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Targeted, Image Guided & Dually Locked Photoresponsive Drug Delivery System

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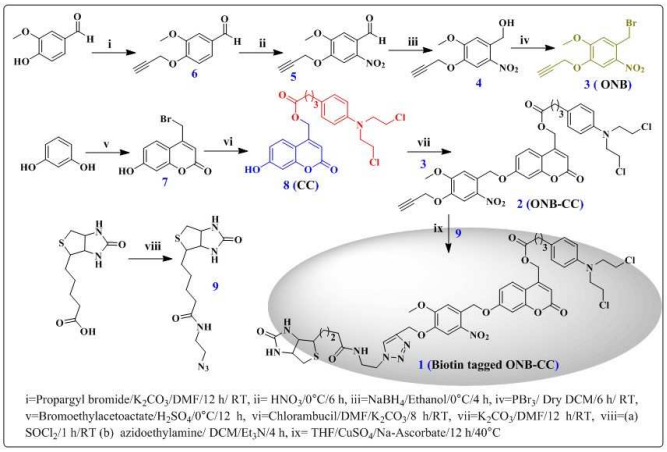
We developed a new targeted image guided photoresponsive DDS based on "dually locked" strategy. On excitation of DDS by light resulted in first unlocking leading to fluorescent turn on in living cells which was employed for real time monitoring of prodrug, the extended irradiation resulted in second unlocking leading to drug release inside the cancer cells.

Cancer targeted theranostic drug delivery systems (DDSs) captured enormous interest since it combines two important features into one delivery system: imaging and delivering anticancer drugs specifically to the cancerous cells.[1] Hence, several targeted theranostic drug delivery systems have been developed to actively target the cancerous cells either by embryonic with folic acid or biotin to achieve efficient receptor mediated endocytosis.[2] In recent years photoresponsive theranostic drug delivery systems have become more interesting because they allow precise control over the drug release including location, timing and dosage through their active ingredient "phototrigger".[3-6] Hence, several photoresponsive DDSs have been developed based on phototriggers *o*-nitrobenzyl and coumarinyl derivatives for efficient drug and gene delivery. Though, Photoresponsive DDSs are extensively utilised it faces a major drawback, generally phototriggers are activated and armed with drugs. Since phototrigger are activated undesirable firing can occur when exposed to ambient light and thus results in premature release of the drug before they reach their target site.[7] To overcome the premature release of drug by photoresponsive DDS it requires an additional step of locking the phototrigger. Recently double prodrug also called as "pro-prodrug" or "cascade-latentiated prodrug" has become a promising technique since it prevents premature drug release by prodrug in-vitro condition by providing additional lock to the prodrug. In case of double prodrug technique the prodrug is locked in a fashion such that only conversion to prodrug is possible first before the prodrug can cleave to release the active drug. In most of the double prodrug forms, the first step usually being an enzymatic cleavage and the second a non-enzymatic hydrolysis.[8] However other type of chemical reaction combination were also explored such as combination of an oxidation and hydrolysis, and both enzymatically mediated cleavage. Recently, Zhu *et.al.,* developed a target activated photoresponsive DDS in which coumarin phototrigger is locked by nitroimidazole. On specific activation by hypoxia the coumarin phototrigger gets unlocked and subsequently releases the drug. [9] Further, You *et.al.,* also developed a double activatable prodrug system containing a photoactivatable aminoacrylate linker and a deactivated photosensitiser. In this work the activation of the photosensitiser was carried out first by cellular esterase and then drug release via photo unclick chemistry. [10] Herein, we report a new photoresponsive DDS in which the phototrigger-I (phototrigger attached directly to the drug) is locked by another phototrigger- II, which will have a greater release ability compared to phototrigger-I. Thus on excitation by light, we can expect first unlocking of phototrigger I to occur, which then subsequently releases the drug. For this strategy, we first caged the anticancer drug chlorambucil using 7-hydroxycoumarin (phototrigger-I) and then the phototrigger-I was locked using o-nitrobenzyl (phototrigger-II). We locked phototrigger 7-hydroxycoumarin by o-nitrobenzyl, since o-nitrobenzyl has rapid release ability in comparison to 7-hydroxy coumarin. Further locking 7-hydroxy

coumarin by o-nitrobenzyl will switch off the fluorescence of 7-hydroxy coumarin and then on brief light illumination the florescence can be reverted back, by this approach we can monitor in real time the unlocking of phototrigger-1. Once the phototrigger is unlocked, and then on extended irradiation will help us to achieve our drug release. Finally, to provide cancer target ability to our DDS, biotin was used as the cancer targeting ligand.



