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ARTICLE

Spiropyran-Coumarin Platform: An Environment Sensitive Photoresponsive Drug Delivery System for efficient cancer therapy

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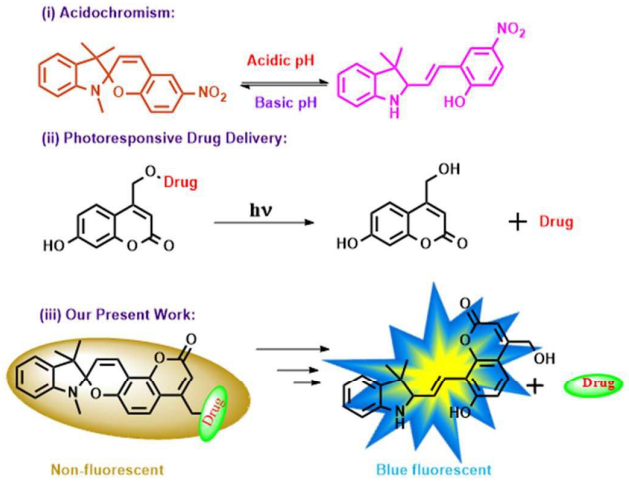
Abstract: In spite of inventing several anticancer agents the clinical payoff still remains unsatisfactory because of their severe host toxicity due to the nonspecific biodistribution in the body. To achieve high tranquility in the anti-cancer drug delivery, thus, we designed and developed a single component photoresponsive drug delivery system, a fusion of two platforms spiropyran and coumarin, which synchronizes two controlling factors: first, the lower pH of cancer tissue, acts as internal control, leads to the ring opening of spiropyran resulting in distinct colour change and fluorescence activation of coumarin and second, the release of anti-tumor drug by the externally controlled light. High fluorescent nature and promising biocompatibility make SP-Cou-Cbl system suitable for cell imaging and in vitro studies.

Introduction:

Cancer is the most breakneck threat to the mankind at present. In spite of inventing several anticancer agents the clinical payoff still remains unsatisfactory because of their severe host toxicity due to the nonspecific biodistribution [1] in the body. Vivid discrimination between the cancer cells and the healthy cells followed by highly precise drug delivery to the diseased cells is one of the best lead to an efficient chemotherapy.[2]

In this context, stimuli-responsive drug delivery systems have proved their potential to optimize therapeutic efficacy and alleviate unwanted side effects by recognizing tumor microenvironment followed by targeting cancer cells in a highly selective manner.[3] These intelligent carriers release cargo as a counter interaction with the intracellular stimuli such as temperature,[4] pH,[5] redox,[6] enzyme[7] etc. The pH-sensitive drug delivery systems attracted a great deal of attention among all other stimuli-responsive systems because of the acidic microenvironment of the tumor tissue compared to the normal tissue. In addition, intervention of an extracorporeal stimulus[8] such as light will lead to high spatiotemporal[9] control over the therapeutic delivery [10]and extreme dose control.[11]

Spiropyran is a well-known acidochromic[12] compound which shows reversible transformation between two species having distinguishable absorption spectrum by varying the pH of the solution. The closed form of spiropyran (SP) contain a C_{spiro}-O bond that cleaved under acidic pH to its zwitterionic merocyanin (MC) isomer and both of these two form (SP and MC) are structurally and property wise distinct from each other (**Scheme 1 (i)**).[13] This potential changing ability of structural properties and optical signal output makes spiropyran the ideal system for optical storage,[14] polymer viscosity control,[15] photomechanical transduction and actuation,[16] bioactivity switching of proteins,[17] tissue engineering[18] and drug delivery.[19] On the other hand, Coumarin has been tremendously used as photoresponsive choromophore for drug delivery (**Scheme 1 (ii)**) [20] because of its natural abundance, non-toxicity, bio-compatibility and highly fluorescent nature.[21] Though the externally triggered photocleavable protecting group, [22] coumarin, have several advantages but the main drawback is the fluorescent signal is not target specific leading to zero discrimination between healthy and tumor cells. To achieve high tranquility in the anti-cancer drug delivery, thus, an ideal system is needed by virtue of which clean release of drug can be done only at the tumor.



Scheme 1: Schematic representation of (i) Acidochromism; (ii) Photoresponsive drug delivery from Coumarin moiety; (iii) Spiropyran locked coumarin drug delivery system.

