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Two-photon responsive Napthyl tagged p-hydroxyphenacyl based drug delivery system: uncaging of anti-cancer drug in the phototherapeutic window with real-time monitoring

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We report a two-photon responsive drug delivery system (DDS) namely, p-hydroxyphenacyl-naphthalene-chlorambucil (pHP-Naph-Cbl) having two-photon absorption (TPA) cross-section of ≥ 20 GM in the phototherapeutic window (700 nm). Our DDS exhibited both AIE and ESIPT phenomena, which were utilized for the real-time monitoring of anti-cancer drug release.

Light-induced drug delivery systems (DDSs) have recently gained significant importance in the area of biological sciences because of their ability to provide spatial and temporal control over the drug release. To date, different types of light-induced DDSs have been developed using the well-known phototriggers like o-nitrobenzyl,[1] coumarinyl,[2] quinolinyl,[3] p-hydroxyphenacyl,[4] anthracenyl[5], and *o*-hydroxy cinnamate[6]. Among them, photoresponsive DDSs constructed based on p-hydroxyphenacyl phototrigger have captured great attention due to its unique properties which include: (i) large Stokes shift (ii) fast release (iii) high photochemical quantum yield and (iv) formation of biologically benign photoproduct.

Because of the aforementioned unique properties of p-hydroxyphenacyl phototrigger, several phenacyl based DDSs have been developed for the release of various important biomolecules like neurotransmitters, enzyme switches, ATP/DEP etc.[7-9] In 2016, Singh group developed p-hydroxyphenacyl–benzothiazole as a DDS for the release of anti-cancer drug chlorambucil in the visible wavelength region (λ ≥410 nm) using excited-state intramolecular proton transfer (ESIPT) phenomenon.[10] In continuation, the same group also developed another DDS based on p-hydroxyphenacyl namely, tetraphenylene-*p*-hydroxyphenacyl-chlorambucil[11] conjugate which released the anti-cancer drug only in the aggregated state on exposure to visible light (λ ≥410 nm) with real time monitoring ability. Though, several visible light activated p-hydroxyphenacyl based DDSs have been reported. To the best of our knowledge, p-hydroxyphenacyl based DDS with uncaging ability in the phototherapeutic window (650-950 nm) is not still known, which hinders their practical usage in the biological applications.[12] To overcome the above-mentioned limitation, two-photon responsive p-hydroxyphenacyl based DDS has been targeted. Elles *et al.* reported two-photon activated p-hydroxyphenacyl based DDS for the release of ATP or DEP on exposure to light of wavelength 570 nm.[13] But the above designed two-photon responsive DDS didn't exhibit promising uncaging ability in the region of 650-950 nm.[14] Hence, there is a need to develop two-photon responsive p-hydroxyphenacyl based DDS having large two-photon absorption (TPA) cross-section in the phototherapeutic window.[15-19]

Recent studies show that enhancing the internal charge transfer (ICT) in a π conjugated donor (D) and acceptor (A) (D-π-A) systems leads to large TPA cross-section.[20-22] Based on the above interesting idea, we intend to construct p-hydroxyphenacyl based DDS with good uncaging ability in the phototherapeutic window. For this purpose, we have incorporated Naphthalene moiety to the p-hydroxyphenacyl group so that a strong ICT can occur, thereby resulting in red-shifted absorption and enhanced two-photon uncaging cross-section (**Fig. 1**). Our newly designed photoresponsive DDS, **pHP-Naph-Cbl** provided the following advantages (i) two-photon absorption in the phototherapeutic window (700 nm), (ii) exhibits AIE phenomenon, thus significantly releases the drug in the aggregated state, (iii) large stokes shift due to the ESIPT process, and (iv) produces a distinct fluorescent colour change on drug release, which enables real-time monitoring ability.

