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Three Arm, Biotin-tagged Carbazole-Dicyanovinyl-Chlorambucil Conjugate: Simultaneous Tumor Targetting, Sensing and Photoresponsive Anticancer Drug Delivery

Yarra Venkatesh,[a] S. Karthik,[a] Y. Rajesh,[b] Mahitosh Mandal,[b] Avijit Jana*[c, d] and N. D. Pradeep Singh*[a]

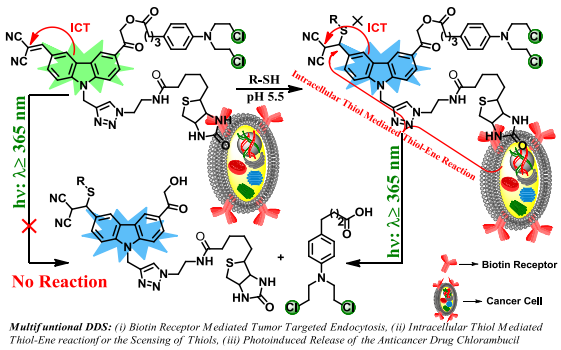
Abstract: Here we report the design, synthesis and in vitro biological studies of a Biotin-Carbazole-Dicyanovinyl-Chlorambucil conjugate (Bio-CBZ-DCV-CBL). This conjugate (6) is a multifunctional single molecule appliance composed of thiol-sensor Dicyanovinyl functionality, a Carbazole derived phototrigger and the fluorescent reporter, chlorambucil (CBL) as the anticancer drug, and biotin as the cancer-targeting ligand. In conjugate **6**, the dicyanovinyl bond undergoes thiol-ene click reaction at pH< 7 with intracellular thiols thereby shuttingdown the internal charge transfer between donor carbazole and acceptor dicyanovinyl unit results in change of fluorescence color from green to blue and thereby sense the tumor microenvironment, followed by photoirradiation results in release of the anticancer drug CBL in controlled manner.

Tumor-targetability coupled with stimuli responsiveness of a theranostic drug delivery systems (DDSs) have gained tremendous attention in recent years.[1,2] It offers early detection and diagnosis of cancers along with improved therapeutic efficacy by the means of enhanced spatiotemporal accumulation of the therapeutic payload.[3] Which further lowers the cytotoxic effect of the therapeutic drug other than the tumor sites and also favors overcoming the drug resistance.[4, 5] These class of DDSs are mostly composed of three ingredients (i) a stimuli responsive prodrug (mostly sensitive to either light irradiation,[6] intracellular thiols,[7-9] enzymes,[10,11] or intracellular pH[12,13] etc.) (ii) a tumor targeting ligand and (iii) a monitoring tag which could allow us to visualize, quantify and also track the spatial distribution, localization, and depletion of both the DDS and the therapeutic drug.

Biological thiols, including glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) plays crucial roles in maintaining the appropriate redox status of biological systems. It has already been established that an abnormal levels of cellular thiols are associated with many diseases[14] GSH is the most abundant cellular thiol, which is important for maintenance of cellular defense against free radicals and reactive oxygen species (ROS) and it also maintains exogenous antioxidants in their reduced forms.[15] Further, it is also reported that high level of biological thiols (common in tumor tissues) offers resistance to chemotherapy drugs.[16] Therefore, selective detection and quantification of GSH under physiological conditions are of significant interest in clinical medicine.

In this circumstances recently, several multifunctional disulfide-linkage based bio-conjugates have been investigated either to sense and quantify the intracellular thiols level or to exploit the higher level of thiols in tumor site to trigger the release of payloads from a redox responsive DDS.[17]

Recently, photoresponsive theranostic DDSs have achieved enormous interest since they allow precise control over the drug release, including location, timing and dosage through an active ingredient “phototrigger”. A number of photoresponsive DDSs have been developed, employing *o*-nitrobenzyl,[18] coumarinyl[19] and perylene[20,21] derivatives as phototriggers for efficient drug or gene delivery.



Scheme 1. Schematic Representation of Multifunctional DDS: Tumor Targetability by Biotin Functionalization and Intracellular GSH Sensing by the means of Thiol-Ene Reaction with Dicyanovinyl Group Followed by Photoresponsive Anticancer Drug Release from the Carbazole FPRPG.