Herein, we designed and developed a single component photoresponsive drug delivery system, a fusion of two platforms spiropyran and coumarin, which synchronizes two

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controlling factors: first, the lower pH of cancer tissue, acts as internal control, leads to the ring opening of spiropyran resulting in distinct colour change and fluorescence activation of coumarin and second, the release of anti-tumor drug by the externally controlled light (**Scheme 1** (**iii**)). The lower pH of the cancerous region will trigger the sequence by giving optical output through colour change and fluorescence activation and next drug will be released by irradiation of light on the particular detected area leading to a precise delivery of anti-tumor agents in very controlled manner.

Experimental

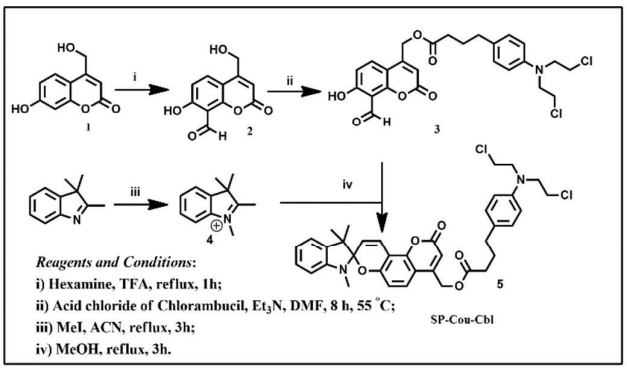
Synthesis of SP-Cou-Cbl conjugate (DDS):

Compound **3** (1 equ.) and **4** (1 equ.) were prepared as per literature procedure.[11, 23] Final compound (**5**) was prepared by refluxing compound **3** and **4** in dry methanol (5 mL) for 3 h, after cooling to room temperature water (100 mL) was added to the reaction mixture. The formed purple solid was recovered by filtration and dried in vacuum, afforded 5. 1H NMR (400 MHz, acetone-d_6): δ = 7.79-7.78 (d, J = 4 Hz, 1H), 7.70- 7.69 (d, J = 4 Hz, 1H), 7.55- 7.46 (t, J = 8 Hz, 2H), 7.48- 7.46 (d, J = 8 Hz, 2H), 7.19- 7.16 (t, J = 4Hz , 1H), 7.12- 7.10 (d, J = 8 Hz, 3H), 6.89-6.85 (t, J = 8 Hz, 1H), 6.77-6.72 (t, J = 8 Hz, 1H), 6.66-6.64 (d, J = 8 Hz, 1H), 6.32 (s, 1H), 6.04-6.01 (d, J = 12 Hz, 1H), 5.36 (s, 2H), 3.80- 3.75 (m, 8H), 2.79 (s, 3H), 2.63-2.60 (t, J = 8 Hz,), 2.55-2.52 (t, J = 4 Hz, 2H), 1.99-1.97 (t, J = 4 Hz , 2H), 1.34 (s, 3H), 1.33 (s, 3H). ^{13}C NMR (100 MHz, *acetone-d_6*): δ = 172.1, 159.3, 157.4, 150.4, 150.0, 147.2, 144.7, 136.2, 130.0, 129.5, 127.6, 125.2, 122.0, 121.4, 120.1, 119.4, 112.2, 111.6, 109.1, 107.1, 106.9, 105.7, 61.0, 53.0, 51.8, 40.7, 33.6, 32.8, 26.7, 25.2, 19.3, 13.4. HRMS cal. For $C_{35}H_{34}Cl_2N_2O_5$ [MH^+]: 632.1845, found: 632.1815.

Result and discussion:

The synthesis of spiropyran-coumarin baesd drug delivery system (DDS) has been outlined in **Scheme 2**. Formylation of 7-hydroxy-4(hydroxymethyl) coumarin (**2**) has been carried as depicted in our previous work.[11] After that, simply by esterification reaction in presence of acid chloride of Chlorambucil and triethylamine (Et_3N) in dimethylformamide for 8 h at 55 °C gave compound **3**. Next, condensation of compound **3** with indole derivative, synthesised as per literature procedure, afforded our targeted DDS, Spiropyran-coumarin-chlorambucil (**SP-Cou-Cbl**). The obtained compounds were characterised by UV-Fluorescence, NMR, HR-MS analysis (**Figure S1-S7**).

