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A fluorescence *off–on* reporter for real time monitoring of gemcitabine delivery to the cancer cells†

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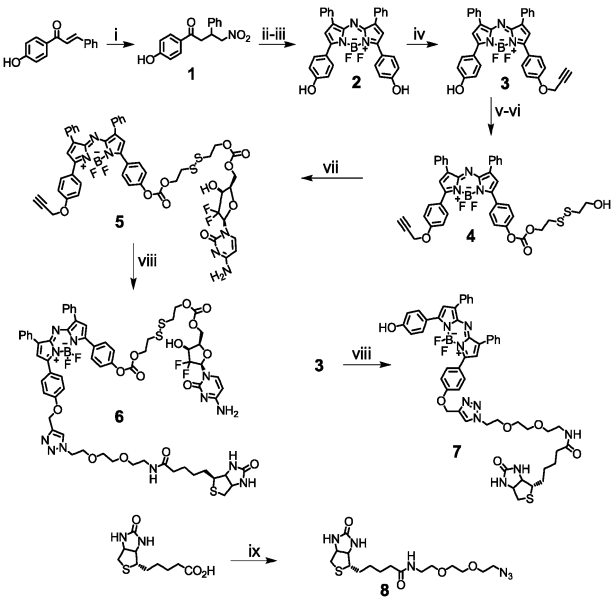
We present the design, synthesis, optical properties and *in vitro* biological assessments of the theranostic prodrug 6 in which a near IR fluorophore is conjugated with a cancer cell-directing biotin unit; further it is linked with the anti-cancer drug gemcitabine *via* a self-immolative spacer, a disulfide bond. The prodrug 6 is able to monitor drug delivery and cellular imaging.

Theranostics, a special type of drug delivery system (DDS) with the capability of real time monitoring of drug release, draws attention from medical or pharmaceutical sciences.[1,2] Its unique feature compared to other DDS systems is that it can generate notable signals upon drug release to provide information regarding the local drug dosage as well as the distribution of the drug in tissues. Moreover, an endowed theranostic DDS with a cancer targeting unit such as biotin or a RGD moiety might be an ideal candidate for the target-selective drug delivery system, which has been demonstrated recently.[3] However, some of them may not be compatible with theranostic DDS since the light for the DDS has to penetrate the tissues which may have strong absorption in the range of 450 to 530 nm, hence tissue penetration depth of light of this range is extremely limited.[4] This problem may be circumvented by use of a near infrared-based theranostic system, which has hardly been reported for theranostic DDS yet. In order to introduce fluorescing theranostic DDSs compatible with the biological tissues, we designed a drug delivery system (Scheme 1) based on a near IR fluorescing BODIPY fluorophore that is conjugated with a cancer cell-directing biotin unit and linked with the anti-cancer drug gemcitabine (GMC)[5] *via* a self-immolative spacer, a disulfide bond. Compound **3** was synthesized starting from 4′-hydroxychalcone following a previously reported protocol.[6] Themono-*O*-DMTr-2-hydroxyethyl disulfide was formed as an activated intermediate by reacting with phosgene which was subsequently reacted with **3** to give a stable compound; isolation and deprotection of the *O*-DMTr group by acetic acid in dichloromethane gave **4**. Compound **4** was further reacted with 4-nitrophenyl chloroformate to give a reactive intermediate; the intermediate was then reacted with gemcitabine to give **5** in moderate yield. Compound **5** was conjugated with biotin derivative **8** to obtain targeted theranostic prodrug **6** followed by the well known "click" reaction.[7]



Scheme 1 Synthesis of theranostic prodrug **6**. Reaction conditions: (i) Et_2NH, $MeNO_2$, EtOH, reflux, 16 h; (ii) NH_4OAc reflux, 24 h; (iii) BF_3, DIPEA, DCM, 12 h; (iv) propargyl bromide, K_2CO_3, acetone, reflux, 12 h; (v) mono-*O*-DMTr (4,4′-dimethoxytrityl)-2-hydroxyethyl disulfide, $COCl_2$, DIPEA, DCM, 0 °C–rt, 12 h; (vi) AcOH, DCM, rt, 24 h; (vii) 4-nitrophenyl chloroformate, DIPEA, DCM, 0 °C–rt, 3 h then gemcitabine, TEA, DMF, rt, 12 h; (viii) **8**, Na-ascorbate, $CuSO_4$, MeOH–H_2O, rt, 4 h; (ix) 2-(2-(2-azidoethoxy)ethoxy)ethanamine, EDCI, DMAP, DMF, rt, 6 h.