Here, in this report, we developed an efficient new theranostic DDS based on carbazole fluorescent Photoremovable protecting group (FPRPG), which consecutively performs three different roles. (i) Firstly it actively targets the cancerous cells, thereby facilitate tumor targeted transportation of the designed DDS. (ii) Secondly it can sense the intracellular thiols level of cancer cells by the means of change in fluorescence color. And (iii) finally, it can release the anticancer drug chlorambucil (CBL) on exposure to UV light. The Bio-CBZ-DCV-CBL conjugate described here composed of four components: (i) a biotin (Bio) unit as the tumor-targeting ligand (ii) a carbazole (CBZ) moiety as the phototrigger and fluorescent imaging agent (iii) a dicyanovinyl (DCV) moiety to sense the intracellular biological thiols at pH< 7, and (iv) light-triggered releasable anticancer drug chlorambucil (CBL).

The design of the present theranostic DDS (Bio-CBZ-DCV-CBL) is rationalize in such a way that initially it exhibits green emission because of the efficient internal charge transfer (ICT) transition from the carbazole donor unit to the dicyanovinyl acceptor unit

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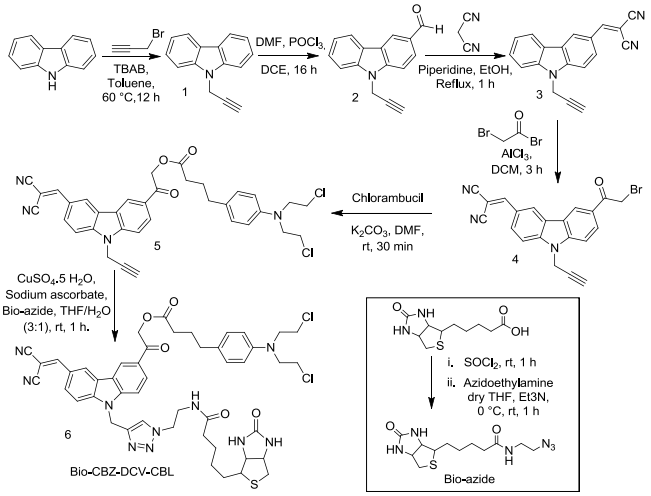
which again restrict the photoinduced drug release process. After internalization of the Bio-CBZ-DCV-CBL by the cancer cells the dicyanovinyl bond will undergo thiol-ene click reaction with intracellular thiols already abundant in higher concentration. Therefore, the extended conjugation and the ICT transition between the acceptor and the donor will be suppressed totally. Which further leads to the radical change in the emission from green to blue. Finally, photoirradiation of CBZ-DCV-CBL-ME conjugate will release the anticancer drug chlorambucil in a control manner as shown in **Scheme 1**.

The Bio-CBZ-DCV-CBL conjugate was synthesized as shown in the **Scheme 2**. First, 9-propargyl carbazole (**1**) was synthesized from 9*H*-carbazole by reacting it with propargyl bromide. Then **2** was obtained by Vilsmeier–Haack reaction of **1** using dry dimethylformamide (DMF) and phosphorus oxychloride ($POCl_3$). Later on, compound **3** was synthesized by the Knoevenagel condensation of **2** with malononitrile in presence of catalytic amount of piperidine and followed by Friedel-Crafts acylation of compound **3** with bromoacetyl bromide in presence of catalytic amount of $AlCl_3$ afforded compound **4**. Then CBZ–DCV-CBL conjugate (**5**) was obtained by reacting compound **4** with chlorambucil. Finally, Biotin-tagged CBZ–DCV-CBL conjugate (**6**) was synthesized by click reaction between **5** and azido-biotin (synthesized separately inset of **Scheme 2**). All the synthesized compounds were characterized by ^{1}H, ^{13}C NMR, IR and mass spectral analysis (**see Figure S1-9 in SI**).



Scheme 2. Synthesis of Multifunctional DDS Bio-CBZ-DCV-CBL conjugate.

