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Redox-responsive Xanthene-coumarin-chlorambucil-based FRET-guided theranostics for "activatable" combination therapy with real-time monitoring

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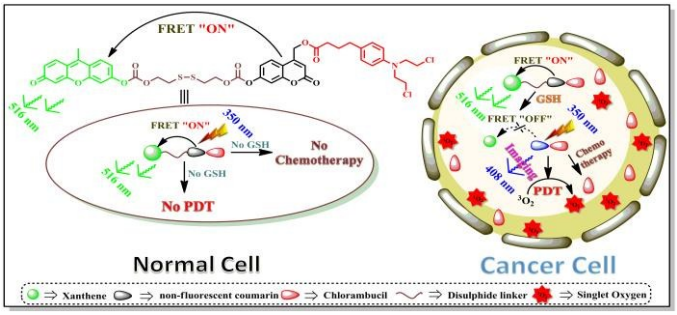
FRET donor-acceptor Xanthene-coumarin conjugate has been designed for redox-regulated synergic treatment of photodynamic therapy and chemotherapy with real-time monitoring. The "locked" FRET pair was selectively "unlocked" by biological reducing thiols via rupture of sacrificial disulfide linker. Distinct change in fluorescence color and selective cancer cell toxicity were observed *in vitro*.

Multimodal therapy i.e. combination of two or more therapies to treat a single disease has now become a well-explored field with regard to efficient cancer healing. There have been ample examples of multimodal therapy involving photothermal therapy (PTT), chemotherapy, and photodynamic therapy (PDT).[1–4] However, most of such multimodal systems involved two or more different drugs for different treatment modalities.[5] Our group has been engaged in developing single chromophore to exhibit synergistic effect of two different treatment modalities for profound tumor eradication.[5,6] The major restraint of previously reported single component multimodal systems is that the chromophore is always in its "unlocked" form to show the synergistic effect leading to undesired side effects.[7] To circumvent this issue, nowadays researchers are developing endogenous stimuli-activated chromophores for target specific cancer therapy.[8–10] With regard to endogenous stimuli-responsive targeting therapy, intracellular pH, specific physiological enzymes, intracellular hypoxia etc. showed remarkable results.[11–13] Recently, redox-responsive chemotherapy is gaining much attention.[14–22] In such cases, the chemotherapeutic drug molecule is attached to a fluorophore by means of a sacrificing disulfide linkage.[23] Such disulfide linkages can be easily cleaved selectively in cancer cells by biological reducing agents viz. glutathione (GSH), thioredoxin (Trx) etc. owing to their higher content in cancer cells (126 nmol/mg-protein) than normal cells (40 nmol/mg-protein).[24] Thus, a thiol-responsive prodrug containing disulfide linkage can serve as an efficient target-specific theranostics for cancer treatment. Though there have been several reports on such "activatable" PDT, development of "activatable" combination therapeutic system is still rare.[25] Real-time monitoring of drug action is a prime concern for target specific cancer treatment. To this end, various energy and electron transfer processes viz. photoinduced electron transfer (PET),[26–28] internal charge transfer (ICT),[29–31] Förster resonance energy transfer (FRET)[32–35] etc. have been extensively explored. Among these, FRET is one of the most promising processes, which depends on the spectral overlap of the emission of the donor with the absorption range of the acceptor moiety.[36] Motivated by these, we aimed to design a FRET-based theranostic, which could show "activatable" synergic treatment of PDT and chemotherapy with real-time monitoring of drug-action. In the present study, we designed 7-hydroxy-(4-methyl)coumarin-xanthene derivative as the FRET donor-acceptor pair. Effective FRET process between coumarin (donor) and xanthene (acceptor) attached through a disulfide linker, makes the whole prodrug system "locked" to show any therapeutic effect (**scheme 1**). On contact with reducing thiols, the prodrug gets "unlocked" releasing coumarin-chlorambucil conjugate as well as the xanthene dye, which could result in simultaneous PDT and photoinduced drug delivery. In addition, such FRET "ON" and "OFF" states lead to the discrete change in fluorescence requisite for real-time monitoring of drug release.



Scheme 1 Redox-responsive FRET-based theranostic Xan-SS-Cou-Cbl mediated bimodal treatment of PDT and chemotherapy.