Figure 1a and **1b** shows the absorption and emission spectra of SP-Cou-Cbl. In neutral pH, absorption spectrum did not show any absorption in visible region and no fluorescent was also noted. On the other hand, a broad absorption band (350 nm to 500 nm) (**Figure 1a**) and appearance of blue fluorescent was observed on gradual lowering of pH (pH 7.4 pH 3.4) (**Figure 1b**).



Scheme 2: Synthesis procedure of **SP-Cou-Cbl**.

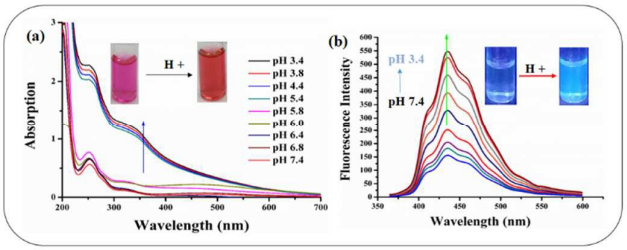


Figure 1: (a) Absorption; and (b) Emission spctra of SP-Cou-Cbl in different acidic pH.

In acidic pH (pH 5 to pH 3), N-atom of indole moiety gets protonated and promoting the ring opening of spiropyran. This phenomenon also assist the localization of π-electrons on the coumarin moiety, exhibiting blue emission (**Figure 1b**). The ring opening of spiropyran ring was further confirmed by the NMR study.

NMR studies showed the molecular level change during the process of acid induced ring opening. The spectral overlay study (**Figure 2**) showed that in the absence of acid (TFA) the spiro bond of SP-Cou-Cbl remains stable in acetone-d_6. While after addition of TFA to SP-Cou-Cbl in acetone-d_6, a distinct spectral change due to the opening of Cspiro-O bond was noted. The chemical shift changes induced by TFA, is the indication of N-protonation. A significant downfield shift from 2.77 to 3.07 ppm of N-methyl group of SP-Cou-Cbl (indicated as a) with addition of TFA due to increased positive charge accompanying N-protonation was observed. The downfield shift of vinylic proton, indicated as b, from 6.02 to 6.48 ppm, can be attributed to the ring opening of spiro ring of SP-Cou-Cbl. The spectral change has been given below in a tabular form (**Table 1**). The acid induced ring opening of SP-Cou-Cbl further confirmed from the RP-HPLC study (**Figure S8**).

To check the photo-induced anticancer drug release property, photolysis of **SP-Cou-Cbl** (1×10^{-4} M solution) was carried out in acetonitrile-water mixture (70:30) using visible light (≥ 410 nm, 125 W, incident intensity (I_0) = 2.886 x 10^{16} quanta s^{-1}). The drug release by photo irradiation was monitored by RP-HPLC. The HPLC chromatogram showed three peaks at tR 4.4 min, tR 5.4 min and tR 7.2 min. Peak at tR 4.4 min corresponds to anticancer drug Cbl, confirmed by injecting authentic sample

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into the HPLC (**Figure S9**). The photorelease of Cbl further supported by NMR spectra (**Figure S10**).

Table 1: The spectral change of SP-Cou-Cbl upon addition of TFA

Proton [α]	δ (ppm) values of SP-Cou-Cbl with different Concentration of TFA (M)	
	0 (M)	0.08 (M)
a	2.77	3.07
b	6.02	6.48
c	6.28	6.28

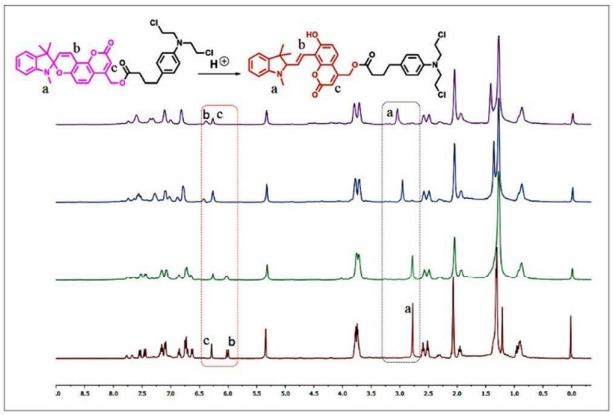


Figure 2: ^{1}H NMR spectral changes of SP-Cou-Cbl (2×10^{-4} M) in *acetone-d_6* in presence of different concentration of TFA (0-0.08 M).

The plot (**Figure 3a**) of percent of drug release against irradiation time (min) showed that about 82% of drug got released within 30 min and the 'ON-OFF' experiment (**Figure 3b**) proves that the light is solely responsible for drug delivery.