After successful synthesis of the Bio-CBZ-DCV-CBL conjugate (**6**) its photophysical properties were investigated in details. UV/Vis absorption and emission spectra of the conjugate **5** were recorded in DMSO/PBS buffer (1:10 v/v) at pH 5.5 in 37 °C. In the UV/vis spectrum, as seen in **Figure 1a**, the conjugate **5** (10 μM) shows two distinct absorption bands at 316 nm and 385 nm respectively. The absorption band at 316 nm corresponded to a localized n–π* transition of acetyl carbazole, whereas the band at longer wavelength of 385 was attributed to intramolecular charge transfer (ICT) transition from the carbazole donor moiety to the dicyanovinyl acceptor. Now, to understand the role of thiols on the absorption and emission profile of CBZ-DCV-CBL conjugate it was titrated against different concentration of GSH. In case of absorption spectra, upon gradual addition of GSH (5.0 mM) to the solution of **5**, the peak at 385 nm corresponds to ICT transition gradually decreases whereas the peak at 316 nm corresponds to the localized n–π* transition of the acetyl carbazole remains almost same (**Figure 1a)**. Similar spectroscopic changes were also observed upon the addition Cys and Hcy **(see Figure S10 in SI)**. Which clearly indicates that a chemical reaction has taken place between **5** and GSH which restricted the extended conjugation between the carbazole donor and the dicyanovinyl acceptor.

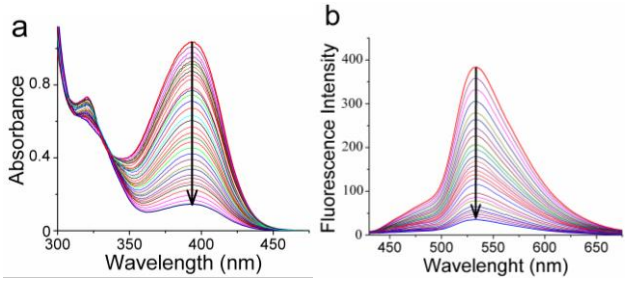


Figure 1. UV-Vis absorption spectra (a) and emission spectra (b) of **5** (10 μM) in DMSO/PBS buffer (1:10) during the course of titration with GSH (5.0 mM at pH 5.5 (Excitation wavelength: 410 nm).

The excitation of the CBZ-DCV-CBL (**5**) at 410 nm produced a dual emission with peaks at 475 and 533 nm respectively. We presume that the appearance of dual emission attributable to the localized π–π* (475 nm)) and ICT (533 nm) excited states originates from the tilted molecular arrangement. Gradual addition of GSH to **5** decreased the intensity of the peak at 533 nm. After the addition of 1.5 equivalent of GSH, the peak at 533 nm was completely quenched as shown in **Figure 1b.** Similar spectroscopic changes were also observed upon the addition Cys and Hcy which further supports the postulation of addition reaction between GSH and **5** and produces an appreciable visual color change from green to blue. However, such spectral changes were not observed upon addition of nonthiol amino acids **see Figure 2** and **Figure S11 in SI**.

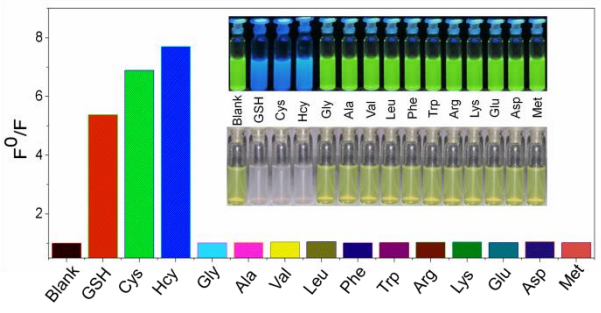
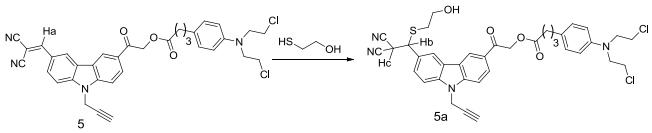


Figure 2. Relative fluorescence color changes of **5** (10 μM) upon addition of 1.5 equiv of GSH, Cys, Hcy and various amino acids at pH 5.5. Insets show the photographs of respective solutions under fluorescent lamp (top) and ambient light (bottom).

Time-dependent fluorescent spectra of CBZ-DCV-CBL (10 μM) were monitored in the presence of GSH (5 mM) in DMSO/PBS buffer (1:10 v/v, pH 5.5). The formation of CBZ-DCV-CBL+GSH was complete within 80 min with a rate constant of $k_{obs} = 4.5 \times 10^{-4}$ s^{-1} under the pseudo first-order reaction conditions (**Figure S12 in SI**).