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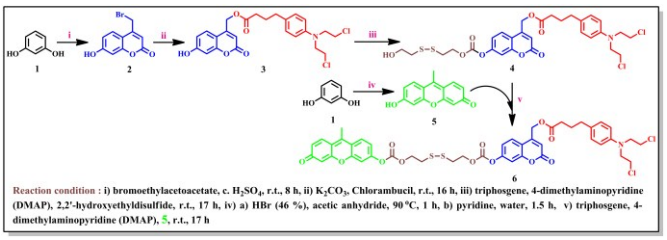
Electronic Supplementary Information (ESI) available: [^{1}H, ^{13}C, DEPT NMR, UV spectra, quantum yield, competing fluorescence spectra, pH dependence of fluorescence property of Xan-ss-Cou-Cbl are discussed in supplementary material.]. See DOI: 10.1039/x0xx00000x

Xanthene-disulphide-coumarin-chlorambucil (Xan-SS-Cou-Cbl) (**6**) was synthesized in three steps. First, coumarin-chlorambucil conjugate (Cou-Cbl) (**3**) and 6-hydroxy-9-methyl-3H-xanthen-3-one (**5**) were synthesized following previous literatures.[37,38] Thereafter, compound **4** was afforded by reacting **3** with 2,2'-hydroxyethyldisulfide in presence of triphosgene and 4-dimethylaminopyridine (DMAP) for 17 h at room temperature.[39] Finally, compound **4** was treated with DMAP and triphosgene at room temperature for 5 h, followed by addition of compound **5** and stirring for 17 h afforded desired compound **6** in 35 % yield as shown in **scheme 2**.



Scheme 2 Synthesis of Xan-SS-Cou-Cbl conjugate (**6**).

The UV-vis spectrum of compound **6** was recorded in a HEPES buffer solution containing 10 % ethanol (pH 7.2) at ambient temperature. It is evident from the UV-vis spectra (**fig. S1)** that the coumarin moiety has absorption maximum at 330 nm and emits in the range of 400-500 nm (as shown in **fig. S1b**). Interestingly, the absorption range of xanthene moiety falls in the same region of coumarin emission (**fig. S1b**). Thus, in the presence of disulfide bridge, coumarin moiety upon excitation with 330 nm light transfers its energy to xanthene moiety serving as a FRET donor. Xanthene, on the other hand, exhibited its characteristic emission maximum at 516 nm upon energy transfer from coumarin, which confirmed the FRET process occurring from coumarin to xanthene. Comparative emission properties (**fig. S2**) of compound **6**, Cou-Cbl and xanthene at excitation wavelength 350 nm were also monitored to further validate FRET process.

Redox-regulated emission was monitored by subjecting a 15 μM of compound **6** to increasing concentration of biological reducing agent glutathione (GSH). Initially, in absence of GSH, compound **6** exhibited strong green fluorescence with an emission maximum at 516 nm upon excitation at 330 nm. This is attributed to the ongoing FRET process from coumarin to xanthene moiety. Upon gradual addition of excess GSH (0-5 mM), the emission maximum at 516 nm (excitation 330 nm) decreased sharply with concomitant increase in the emission intensity at 408 nm (**fig. 1a**). Such observation is indicative of cleavage of disulfide linkage between Cou-Cbl and xanthene moiety. Thus, in presence of excess GSH the fluorophores become independent of each other showing characteristic emission maxima of **3** at 408 nm (excitation 330 nm). Remarkable change in fluorescence from green to blue of compound **6** depending on the varying concentration of GSH made them useful for real-time monitoring of drug delivery processes under biological system. The fluorescence quantum yield (Φ_f) of compound **6** in HEPES buffer containing 10 % ethanol was calculated to be 0.13 taking quinine sulfate in 0.1 M H_2SO_4 (Φ_f = 0.54) as reference.

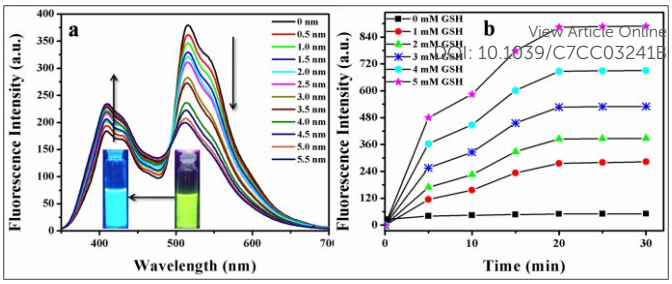


Figure 1 (a) Fluorescence changes of compound **6** (15 μM) after subjecting to increasing concentrations of GSH (0.0-5.5 mM) [excitation wavelength (λ_{ex}) = 330 nm], and **(b)** time-dependent increase of fluorescence at 408 nm upon reaction of **6** (15 μM) with GSH (0.0-5.5 mM) [λ_{ex} = 330 nm]. All data were acquired at 37 °C in HEPES buffer (pH 7.2) containing 10 % (v/v) ethanol.