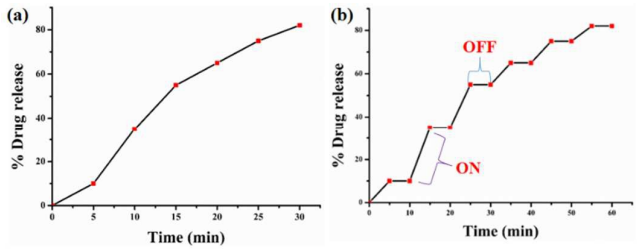
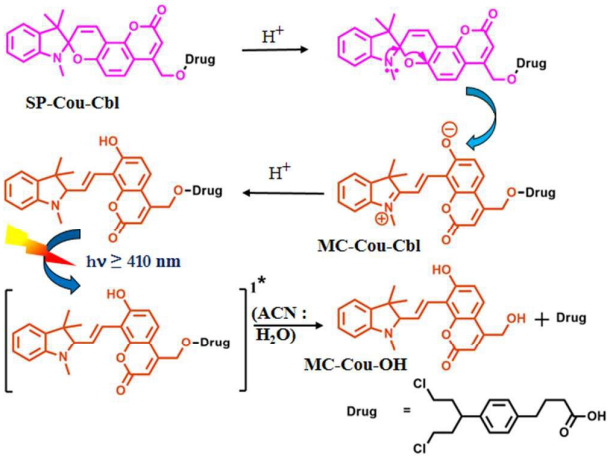


Figure 3: (a) Amount of drug (chlorambucil) released from SP-Cou-Cbl at different UV-light (≥ 365 nm) irradiation time; (b) HPLC peak area of the released chlorambucil *vs* Time curve under bright and dark conditions."ON" indicates the beginning of UV light irradiation;"OFF" indicates the ending of light irradiation.

Based on the literature survey, we can propose that, the possible mechanism (**Scheme 3**) could be i) N-atom of SP-Cou-Cbl gets protonated in acidic pH followed by spiro ring opening of of SP-Cou-Cbl; ii) upon visible light irradiation merocyanin-coumarin-chlorambucil (MC-Cou-Cbl) goes to its singlet excited state and finally, iii) singlet excited state undergoes heterolytic cleavage to form photoproduct merocyanin-coumarin-methanol (**MC-Cou-OH**) and chlorambucil (**Cbl**).



Scheme 3: Possible mechanism of ring opening and photorelease of anticancer drug.

Cellular internalization of SP-Cou-Cbl:

To establish SP-Cou-Cbl as an efficient photoresponsive DDS, the cellular uptake capability was studied against human breast cancer cell line MDA-MB-231, procured from NCCS, Pune and maintained in DMEM media containing 10% FBS (Gibco, USA), 100 U/ml streptomycin and 100 U/ml penicillin. Cells were regularly maintained at 37^0 C in a humidified cell culture incubator with an atmosphere of 5% CO_2. Briefly, 3×10^5 MDA-MB-231 cells were seeded per well of a polystyrene –coated 6-well cell culture plate and incubated for 12 hours for proper attachment and proliferation. Thereafter, cells were incubated with 10µg/ml of the conjugate, SP-Cou-Cbl, for 4 h and after irradiation with visible light (≥ 410 nm), analysed on Olympus FV-1000 Confocal microscope. Confocal images of the cells showed blue fluorescence majorly from the cytoplasm and partially from the nucleus of the cell (**Figure 4 d–f**). Notably, no fluorescence was observed in the control cells (**Figure 4 a–c**).

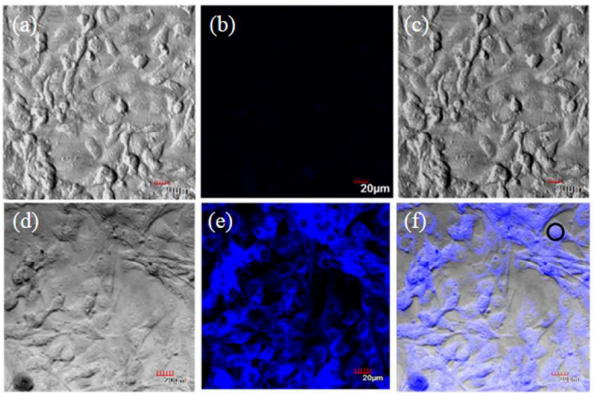


Figure 4: Confocal images from the cells treated and untreated with SP-Cou-Cbl for 4 h : (a–c) control cell; (d–f) SP-Cou-Cbl. (a and d) bright field; (b and e) blue fluorescence;

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(c and f) overlay images of the bright and fluorescence fields (scale bar = 20 µm). The nuclear localization is indicated by a black circle.