In order to see color change from green to blue the excitation of the CBZ-DCV-CBL (**5**) at 380 nm produced a dual emission peaks at 440 and 533 nm (CT emission) respectively. Gradual addition of GSH to the solution of **5** results in gradual decrease in the emission intensity at 533 nm with the concomitant increase in the emission peak at 440 nm **(Figure S13 in SI).** It was noted that the addition of 1.5 equivalent of GSH end up with the complete quenching of the peak at 533 nm whereas the maximum intensity at 440 nm was noted. Therefore the addition of GSH to the solution of CBZ-DCV-CBL resulted complete disappearance of the CT green emission and therefore the localized π–π* transition with strong blue emission becomes the only emissive band which could be clearly visible in naked eye under the irradiation of a hand held UV-lamp at 365 nm.

Further, to validate the above hypothesis, a model reaction between CBZ-DCV-CBL and a thiol, 2-mercaptoethanol (ME) was performed (**Scheme 3)** and the course of the reaction was monitored by ^{1}H NMR spectroscopy (**Figure S14 in SI**). Upon addition of ME, the olefinic proton (H_a) of CBZ-DCV-CBL with chemical shift of ~ 8.60 ppm vanished with the appearance of two new peaks H_b at 5.08 ppm and H_c at 5.68 ppm which confirms the thiol-ene addition reaction between the thiol group and the dicyanovinyl group. Again, the mass spectral analysis also shows the molecular ion peak of the addition product of CBZ-DCV-CBL and ME (**Figure S15 in SI**) further support the formation of addition product. Then we separated CBZ-DCV-CBL-ME by reverse phase HPLC using acetonitrile as a mobile phase. The fluorescence quantum yield (Φ_f) of CBZ-DCV-CBL-ME in absolute ethanol was calculated using 9, 10-Diphenylanthracene as the standard (Φ_f = 0.95 in ethanol) and found to be Φ_f = 0.12.



Scheme 3. Model reaction between CBZ-DCV-CBL and 2-mercaptoethanol (ME) in DMSO-d_6.

To investigate the drug release ability from conjugate **5a**, a solution of CBZ-DCV-CBL-ME conjugate (1×10^{-4} M, 5 ml) in ACN/PBS buffer (3:7 v/v) was irradiated with the 125 W medium pressure Hg lamp as the source of UV light (λ ≥ 365 nm) using a suitable UV cut-off filter (0.1 M $CuSO_4$ solution) with continuous stirring for 60 min.The release of the anticancer drug, chlorambucil was monitored by RP-HPLC. The HPLC data analysis demonstrates that, with increasing irradiation time, there is a gradual decrease of the peak at t_R 4.25 min, indicating the photocleavage of CBZ-DCV-CBL-ME conjugate. Meanwhile, we also observed a gradual increment of two additional new peaks at t_R 2.35 min and t_R 5.72 min, corresponding to the photoproduct (PP1) and chlorambucil (PP2) respectively (**Figure 3**).

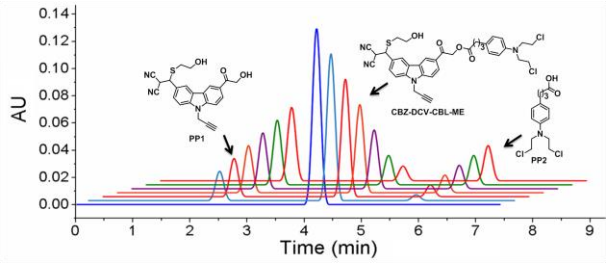


Figure 3. HPLC overlay chromatogram of CBZ-DCV-CBL-ME conjugate at different time intervals of light irradiation (0-60 min).

The light-induced release of the anticancer drug was then quantified by plotting the HPLC peak area obtained for chlorambucil *vs*. different irradiation times (**Figure 4a**). The photolysis was followed until 90% of chlorambucil was released from the CBZ-DCV-CBL-ME conjugate. Further, to demonstrate the precise control over the drug release by light, we monitored the release of chlorambucil by periodically switching the UV light source on and off. **Figure 4b** clearly indicates that whenever the light source was switched off, the drug release stopped, which clearly indicates that external stimulus light only induces the anticancer drug release. Further, we calculated the rate constant (K_p) for the drug release and is found to be 2.59536×10^{-4} s^{-1}. From this, the photochemical quantum yield was calculated by using potassium ferrioxalate as an actinometer and found to be Φ_p= 0.099.

