of **6a** and **6b** were also recorded upon exposure to different concentrations of DTT over 24 h (Fig. S4, ESI‡). The sharp Q band was virtually unchanged in all the cases, which suggested that both compounds and the phthalocyanine fragment of **6a** remained non-aggregated, and the fluorescence enhancement for **6a** was due to relaxation of the quenching effect upon reductive cleavage of the disulfide linker rather than the change in aggregation state.

DTT-mediated production of singlet oxygen by these compounds was also investigated. Fig. 2 compares the rate of photodegradation of DPBF sensitised by **6a** upon exposure to different concentrations of DTT for 24 h in PBS. In the dark, the compound could not produce singlet oxygen even in the presence of 10 mM of DTT. Upon illumination and at up to 2 μM of DTT, the rate of singlet oxygen production was still very slow. However, when the concentration of DTT was increased to 5 or 10 mM, the singlet oxygen production efficiency was remarkably enhanced. Clearly, the photosensitisation ability of **6a** was restored upon reductive cleavage of the disulfide linker. This was supported by the response of **6b**, for which the rate of singlet oxygen production remained slow even in the presence of 10 mM of DTT. By contrast, for the non-ferrocene-containing reference compound **7**, the efficiency was remarkably high and very similar to that of **6a** under the same conditions (Fig. S5, ESI‡).

The therapeutic efficacy of all these compounds was investigated against MCF-7 human breast cancer cells. Fig. S6 (ESI‡) shows the dose-dependent survival curves for phthalocyanines **6a**, **6b** and **7**, and the ferrocenyl chalcone analogue **5a** after incubation for 6 or 24 h and with prior treatment with different concentrations of DTT for 1 h, both in the absence and presence of light. All these compounds were non-cytotoxic in the dark. Upon illumination (λ > 610 nm, 40 mW cm^{-2}, 48 J cm^{-2}) and without prior treatment with DTT, **6a** and **6b** exhibited similar photocytotoxicity. Both of them were less potent than **7** probably due to the quenching effect by the ferrocenyl units. Unexpectedly, compound **5a** was not cytotoxic up to a concentration of 1 mM. Similar results were obtained after the cells were pre-treated with 2 μM of DTT. However, when 4 mM of DTT was used, the photocytotoxicity of

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Table 1 Comparison of the IC_{50} values of **6a**, **6b** and **7** against MCF-7 cells after incubation for 6 or 24 h and with prior treatment with different concentrations of DTT for 1 h

	IC_{50} (nM)					
	6 h Drug incubation			24 h Drug incubation		
	0 μM DTT	2 μM DTT	4 mM DTT	0 μM DTT	2 μM DTT	4 mM DTT
6a	160 ± 5	150 ± 5	81 ± 5	124 ± 2	119 ± 2	50 ± 1
6b	179 ± 8	162 ± 4	163 ± 6	149 ± 6	135 ± 5	130 ± 9
7	80 ± 7	75 ± 6	76 ± 6	52 ± 2	51 ± 2	50 ± 2

6a was significantly enhanced, while that of **6b** and **7** remained essentially unchanged. Table 1 compares their IC_{50} values, defined as the drug concentration required to kill 50% of the cells, under different conditions. It can be seen that only **6a** is DTT-responsive. For 24 h drug incubation, pre-treatment with 4 mM of DTT reduced its IC_{50} value by more than 2-fold from 124 to 50 nM, which was almost the same as that of **7**. On the basis that the intracellular thiol level is in millimolar range and tumour tissues generally have an elevated thiol concentration compared with normal tissues,[13] these results demonstrate that **6a** is a promising photosensitiser for targeted PDT, which can be activated in a reducing environment, such as tumour, leading to enhanced fluorescence emission, singlet oxygen production and *in vitro* photodynamic activity.

To further investigate the reductive disulfide cleavage of **6a** at the cellular level, the intracellular fluorescence of this compound was examined under different conditions. MCF-7 cells were first treated with different concentrations of DTT for 1 h followed by incubation with **6a** for 24 h. As shown in Fig. 3a, the cells being pre-treated with 4 mM of DTT show strong intracellular fluorescence, while the fluorescence is hardly observed for the cases of 0 and 2 μM of DTT. For the non-cleavable analogue **6b**, the intracellular fluorescence remained extremely weak for all the three cases (Fig. S7, ESI‡). The average relative fluorescence intensity per cell for these compounds was also measured and is compared in Fig. 3b. Only **6a** at high concentration of DTT (4 mM) shows high intracellular fluorescence, which is about 9-fold higher than that of the other cases. These results are also in accord with the cleavage of the disulfide linkage in **6a** by DTT inside the cells.

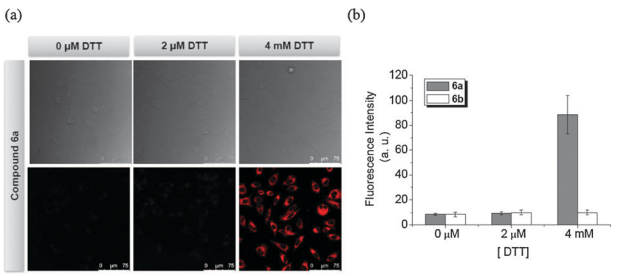

Fig. 3 (a) Confocal fluorescence images of MCF-7 cells pre-treated with different concentrations of DTT followed by incubation with **6a** (1 μM) for 24 h (lower row). The corresponding bright field images are shown in the upper row. (b) Comparison of the intracellular fluorescence intensity for **6a** and **6b** at different concentrations of DTT. Data are expressed as mean ± standard deviation (number of cells = 30).

To reveal the subcellular localisation of **6a** after disulfide cleavage, we coincubated the MCF-7 cells, which were pre-treated with DTT (4 mM) for 1 h, with **6a** and ER-Tracker Green, LysoTracker Green DND 26 or MitoTracker Green FM, which are specific fluorescent dyes for endoplasmic reticulum, lysosomes and mitochondria respectively. As shown in Fig. S8 (ESI‡), the fluorescence caused by the ER-Tracker can superimpose with the fluorescence caused by **6a** after disulfide cleavage. This observation suggests that the phthalocyanine fragment of **6a** is localised in the endoplasmic reticulum of the cells. By contrast, the fluorescence images could not be overlapped for the cases of MitoTracker and LysoTracker (Fig. S9, ESI‡), indicating that the fragment is not preferentially localised in the mitochondria and lysosomes of the cells.

In summary, we have prepared a novel disulfide-linked ferrocenyl phthalocyanine which can be activated by DTT at millimolar concentrations. On the basis that the intracellular thiol level is in this range and tumour tissues generally have an elevated thiol concentration compared with normal tissues, this compound is a promising photosensitiser for targeted PDT.

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