Anticancer efficacy of SP-Cou-Cbl before and after UV light irradiation:

The anticancer activity of SP-Cou-Cbl was evaluated against human breast cancer cell line MDA-MB-231 by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 10^4 MDA-MB-231 cells per ml were seeded in 96 – well cell culture plates and incubated for 12 h for proper attachment. Thereafter, various treatments, viz., photoproduct (PP:MC-Cou-OH), Caged compound (SP-Cou-Cbl) and Cbl at different concentrations (1.25, 2.5, 5, 10, and 20 µg/ mL) were added in triplicates and appropriate wells were irradiated by visible light (≥ 410 nm) for 30 min. The plate was then incubated for 72 hours at 37 ˚C. MTT was then added to the wells at 4 mg/ml and absorbance data recorded at 595 nm using a microplate spectrophotometer, MultiSkan Go (Thermo Fisher Scientific, USA).

The cell viability tests of the before and after photolysis are shown in **Figure 5(a)** and **Figure 5(b)** respectively. The cytotoxicity of PP and SP-Cou-Cbl was significantly lower than Chlorambucil at any given concentration, for non-irradiated samples. However, for irradiated samples, SP-Cou-Cbl showed a higher cytotoxicity than free chlorambucil (**Figure 5b**). This may be due to the efficient release of the anticancer drug chlorambucil inside the cancer cell. On the other hand, cells incubated with PP only, post irradiation, showed insignificant cell death, indicating that the photoreleased anticancer drug Chlorambucil is solely responsible for the cytotoxicity. IC50 of SP-Cou-Cbl was obtained close to 10 µg/ml. To further confirm the results, 3×10^5 MDA-MB-231 cells per well of a 6-well plate were incubated with 20 µg/ ml of SP-Cou-Cbl for 4 hours and irradiated by visible light (≥ 410 nm) for varying lengths of time (5, 10, 20 and 30 min). The plate was then incubated for 24 hours at 37 ◦C in a humidified cell culture incubator with an atmosphere of 5% CO_2. Confocal microscopy images (**Figure 6**), revealed that cells incubated with SP-Cou-Cbl showed a decrease in cell viability (as indicated by increased round floating cell population) with an increase in the irradiation time (0–30 min), confirming the controlled drug release. The higher cytotoxicity of SP-Cou-Cbl treated cells compared to free chlorambucil treated cells, after UV light irradiation suggests that SP-Cou-Cbl can effectively enhance the efficiency of the intracellular delivery of the drug chlorambucil and be an excellent drug delivery system.

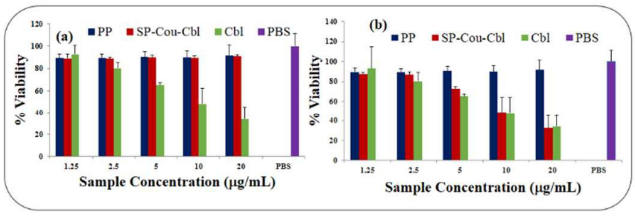

Figure 5: The cell viability studies of SP-Cou–Cbl, MC-Cou-OH and Cbl (0.1–10 mg mL-1) was depicted (a) before photolysis; and (b) after photolysis.