Scheme 1.Synthesis of targeted dually locked photoresponsive DDS.

The targeted dually locked photoresponsive DDS has been synthesized following a sequence of chemical reactions as depicted in the Scheme 1. First, the o-nitrobenzylbromide derivative (3) was synthesized starting from vanillin in four steps. On the other hand photocaged coumarin chlorambucil (8) was synthesized starting from resorcinol in two steps. Then compounds 3 and 8 were coupled in presence of K2CO3 in dry DMF to yield locked phototrigger, "O-nitrobenzyl caged coumarin-chlorambucil" (ONB-CC) 2. Finally, the biotin tagged ONB-CC (1) was synthesized by carrying out click reaction between prodrug (2) and azido-biotin (synthesized separately) in the presence of CuSO4/Na ascorbate in dry THF at 40°C. All the synthesized compounds were characterized by 1H &13C NMR (Supporting Information, SI).

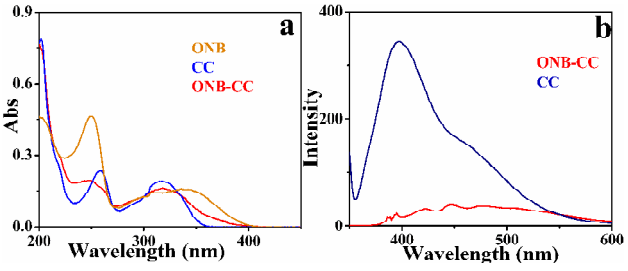


Figure 1:(a)Absorption spectra of O-nitrobenzyl (ONB), Coumarin chlorambucil (CC), and O-nitrobenzyl caged Coumarin Chlorambucil (ONB-CC). (b) Emission spectra of Coumarin chlorambucil (CC) and O-nitrobenzyl caged Coumarin Chlorambucil (ONB-CC).

Photochemical and Fluorescent Properties of locked photocaged ONB-CC: O-nitrobenzyl (ONB), coumarin chlorambucil (CC) and o-nitrobenzyl caged coumarin chlorambucil (ONB-CC) exhibited similar absorption maxima at around 360 nm (Figure 1a). As anticipated the prodrug coumarin chlorambucil (CC) exhibited strong emission peak at 420-500 nm. But locked prodrug (ONB-CC) does not show any emission. This can be explained due to the presence of strong electron withdrawing *o*-nitrobenzyl substituent at 7- position of coumarin (Figure 1b). Further, the fluorescent quantum yields of CC and ONB-CC were found to be Φf = 0.4856 and 0.0096, respectively (quinine sulphate quantum yield: 0.54 as a standard). Hence, the above spectral studies indicate that our ONB-CC can be exploited for real time monitoring using their fluorescent "turn on" ability and drug release using one photon excitation (1PE) and 2PE.

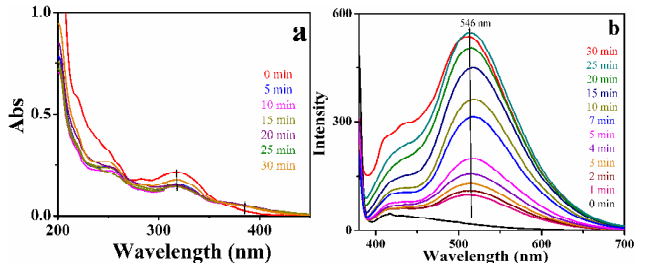
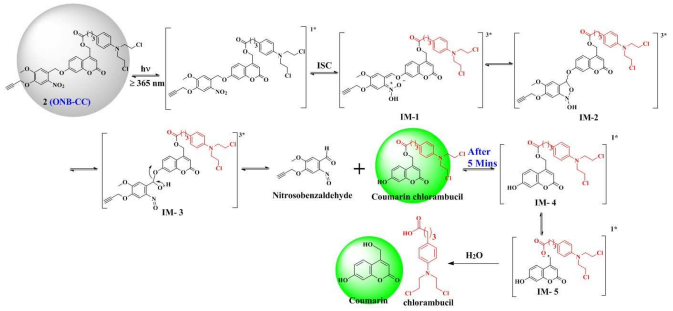


Figure 2: Course of photolysis of ONB-CC in acetonitrile /water (80/20) (a) absorption spectra (b) emission spectra.