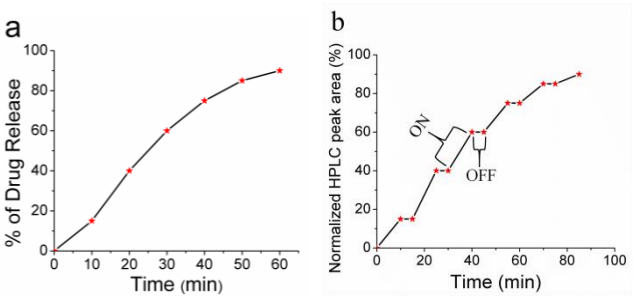


Figure 4. (a) Amount of chlorambucil released by CBZ-DCV-CBL-ME at different irradiation times (0-60 min) using UV light (λ≥365 nm), (b) progress of the release of chlorambucil under bright and dark conditions (‘‘ON’’ indicates the start of UV light irradiation and ‘‘OFF’’ indicates the end of UV light irradiation.

To investigate the cellular uptake property of CBZ-DCV-CBL and Bio-CBZ-DCV-CBL, the cell imaging studies were performed by HeLa cells. Confocal microscopy study confirms the internalization of Bio-CBZ-DCV-CBL is more compare to CBZ-DCV-CBL as observed from **Figure 5 iib** and **iiib, ivb**. The two major features of DDS, (i) ability of biotin moiety guiding towards

biotin receptor and (ii) responsiveness of the system towards glutathione has been assessed here. Since, cancer cells highly express glutathione and biotin receptors. The concept of targeted delivery has been appreciably showed in case of Bio-CBZ-DCV-CBL. The time dependent increase in internalization of the DDS [increase in blue fluorescence – biotin moiety targeting tumor cells (as shown in **Figure 5 iiib and ivb**)] and reduction in green fluorescence – glutathione sensing (as shown in **Figure 5 iiic and ivc**). The dual characteristic of the DDS (Bio-CBZ-DCV-CBL) serves the purpose of targeting and sensing, thus enhancing the internalization on tumor cells.

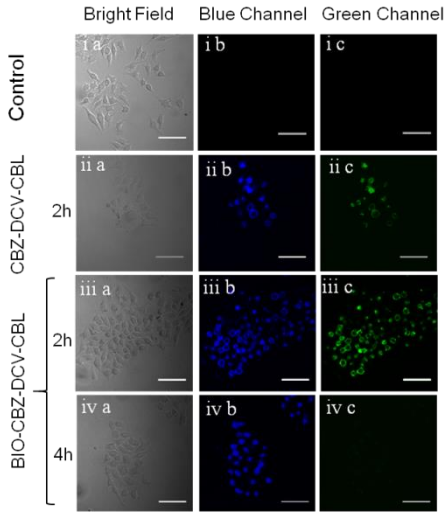


Figure 5. Confocal microscopy images of HeLa cells: (i) untreated cells; (ii) cells treated with CBZ-DCV-CBL after 2 h of incubation shows both blue and green fluorescence illustrating internalization and glutathione sensing. (iii and iv) cells treated with Bio-CBZ-DCV-CBL showing time dependent increase in internalization of the DDS illustrating increase in blue fluorescence (iii b and iv b)–biotin moiety targeting tumor cells and reduction in green fluorescence (iii c and iv c) glutathione sensing). (Scale bar = 20 μm).

Further, to validate the concept of GSH sensing mediated cancer cell detection over the normal cell, cell imaging was performed with Bio-CBZ-DCV-CBL conjugate on normal cell line NIH 3T3. NIH 3T3 cells were treated with Bio-CBZ-DCV-CBL for 2h and 4h respectively following the same conditions to that of HeLa cells. Followed by confocal fluorescent images (**Figure 6**) were taken which clearly displays that after 2h and 4h of treatment Bio-CBZ-DCV-CBL conjugate was readily internalized. From the **Figure 6** it could be clearly observe that even after 4h of treatment green emission from Bio-CBZ-DCV-CBL conjugate was visible (**Figure 6 iib**) which is contrary to the images obtained in case of cancerous HeLa cells where green emission of Bio-CBZ-DCV-CBL conjugate was totally quenchehed owing to the already mentioned thiol-ene reaction. Therefore, the above result clearly display that the Bio-CBZ-DCV-CBL conjugate can precisely distinguish the cancer cells and the normal cells. However, reduced intensity of green emission (**Figure 6 iib)** in case of 4h incubation in comparison to 2h incubation (**Figure 6 iia**) is may be because of the same thiol-ene reaction where normal level of GSH present in NIH 3T3 cells reacted with Bio-CBZ-DCV-CBL conjugate.