To justify the presumed concomitant release of the fluorophore and GMC, we have carried out UV-Vis and fluorescence study in the presence of thiols such as DTT. From Fig. 1, it is observed that as the UV absorption at 700 nm gradually increases upon addition of DTT (Fig. 1a), the fluorescence intensity increases at 720 nm

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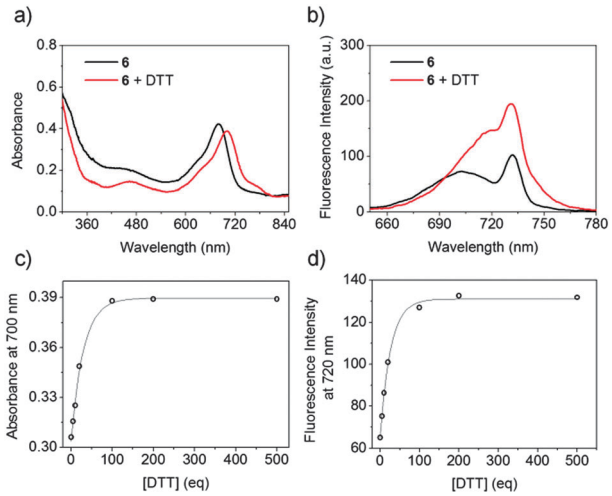


Fig. 1 (a) UV-Vis absorption and (b) fluorescence spectra of **6** (10.0 μM) in the absence and presence of DTT (5.0 mM). Changes in absorbance at 700 nm and fluorescence intensity at 720 nm as a function of concentration of DTT (0–500 eq.; 0–5.0 mM). All the spectra were acquired 30 min after addition of DTT at 37 °C in PBS buffer (pH 7.4) containing 4% (v/v) of DMSO and 0.4% (v/v) of Cremophor EL with excitation λ_{max} = 700 nm.

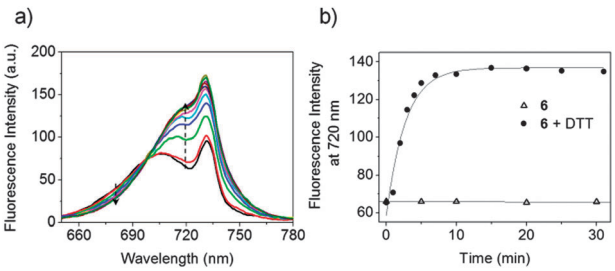
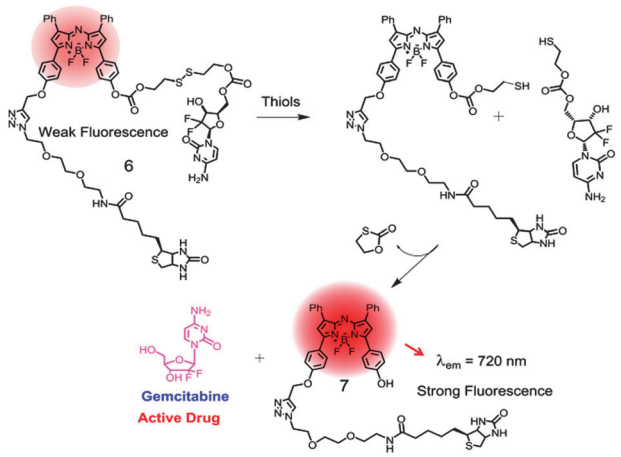