Increase in fluorescence intensity at 408 nm with respect to time was monitored in presence of varying concentration of GSH. It was observed that maximum increase in fluorescence intensity was observed after 20 min of addition of 5 mM GSH, after which it reached saturation point (**fig. 1b**). As 5 mM is the average GSH concentration in cancer cells,[40] compound **6** could be used as a competent redox-responsive prodrug for target-specific cancer treatment. The change in fluorescence property of compound **6** in presence of various biologically relevant metal ions viz. Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} (**fig. S3a, S4a**) and amino acids (**fig. S3b, S4b**) was carried out to establish the selectivity of compound **6** towards biological reducing thiols. The change in fluorescence property was observed only for thiol containing biological reducing agents viz. GSH, cysteine (Cys), homo-cysteine (Hcy) and dithiothreotol (DTT). Effect of pH (**fig. S5**) on the fluorescent nature of compound **6** was also studied by gradually adding 1 M NaOH or 1 M HCl into a solution of compound **6** in ethanol:HEPES buffer (pH 7.2).

The process (**fig. S6**) of redox-triggered release of Cou-Cbl from Xan-SS-Cou-Cbl was supported by mass spectral studies and HPLC analysis. Compound **6** showed a single peak at m/z 910.1529 ($M + H^+$) and a single peak at retention time (t_R = 3.1 min) in HPLC chromatogram implying undisturbed prodrug system (**fig. S7**). However, after treatment with 5 mM GSH, two discrete peaks were seen at m/z 478.1192 ($M + H^+$) and 227.0712 ($M + H^+$) corresponding to compounds **3** and **5** respectively (**fig. 2a**). After GSH treatment, HPLC showed two peaks at t_R = 3.9 and 6.8 min (**fig. 2b**) for xanthene (**5**) and Cou-Cbl (**3**) respectively.

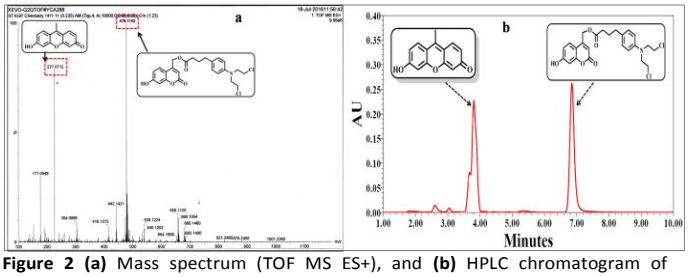


Figure 2 (a) Mass spectrum (TOF MS ES+), and **(b)** HPLC chromatogram of compound **6** after addition of GSH.

Redox-responsive singlet oxygen generation from compound **6** was ascertained by a well-known photodegradation study of 1,3-diphenylisobenzofuran (DPBF).[41] The decrease in the absorption maximum of DPBF at 425 nm after 1 h treatment of GSH on compound **6** solution with respect to time indicated

redox-activated singlet oxygen generation. Upon irradiation with ≥ 350-nm light (20 mW/cm^2) in an HEPES buffer solution containing 10 % EtOH, compound **6** showed singlet-oxygen quantum yield (Φ_Δ) ~ 0.38 in presence of GSH. This can be attributed to the "unlocking" of Cou-Cbl moiety from compound **6** after cleavage of disulfide linker by GSH. Conversely, when Cou-Cbl was attached to xanthene moiety by disulfide linker, coumarin served as the energy donor (FRET donor) to xanthene (FRET acceptor). Hence, coumarin was in its "locked" state during such FRET process and was unable to generate singlet oxygen. Without treatment with GSH, compound **6** showed comparatively low Φ_Δ ~ 0.15 (**fig. 3**) owing to weak singlet oxygen generation from 6-hydroxy-9-methyl-3H-xanthen-3-one (**5**) moiety. Comparative singlet oxygen generation abilities of Xan-SS-Cou-Cbl upon treatment with GSH, free xanthene, and free Cou-Cbl were also monitored (**fig. S8**).