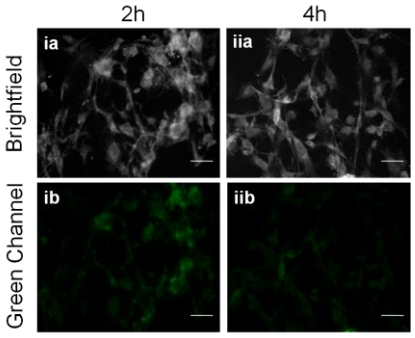


Figure 6. Confocal microscopy images of normal cells (NIH 3T3): (ia and iia) Brightfield images; (ib) cells treated with Bio-CBZ-DCV-CBL after 2 h of incubation: green emission illustrating internalization of Bio-CBZ-DCV-CBL conjugate. (iib) cells treated with Bio-CBZ-DCV-CBL after 4 h of incubation showing less reduction in green fluorescence compare to cancer cells (Scale bar = 20 μm).

The *in vitro* anticancer activity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide,[22] a yellow tetrazole) assay in HeLa cells. Different concentration of compounds CBL, CBZ-DCV-CBL and Bio-CBZ-DCV-CBL were added in the wells and subjected to photolysis. The percentages of cell viability vs. concentration of compounds at a different time interval before and after photolysis are presented in **Figure 7a** and **Figure 7b** respectively. From results, it is evident that Bio-CBZ-DCV-CBL after photolysis possesses potent anticancer activity because of biotin receptor targeting and glutathione sensing, in terms of cell viability. The CBL in Bio-CBZ-DCV-CBL is able to target the cells efficiently in comparison to only CBL and CBZ-DCV-CBL. From these results, it is clearly confirmed that the biotin moiety and glutathione sensing plays a dual role of targeting and sensing unit to tumor cells in this DDS.

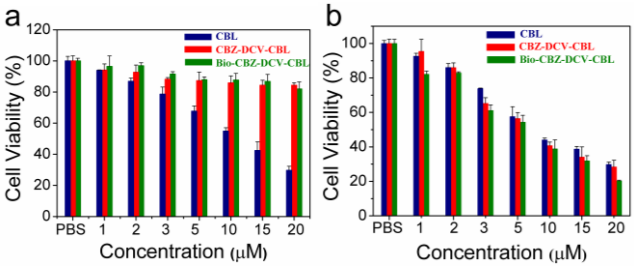


Figure 7. (a−b) Cell viability assay of Bio-CBZ-DCV-CBL in HeLa cell line: (a) before photolysis, (b) after photolysis. Values are presented as mean ± SD.

In conclusion, we have developed a new theranostic agent, Bio-CBZ-DCV-CBL conjugate (**6**) and its synthesis, characterization, spectroscopic properties and anticancer activity as well as biological applications has been investigated. Upon addition of free thiol to compound **6**, the dicyanovinyl bond break down *via* thiol-ene click reaction at pH < 7, restricting the intramolecular charge transfer between donor and acceptor. This leads to

change in fluorescence emission from green to blue and facilitate releasing of the active drug CBL upon irradiation. The confocal microscopic experiment reveals that the present theranostic DDS effeciently internalized by the biotin receptor-positive HeLa tumor cells, sense the intracellular thiols of cancer microenvironment by thio-ene reaction and thereby reduces resistance to chemotherapy drugs and showed increased therapeutic effect. Therefore, our DDS demonstrated a new and efficient strategy for the specific tumor targeted drug delivery and tumor imaging.

Experimental Section

Experimental Details.

ACKNOWLEDGMENT

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Keywords: Carbazole • Photoremovable Protecting Group • Tumour Targeted • Glutathione Sensing • Photoresponsive Anticancer Drug Delivery

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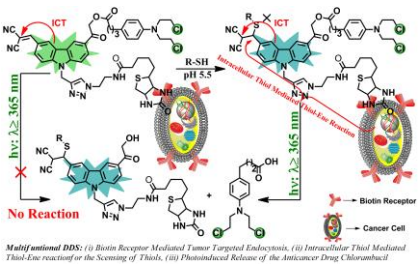

Multifuntional DDS: (i) Biotin Receptor Mediated Tumor Targeted Endocytosis, (ii) Intracellular Thiol Mediated Thiol-Ene reactionfor the Seensing of Thiols, (iii) Photoinduced Release of the Anticancer Drug Chlorambucil

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