Fig. 2 (a) Time-dependent fluorescence spectral changes observed when **6** (10.0 μM) was treated with DTT (5.0 mM). (b) Changes in fluorescence intensity at 720 nm recorded as a function of time with (●) and without (△) DTT. All the spectra were acquired 30 min after addition of DTT at 37 °C in PBS buffer (pH 7.4) containing 4.0% (v/v) of DMSO and 0.4% (v/v) of Cremophor EL with excitation λ_{max} = 700 nm.

and reaches the saturation point in the presence of 100 equiv. of DTT (Fig. 1c and d). Furthermore, we have evaluated the theranostic efficacy of **6** at a fixed concentration of DTT (5.0 mmol) with respect to time.

From Fig. 2a and b, it is observed that the absorbance and the fluorescence of probe **6** are fully consumed and reach the saturation point within 10 min. This observed result implies that by the action of DTT, the vulnerable S–S bond is cleaved and subsequently active GMC and the fluorophore (**7**) (Scheme 2) are generated as observed previously by us in our another study.[8] Compound **6** was treated with 5.0 mmol GSH at 37 °C for 2 h and then the aliquot was subjected to MS analysis; we obtained mass for gemcitabine (M_w = 262) and **7** (M_w = 966.3) as seen in Fig. S17 (ESI†). These data strongly support the drug release upon GSH treatment.

To evaluate the possibility of interferences from biologically relevant analytes, the reactions of **6** with various thiols, nonthiol amino acids, and metal ions were investigated under



Scheme 2 Proposed mechanism for drug release.

the similar conditions. Upon the addition of cysteine (Cys) or homocysteine (Hcy) to **6** changes in the absorption and emission features similar to those in the case of DTT are seen (Fig. S16, ESI†).

On the other hand, no appreciable spectroscopic changes were seen upon exposure to non-thiol amino acids or biologically relevant metal ions. Additionally, we have carried out time-dependent drug release in the presence of biologically available thiols such as Cys, Hcy, GSH, Trx as shown in Fig. S18 (ESI†). We observed that the reactivity order was Cys > Hcy > GSH > Trx. To confirm the drug release and fluorescence change at the cellular level, we have carried out *in vitro* cellular imaging in A549 cells with treatment of **6**. Fig. 3b displays a strong fluorescence response when excited at 740 nm using a two photon laser. In contrast to that observed in Fig. 3c little fluorescence intensity was observed as the cells were pretreated with NEM, a strong modifier of the reactive biological thiols.[8] This experimental result implied that only biologically available thiols can trigger the drug release as suggested by *in vitro* solution tests.

In order to demonstrate the preference of the prodrug **6** to the cancer cells, we investigated the fluorescence enhancement of **6** with A549 cells[9] and WI38 cells,[10] the biotin receptor positive and negative cell lines, respectively. As shown in Fig. 4a, the fluorescence enhancement was dramatically affected in the A549 cells and a strong fluorescence signal concomitantly appeared. In contrast, the negative cells, WI38 cells (Fig. 4b), remain dark even under similar conditions. The overexpression of the biotin receptors is strongly anticipated for many cancer cells, which

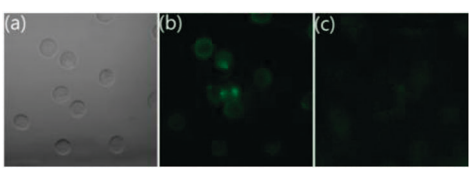


Fig. 3 (a) Phase contrast and (b) confocal microscopy images of A549 cells treated with 5.0 μM of **6** in PBS buffer, total incubation period of 30 min; (c) cells were pretreated with NEM (1.0 mM) in PBS buffer for 30 min, then treated with 5.0 μM of **6** in PBS buffer, incubation period of 25 min. Cell images were obtained using two photon excitation at 740 nm and a long-path (650–720 nm) emission filter.