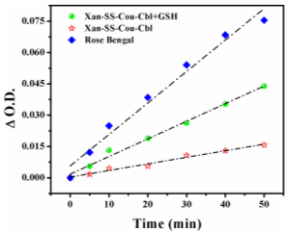


Figure 3 Photodegradation of DPBF at 425 nm by Xan-SS-Cou-Cbl before and after treatment with GSH.

Redox-activatable photoinduced chemotherapy of compound **6** was screened by photolysis study in presence and absence of GSH. Towards this end, compound **6** was treated with GSH. Afterwards, the solution was irradiated with light of ≥ 350 nm (1 M $CuSO_4$ filter) using 125 W medium pressure Hg lamp.[42] The reverse phase HPLC was carried out using 7:3 (acetonitrile:water) as the mobile phase. Before photolysis, the reverse phase chromatogram (**fig. 4**) showed two peaks at retention times (t_R) 6.8 min and 3.9 min corresponding to Cou-Cbl (D) and xanthene moiety (B) respectively, which were formed from **6** upon treatment with GSH. The peak at 6.8 min gradually decreased with increase in irradiation time attributed to the steady decomposition of Cou-Cbl (D). Concomitantly, appearance of two new peaks at t_R = 4.9 and 2.6 min were observed. The peak at retention time 2.6 min corresponded to the successive release of anticancer drug chlorambucil (A); however relatively less polar photoproduct coumarin-based alcohol (C) appeared at 4.9 min. However, the peak corresponding to xanthene moiety (t_R = 3.9 min) remained same throughout the photolysis. Such chromatogram precisely demonstrated the light-induced chemotherapeutic application. The uncaging of Cou-Cbl proceeds via singlet excited state of coumarin, wherefrom it undergoes heterolytic bond scission leading to release of clorambucil.[42]

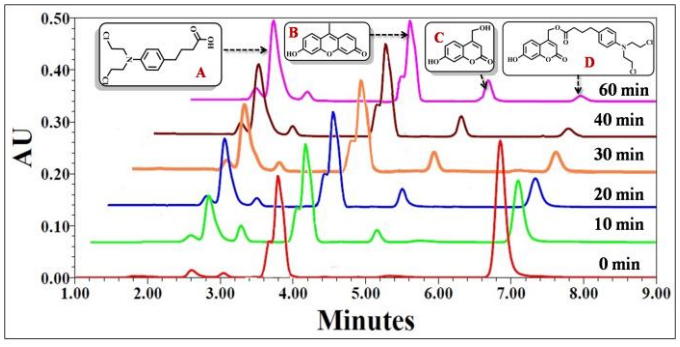


Figure 4 HPLC overlay chromatogram (Wavelength of detection = 254 nm) of the compound **6** after treatment with GSH at different time intervals of light irradiation (0–60 min). Photolysis was carried out after 1 h treatment of GSH. [A = anticancer drug chlorambucil, B = xanthene, C = photoproduct coumarin alcohol and D = Cou-Cbl].

It was observed that upon GSH treatment, compound **6** showed almost 80 % drug release after 60 min of illumination with a quantum yield of 0.039; however, in absence of GSH, drug release was negligible (**fig. S9a**). Due to FRET, Cou-Cbl remained "locked" in compound **6** in absence of GSH and gets "unlocked" only on exposure to reducing thiol. This kind of "activatable" photoinduced chemotherapeutic property of compound **6** made them an excellent choice for targeted cancer treatment. Precise control of the photolytic release of chlorambucil was demonstrated by monitoring the release of chlorambucil after periods of exposure to light and dark conditions (**fig. S9b**).

In vitro redox-responsiveness was monitored by incubating HeLa cell line with compound **6** for 4 h at 37 °C.[43] The cells showed strong blue fluorescence (**fig. 5(b1)**) under confocal laser scanning microscopy (CLSM) upon excitation at 330 nm attributed to presence of free Cou-Cbl following reaction between compound **6** with GSH that is inherently present in cancerous cells like HeLa. However, a strong green fluorescence (**fig. 5(c1)**) was observed upon excitation at 450 nm for HeLa cell line owing to the presence of free xanthene molecule. Thus, confocal images revealed successful rupture of disulfide bond between Cou-Cbl and xanthene upon interaction with biological reducing thiols showing their characteristic emission properties upon excitation at 330 (blue fluorescence) and 450 nm (green fluorescence) respectively.