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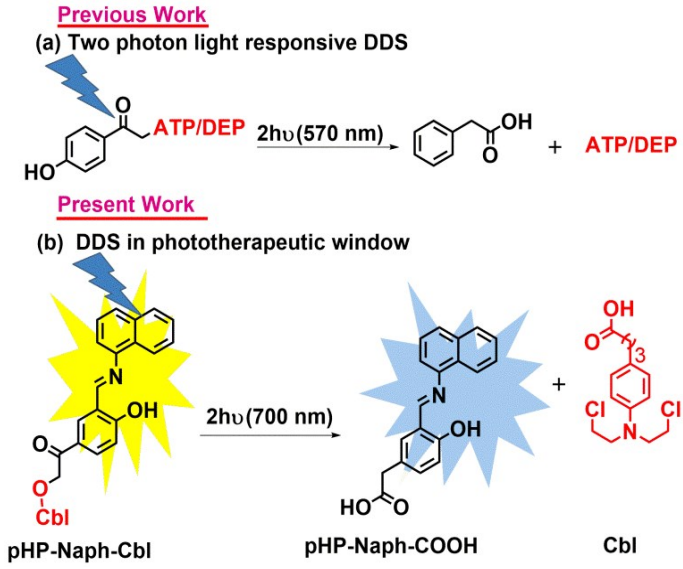
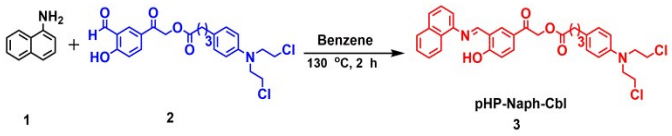


Fig.1 (a) Christopher G. Elles and co-workers reported two-photon activated pHP phototriggers for uncaging of the bioactive molecule, and (b) Working protocol of DDS **pHP-Naph-Cbl** in phototherapeutic window.

Our DDS, **pHP-Naph-Cbl**, was synthesized according to the following protocol (**Scheme S1**). The synthesis of compound **2** was carried out as reported in our earlier work.[10] The condensation reaction of **2** with 1-Naphthylamine on heating at 130 °C for 2 h in benzene solvent furnished the desired compound **3**. The desired DDS **3** was characterized by NMR (^{1}H and ^{13}C) (**Fig. S1-S2**), and mass spectrometry (**Fig. S3**).



Scheme 1. Synthesis of photoresponsive DDS, **pHP-Naph-Cbl**.

The photophysical properties of our DDS, **pHP-Naph-Cbl,** was noted to be sensitive with respect to different solvents. The UV spectrum of our DDS (1 x 10^{-4} M) showed an absorption maximum at around 350 nm (**Fig. 2b**) in studied solvents. The influence of the ESIPT process was demonstrated by emission spectroscopy (**Fig. 2c**). In the case of non-hydrogen bonding solvents(benzene, chloroform), our DDS showed emission maximum at around 550 nm, due to the keto form (**5**) which can be attributed to the ultrafast proton transfer from the hydroxyl group of pHP to imine nitrogen atom of Napthyl moiety (ESIPT). Whereas, in the polar aprotic solvents (DMSO, EtOAc), our DDS showed blue-shifted emission maximum at 425 nm, due to the enol form (**4**) of **pHP-Naph-Cbl**. Interestingly, in polar protic solvents (MeOH, ACN) our DDS provided two emission maxima, one at 550 nm and another at around 425 nm because of the keto-enol tautomerization of **pHP-Naph-Cbl**. However, in MeOH and DCM our DDS exhibited two humps around 425 nm which can be due to the presence of both cis- and trans-enolic forms (**Fig. S4**).

Furthermore, we also investigated the AIE properties of our photoresponsive DDS, **pHP-Naph-Cbl**. The emission spectra of our DDS (1 X 10^{-4} M) were recorded in an ACN–Water, binary mixtures with different water fractions (f_w). In pure ACN solution, the DDS exhibited very weak fluorescence. But with a slight increment in the water fraction, the fluorescence intensity of the DDS increased slowly. The emission intensity of

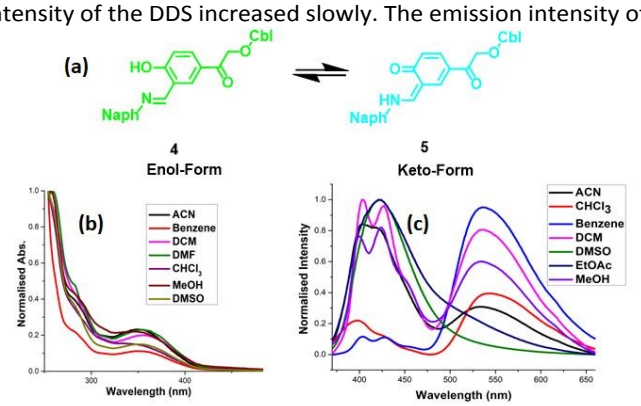


Fig.2 (a) Existence of keto and enol forms of **pHP-Naph-Cbl**, (b) Normalized UV-Vis, and (c) Emission spectra of **pHP-Naph-Cbl** (1 x 10^{-4} M) in different solvents).Excitation wavelength 350 nm.

the DDS abruptly increased from f_w = 85 to 95 vol % (**Fig. 3a**), which is associated with the formation of aggregates of DDS due to its poor solubility in the aqueous medium, and fluorescence quantum yield of DDS (1 x 10^{-4} M) in the above ACN-Water binary (1:9 v/v) mixture was found to be 0.22 (**Table S1**). We also estimated the stability of DDS in the biological medium and at different pH in dark conditions (**Table S2**).