To demonstrate the fluorescence "turn on" ability of locked photocaged ONB-CC, 5 mL of 1.0×10–5 M aqueous acetonitrile solution (80/20 v/v) of ONB-CC was irradiated using 125W medium pressure Hg lamp ≥ 365 nm using 1 M $CuSO_4$ solution as UV cut-off filter. Upon irradiation, the non-fluorescent ONB-CC gets excited to its singlet state, which then undergoes rapid singlet-triplet intersystem crossing. Subsequently from the triplet exited state it unlock to release fluorescent prodrug coumarin chlorambucil (CC) in 5 min via aci-nitro intermediate as depicted in the scheme 2 (the photorelease was confirmed by 1H NMR spectroscopy Figure 4a).



Scheme 2: Proposed mechanism for fluorescence "turn on" ability of double photocaged ONB-CC.

The exemplary change of absorption and emission spectra of ONB-CC at regular intervals of irradiation were shown in Figure 2a-b. The initial fluorescence intensity of ONB-CC at 546 nm was extremely week or negligible in acetonitrile/water solution, upon irradiation the fluorescence intensity drastically increased in 5 min and reached the maximum in 30 min (Figure 2b). This robust fluorescence "turn on" ability of ONB-CC will be important for generating the information of prodrug *in-vitro* studies.

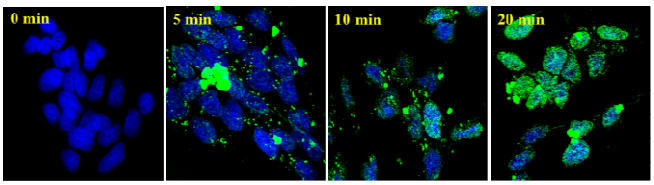


Figure 3: Cellular uptake images of MDA-MB-231 cells treated with DAPI to stain the nucleus, Biotin tagged ONB-CC (1) after irradiation ≥ 365 nm at varying time periods (5, 10 and 20 min).

To confirm the cellular uptake efficiency and fluorescence "turn on" ability of ONB-CC in vitro, we used breast cancer cells (MDA-MB-231). Cells were incubated with DAPI and 50μM biotin tagged ONB-CC (1) for 4h. At 0 min cells showed strong blue fluorescence, due to DAPI stain, then the cells were exposed to UV light (≥ 365 nm) at regular intervals and the fluorescent intensity were monitored by fluorescence microscopy. After 5min of irradiation, we noticed cancer cells glows green fluorescent which indicated the unlocking of non-fluorescent ONB-CC to release fluorescent prodrug coumarin chlorambucil. Further, fluorescence intensity was also found to be increased with respect to irradiation time (0, 5, 10 and 20 min) indicating larger amount of release of prodrug by ONB-CC. Furthermore, after 20 min of irradiation fluorescence intensity was found to be greater in the cytoplasm and nuclear periphery of cancer cells revealing that the prodrug coumarin chlorambucil is accumulated mainly in these regions. Thus the robust change in fluorescent intensity of ONB-CC will guide us to image the prodrug more preciously in the targeted site.