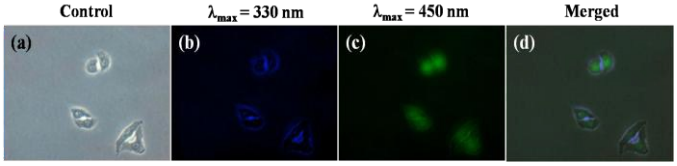


Figure 5 Confocal fluorescence images of compound **6** (10 μM) in HeLa cell line under **(a)** brightfield, **(b)** excitation at 330 nm [emission channel = 408 nm], **(c)** excitation at 450 nm [emission channel = 520 nm], and **(d)** overlay of **b** and **c**. [Scale bar = 50 μm]

A dose-dependent study was carried out by incubating both cell lines with compound **6** for 4 h. After 10 min of irradiation with UV-vis light of ≥ 350 nm wavelength using 125-W medium-pressure Hg lamp, only 12 % of the cancerous HeLa cells survived in a 10-μM solution of compound **6** (**fig. 6b**). However, under the same conditions, compound **6** showed ~ 75 % cell viability after 60 min of irradiation in the non-cancerous HEK 293 cells. Hence, it was evident from the MTT assay data that compound **6** showed extremely high cytotoxicity to cancerous HeLa cells by means of combined treatment of PDT and chemotherapeutic effect of chlorambucil; however, it remained significantly non-toxic to a non-cancerous cell line. When we performed the same MTT assay in the dark **(fig. 6a)**, compound **6** did not show any significant cytotoxicity in either HeLa or HEK 293 cells.

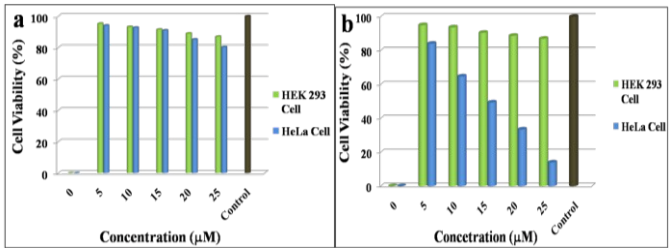


Figure 6 Comparative cell viability study of compound **6** on HeLa **(blue bar)** and HEK 293 cell lines **(green bar) (a)** before irradiation, and **(b)** after irradiating 60 min with UV-vis light (≥ 350 nm wavelength) at different concentrations. Values are presented as means ± standard deviations of three different observations

In conclusion, we have presented redox-triggered "activatable" synergic treatment of PDT and chemotherapy using FRET-based disulfide-linked xanthene-coumarin-chlorambucil conjugate (Xan-SS-Cou-Cbl) enabling real-time monitoring of drug delivery. The prodrug, Xan-SS-Cou-Cbl containing a FRET donor-acceptor pair of coumarin and xanthenes, showed a remarkable FRET-induced change in fluorescence property before and after GSH treatment. Comparative *in vitro* application in normal and cancerous cells revealed the intracellular redox-responsiveness of Xan-SS-Cou-Cbl by the prominent color change from green to blue under confocal microscope. Photocytotoxicity assay demonstrated the selective toxicity of Xan-SS-Cou-Cbl only in case of cancer cells due to presence of excess GSH; however remained non-toxic in normal cells.

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Redox-responsive Xanthene-coumarin-chlorambucil-based FRET-guided theranostics for "activatable" combination therapy with real-time monitoring

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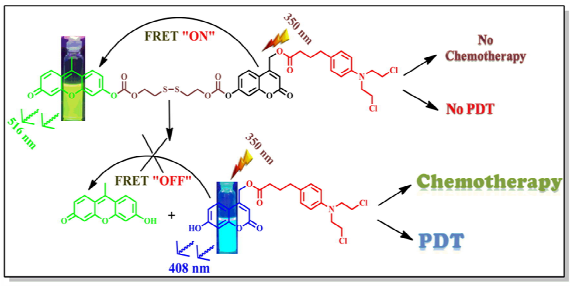

FRET-based theranostic, xanthene-coumarin-chlorambucil, exhibited redox-responsive "activatable" synergic treatment of PDT and chemotherapy with fluorescence-change from green to blue.