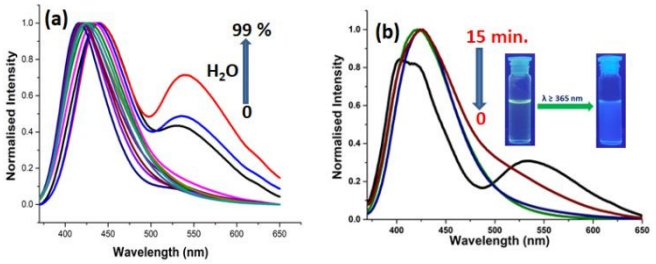


Fig.3 (a) Fluorescence spectrum of DDS, **pHP-Naph-Cbl** (1 × 10^{-4} M) in ACN–water binary mixture with different water fractions (f_w), and **(b)** Fluorescence spectrum of DDS, pHP-Naph-Cbl (1 × 10^{-4} M) in ACN–PBS buffer of pH 7.4 at different intervals of photoirradiation (0–15 min).

To demonstrate the photorelease capability of our photoresponsive DDS (1 × 10^{-4} M), in ACN–PBS buffer of pH 7.4 (1: 9 v/v) was exposed to a medium pressure mercury lamp (125 W) as the source of light ($\lambda \geq 365$ nm) using a 1 M aqueous solution of $CuSO_4$ as the UV cut-off filter with constant stirring for 15 min. The photorelease was monitored using reverse-phase HPLC (**Fig. 4**). The gradual depletion of the peak at retention time (t_R) **4.7** min with increasing time of irradiation indicates the photodecomposition of **3**, whereas, the appearance of two new peaks at **3.9** min and **3.2** min denotes the formation of the photoproduct (**pHP-Naph-COOH**) and chlorambucil (assigned by injecting the authentic sample), respectively. Formation of photoproduct **Cbl** and **pHP-Naph-COOH** from **pHP-Naph-Cbl**, after 15 min of photoirradiation was characterized by HRMS (**Fig. S5-S6**).

Interestingly, we noted a distinctive fluorescence colour change from greenish-yellow to blue during the photolysis of our DDS, **pHP-Naph-Cbl** (**Fig. 3b**). Initially, the emission spectrum showed the band at around 550 nm (due to AIE) corresponding to **pHP-Naph-Cbl** (at 0 min). When the irradiation time was gradually increased (0–15 min), the intensity of the yellow-emission band was gradually decreased and a new blue-emission band at 430 nm (Stokes Shift 120 nm) corresponding to the photoproduct, **pHP-Naph-COOH** gradual increased in intensity. The blue shift in the fluorescence spectrum of the photoproduct, **pHP-Naph-COOH**, is due to the loss of internal charge transfer (ICT) from Napthyl to phenacyl group (as the photoproduct has no phenacyl functionality). Thus our DDS is capable of monitoring the drug release in real-time.

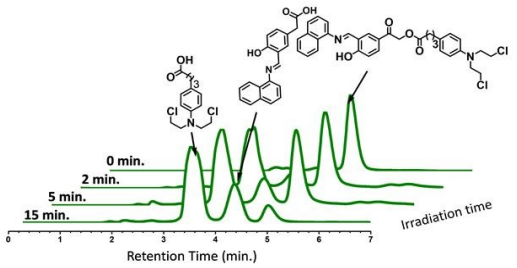
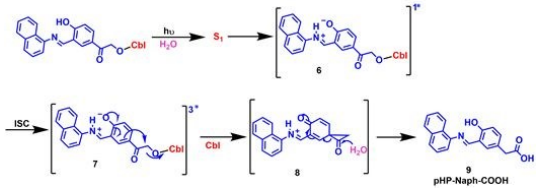


Fig.4 Overlay HPLC chromatogram of **pHP-Naph-Cbl** in ACN/PBS buffer of pH 7.4 (1:9 v/v) at different time intervals (0–15 min) of photoirradiation.

We also noted that the drug release solely depends upon the incident light, thus we carried out a 'light on-off' experiment, in which we exposed our DDS, **pHP-Naph–Cbl** solution to light and dark conditions for different time intervals.[23] We observed that our DDS released anti-cancer drug chlorambucil only on exposure to light. In addition, we also found that our DDS on exposure to light for 15 min released 95% of anti-cancer drugs (**Fig. S7 a** and **b**).