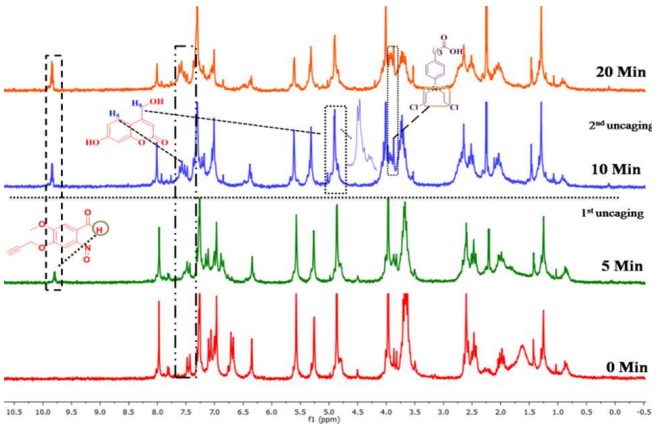


Figure 4: photolysis of ONB-CC was monitored by 1HNMR.

In order to demonstrate the photoinduced anticancer drug release behavior of DDS, ONB-CC was excited using one-photon and two–photon irradiation. The course of photocleavage was monitored by 1H NMR and reverse phase HPLC. Based on 1H NMR spectroscopy, it is clearly observed that first unlocking of ONB-CC produced nitrosobenzaldehyde and prodrug (CC) on irradiation for 5 min. The benzylic proton intensity of ONB-CC at 5.67 ppm decreased and a new peak appeared at 9.67 ppm for nitrosobenzaldehyde (Figure: 4a). The HPLC profile also supported the initial uncaging of ONB-CC. At 5 min of irradiation time, the peak at 7.36 Rt corresponding to ONB-CC decreased, while two new peaks appeared at 4.23 and 4.77 Rt, assigned to *o*-nitrosobenzaldehyde and coumarin chlorambucil, respectively. The second uncaging of prodrug (CC) was observed on extended irradiation (20 min) by both HPLC and NMR spectroscopy. 1H NMR revealed that benzylic proton at 4.85 ppm of prodrug (CC) clearly decreased in intensity and a new proton signals corresponding to the photoproduct (coumarin-4-yl-methanol) emerged at 4.90 (H9) and 7.49-7.76 ppm (doublet, aromatic proton H5). Further, we also noted appearance of new multiplet at 3.75 ppm corresponding to the methylene protons (N-CH_2) of released chlorambucil. Further, we noticed by HPLC 90 % of first unlocking and 70% of second unlocking was achieved by our DDS after 5 and 20 min of irradiation by soft UV light (≥365 nm by 125 W medium pressure Hg vapor lamp). However less than 7% of the drug release was noted using diode laser (730 nm, 30mW/cm2), suggesting that external light dose could regulate the drug release.

We evaluated the cytotoxicity of biotin tagged ONB-CC through MTT assay on MDA-MB-231 cell line. The cells incubated with biotin tagged ONB-CC with different concentration (0-100 μM) was irradiated for 30 min under UV light (≥ 365 nm). Cell viability of 40% was observed at the concentration of 60μM upon 48 h treatment whereas the viability was shifted to 50% at 50μM concentrations (Figure S3). There was a significant reduction in the drug concentration upon carrier mediated delivery when compared to native chlorambucil (the IC50 of native chlorambucil was 67.38 μM as reported previously).[11] On the other hand, cell viability was found to be largely unaffected by biotin tagged ONB-CC which was unexposed to light, indicating the cytotoxicity was likely caused by the released drug chlorambucil upon light irradiation. Further, the cell cycle analysis showed higher apoptotic activity for biotin tagged ONB-CC compared to free chlorambucil and ONB-CC (Figure S4) suggesting that biotin mediated accumulation can significantly enhance the efficiency of the intracellular drug delivery. This explains the possible reason for high efficiency of our DDS against breast cancer than native chlorambucil.

In conclusion, we have developed a double locked phototriggered for targeted image guided DDS. One- and two- photon excitation (1PE and 2PE) of DDS rapidly activated fluorescence enhancement in living cells which was employed for the real time monitoring of the prodrug and *in-vitro* cellular imaging application. Further, extended irradiation of DDS resulted in release of anticancer drug chlorambucil which enhanced cytotoxicity compared to free drug towards MDA-MB-231 line.

Notes and references

[a]Department of Chemistry, [b]School of Medical Science & Technology. Indian Institute of Technology Kharagpur, Kharagpur, India. DST-SERB for financial support. DST-FIST for 200 MHz NMR. S. K and M. G is thankful to IIT KGP for their fellowship.†Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

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