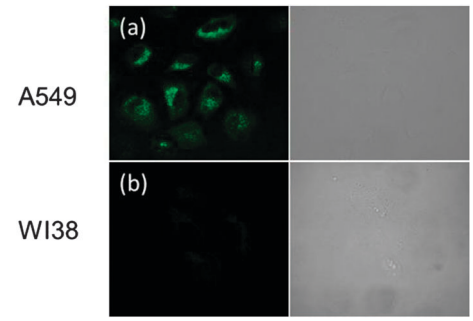


Fig. 4 (a) A549 cells were treated with 5.0 μM of **6** in PBS buffer, total incubation period of 25 min; (b) WI38 cells were treated with 5.0 μM of **6** in PBS buffer, incubation period of 25 min. Cell images were obtained using two photon excitation at 740 nm and a long-path (650–720 nm) emission filter. The left side panels show the confocal microscopy images of A549 and WI38 cells; the right side panels show nonconfocal phase contrast images.

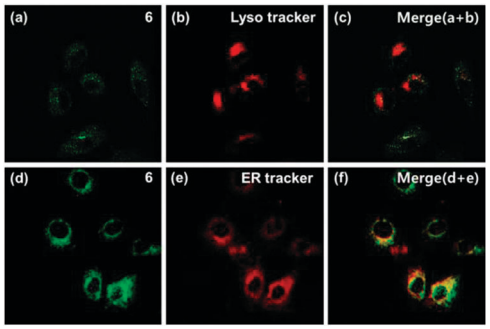


Fig. 5 Confocal microscopic images of colocalized experiments in A549 cells. (a) and (d) Fluorescence images of A549 cells incubated with **6** (10.0 μM) for 25 min. (b) Fluorescence image of A549 cells incubated with **6** (10.0 μmol) as well as Lyso Tracker Blue DND-167 (0.05 μM) for 25 min. (c) Overlay of the merged images of (a) and (b). (e) Fluorescence image of A549 cells incubated with **6** (10.0 μM) and ER Tracker Red (1.0 μM) for 25 min. (f) Overlay of the merged images of (d) and (e). Images of the cells were obtained using excitation wavelengths at 740 nm, and a band path emission filter (370–450 nm, Lyso Tracker), (530–570 nm, ER Tracker), and (650–720 nm), respectively.

inferred that the theranostic prodrug **6** would be desirable for drug delivery systems toward anti-cancer therapy.

To identify the intracellular location of GMC release from **6**, co-localization experiments were performed using fluorescent endoplasmic reticulum (ER)- and lysosome-selective markers. As seen in Fig. 5e, the fluorescence ascribable to **6** colocalizes apparently with the endoplasmic reticulum (ER). However, it failed to show its coexistence with the lyso-tracker as shown in Fig. 5c. Therefore, it is noteworthy that thiol-induced disulfide cleavage of **6** occurs in the endoplasmic reticulum (ER), serving to release the GMC molecule, which presumably diffuses into the cell nucleus, the final location of the GMC acting as a pro-apoptotic agent.[11,12]

Additionally, the cytotoxicity of **6** was evaluated by determining the viability of cells after incubation with **6** using MTT assay as shown in Fig. S19 and S20 (ESI†). The cell viability was decreased in a dose-dependent manner. This observation implied that prodrug **6** is able to release active drugs and it is applicable, as a useful, activatable theranostic prodrug, in non-invasive imaging and delivery of a potent drug.

In conclusion, we have reported an activatable theranostic agent, **6** and its synthesis, characterization, spectroscopic properties, anti-cancer effects, as well as biological applications. The disulfide bond cleavage by intracellular thiol species leads to release of the active GMC and fluorescence enhancement. The co-localization experiments using a commercially available lysosome-selective dye as well as an endoplasmic reticulum-selective dye demonstrated that compound **6** localized to the endoplasmic reticulum presumably through the receptor mediated endocytosis. According to confocal microscopic experimental studies, our final prodrug only goes to biotin receptor-positive A549 tumor cells, compared to biotin receptor-negative WI38 cells. Therefore, our drug delivery system (DDS) could provide a powerful new strategy for the specific tumor targeting drug delivery and non-invasive cellular imaging.

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