The mechanism of photorelease by **pHP-Naph-Cbl** is shown in **scheme 2**. Upon exposure to light (λ ≥365 nm), our DDS, **pHP-Naph-Cbl** (1×10^{-4} M) gets excited from the ground state (S_0) to its singlet excited state (S_1). In the S_1 state, DDS undergoes strong intramolecular hydrogen bonding between the imine nitrogen and **-OH** group of **pHP** (CH=N----HO), which facilitates ESIPT behaviour in the excited state, thus resulting in deprotonation of the **pHP** group to give intermediate **6**. The intermediate **6** then, undergoes efficient ISC to its triplet excited state **7** (T_1). From the T_1 state, DDS undergoes photo-Favorskii rearrangement[24] to give spirodiketone intermediate **8** leading to the concomitant release of anti-cancer drugs. The intermediate spirodiketone is then subjected to hydrolysis in the aqueous medium to give the rearranged photoproduct **9** (**pHP-Naph-COOH**).



Scheme 2 Proposed photorelease mechanism of the **pHP-Naph–Cbl**.

To support our argument that the drug release occurs from T_1 state, we carried out the photolysis in the presence of a triplet quencher, potassium sorbate (PoS). We noted the complete arrest of drug release at 200 μM concentration of PoS (**Fig. S8**).

Further, we were also interested to investigate whether the drug release happens only in the aggregate state. Hence, we performed irradiation of our DDS in different water fractions (f_w). We observed that the anti-cancer drug release reached a maximum of 95 % (photochemical quantum yield 0.49) with a gradual increase in f_w, in compound **3** (1×10^{-4} M) in ACN–PBS buffer, at pH 7.4. We noted no substantial anti-cancer drug release was observed below f_w 80 % and in pure ACN (**Fig. S9**). This indicated that the aggregation phenomenon initiates the anti-cancer drug release (**Table S3**). We also evaluated the photolysis of **pHP-Naph-Cbl** at different pH medium (**Table S4**), the results support that ESIPT process assist the photorelease (**Fig. S10**).

After a single-photon uncaging of **pHP-Naph-Cbl**, we were interested to study the two-photon uncaging cross-section (δ_u)[25] of our DDS, **pHP-Naph-Cbl** (1×10^{-4} M) in DMSO/Water (1:9 V/V). First δ_a of our DDS (1×10^{-4} M) in DMSO/Water, (1:9 V/V) was determined using open aperture Z-scan technique with pulsed laser (pulse width 100 fs, repetition rate 80 MHz at 700 nm with 1.0-Watt power) (**Fig. S11**), and the result was found to be $\delta_a \geq 20$ GM (**Fig. 5**). Then δ_u of **pHP-Naph-Cbl** was calculated using the formula $\delta_u = \varphi_u \delta_a$, thus δ_u of **pHP-Naph-Cbl** was found to be 10 GM (1 GM = 10^{-50} cm^4 s/photon). The two-photon release of our DDS was performed with a laser of 100 fs pulse at the 80 MHz repetition rate at the wavelength of 700 nm. The result indicated that 25 % of drug release was noted after 3 h of irradiation (**Fig. S12**). Furthermore, we also evaluated the δ_u of our DDS at different wavelengths and we noted that δ_u of DDS was maximum at 700 nm (**Fig. S13**).

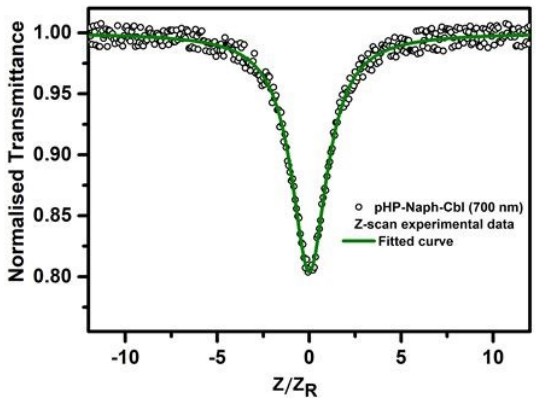


Fig.5 Open aperture (OA) Z-scan measurement (at 700 nm) of **pHP-Naph-Cbl** of normalized transmittance. The discrete points represent experimental data and the solid line represents theoretical fitting.

Based on the promising photorelease ability of our DDS, we were keen to carry out the *in vitro* studies of the DDS using breast cancer cell line MCF-7. Cellular uptake studies showed that DDS, **pHP-Naph-Cbl** got internalized in the cell after 12 h of incubation. **Fig. 6** (**ia, ib,** and **ic**), shows that **pHP-Naph-Cbl** is uniformly distributed inside the cell. Real-time monitoring of

drug release by our DDS (**pHP-Naph-Cbl**) inside breast cancer cell line MCF-7 was analyzed by fluorescence confocal images (**Fig. 6**). Initially, the cells showed yellow fluorescence due to cellular uptake of **pHP-Naph-Cbl**. After exposure to light of wavelength ≥365 nm for 15 min, we observed a complete fluorescent colour change from yellow to blue, suggesting a greater extent of photorelease of chlorambucil by our DDS (**pHP-Naph-Cbl**).

Further, we evaluated the cytotoxicity of **pHP-Naph-Cbl** using the MTT assay [MTT=3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] in the cell line MCF-7. Cell viability remained above 95 % at different concentrations of **pHP-Naph-Cbl**. For the light-exposure experiment, cells were incubated with different concentrations of **pHP-Naph-Cbl** and after 15 min of irradiation (λ ≥ 365 nm), **Cbl** was released thereby causing toxicity to breast cancer cell line MCF-7 (IC_{50} 31 μM) as validated by the MTT toxicity data (**Fig. S14**)**.** Further, we also noted that our DDS (**pHP-Naph-Cbl**) showed enhanced cytotoxicity in the presence of light compared to the free **Cbl** at given concentration**.**

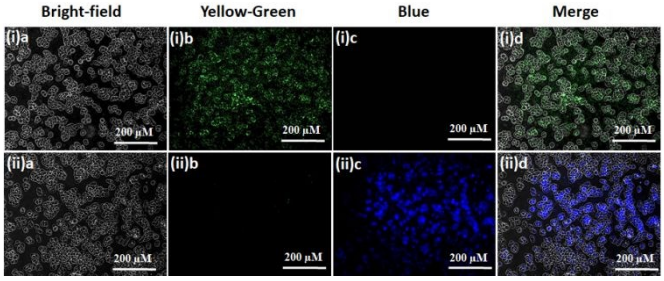


Fig.6 Fluorescence confocal images of cellular internalization of **pHP-Naph-Cbl** in breast cancer cell line MCF-7 (i)a bright-field image, at 0 min., (i)b fluorescence image of bright field at 475-550 nm, (i)c fluorescence image of bright field in blue channel, (emission wavelength range 420-450 nm), (i)d merge image of bright-field, yellow-green and blue channel, (ii)a bright-field image at 0 min. (ii)b fluorescence image of bright field at 475-550 nm after 15 min. of photoirradiation, (ii)c fluorescence image of bright field in blue channel after 15 min. of photoirradiation (emission wavelength range 420-450 nm), and (ii)d merge images of bright-field, yellow-green and blue channel of **pHP-Naph-Cbl**. Excitation wavelength for yellow-green and blue channels are 460 nm and 350 nm, respectively. Scale bar = 200 μM.

In conclusion, two-photon responsive p-hydroxyphenacyl based DDS with uncaging ability in the phototherapeutic window was developed by simple tagging with Napthyl moiety. The designed DDS showed the TPA cross-section to be greater than 20 GM and TP uncaging of 10 GM at 700 nm region. The DDS released the anti-cancer drug only in the aggregated state and exhibited real time monitoring ability of drug release. In future, we want to explore our **pHP-Naph-Cbl** as a two-photon responsive nano DDS for the efficent release of anticancer drug for in vivo applications.

Conflicts of interest

There are no conflicts to declare**.**

Notes and references

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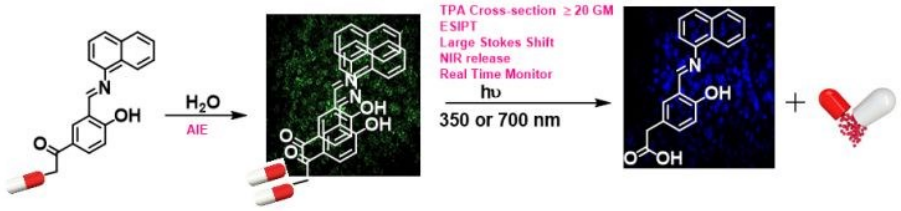

Two-photon responsive drug delivery system having two-photon absorption (TPA) in the phototherapeutic window with two-photon uncaging cross-section ≥ 10 GM and exhibiting real-time monitoring of anti-cancer drug release.