Cell cycle analysis

The mode of action of SP-Cou-Cbl in MDA-MB-231 cells was investigated through flow cytometry on a BD FACS Aria flow cytometer. Prior to the experiment, 3×10^5 MDA-MB-231 cells were seeded in each well of a 6-well plate and after proper attachment, they were synchronized by incubation in incomplete DMEM media for 24 hours. Cells were then treated or untreated with 20 µg/ml SP-Cou-Cbl and after 4 h of incubation, irradiated with visible light (≥ 410 nm) for 30 min. After 24 h of irradiation, treated and control cells were analysed by flow cytometry. As shown in **Figure 7a**, 8.6% sub-G0/G1, 57.3% G0/G1, 12.3% S and 21.2% G2/M phase cells were found in the non-irradiated drug treated control whereas, the visible light irradiated drug treated cells (**Figure 7b**) had 12.1% sub-G0/G1, 32.4% G0/G1, 16.3% S, and 38.2% G2/M phase cells after 24 h of incubation. The effect of the photo released drug, chlorambucil was prominent in the SP-Cou-Cbl treated samples where there was a significant decrease in G0/G1 phase and corresponding increase in G2/M population confirming arrest in G2/M phase. A concomitant rise in the sub G0/G1 cell population is indicative of apoptotic cell death, which was clearly observed in the visible light irradiated SP-Cou-Cbl treated cells (**Figure 7b**). In conclusion, SP-Cou-Cbl upon irradiation releases the drug of interest, which induces an arrest in the G2/M phase of the cell cycle with a simultaneous increase in the sub-G0/G1phase of the cells and a decrease in the G0/G1 phase of the cells.

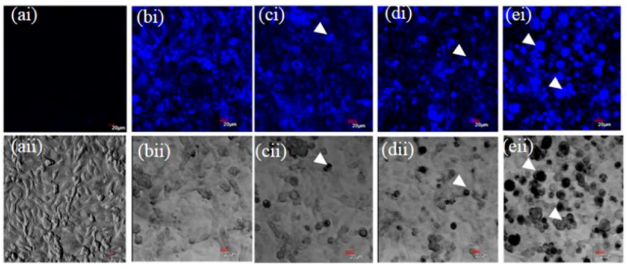

Figure 6: Anticancer activity study of SP-Cou-Cbl in MDA-MB-231 by confocal microscopy: Top panel shows fluorescence images and bottom panel shows bright field images. (a) Cells without irradiation; Cells with visible light (≥ 410 nm) irradiation Cells after (b) 5 min (c) 10 min, (d) 20 min and (e) 30 min. (scale bar = 20 µm). White arrows indicate dead cells. (i) Fluorescent field; (ii) Bright field.

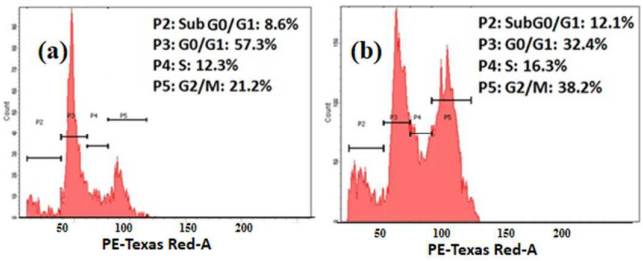

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Figure 7: Cell cycle analysis: Flow cytometry for cell cycle analysis of the (A) control cells after 24 h and (B) cells + 20 µg/mL SP-Cou-Cbl, irradiated for 30 min and incubated for 24 h.

Conclusion:

Here, we have demonstrated for the first time a spiropyran locked coumarin system for efficient photoregulated drug delivery. It can locate tumor by the appearance of blue fluorescent color due to the unlocking of the coumarin ring by the acidic nature of tumor cell. Drug can be released in a dose controlled manner by the use of externally regulated light source. High fluorescent nature and promising biocompatibility make SP-Cou-Cbl system suitable for cell imaging and MDA-MB-231 cells was used for in vitro studies.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [General experimental procedure, Characterization of compounds **2, 3, 4 and 5**, the physical characteristics of compound **5**, HPLC chromatogram of photolysis and ring opening of compound 5.

Reference:

1. W. Arap, R. Pasqualini and E. Ruoslahti, *SCIENCE*, 1998, **278**,377.
2. N. Rapoport, *Prog. Polym. Sci.,* 2007, **32**, 962.
3. F. Danhier, O. Feron and V. Préat, *Journal of Controlled Release*, 2010, **148**, 135.
4. B. Sahoo, K. S. P. Devi, R. Banerjee, T. K. Maiti, P. Pramanik and D. Dhara, *ACS Appl. Mater. Interfaces*, 2013, **5**, 3884.
5. L. Wang, G. Liu, X. Wang, J. Hu, G. Zhang and S. Liu, *Macromolecules*, 48, **2015**, 7262.
6. H. Yang, Q. Wang, S. Huang, A. Xiao, F. Li, L. Gan and X. Yang, *ACS Appl. Mater. Interfaces*, 2016, **8**, 7729.
7. S. Chen, F. Jiang, Z. Cao, G. Wang and Z. M. Dang, *Chem. Commun.,* 2015, **51**, 12633-12636.
8. L. Meng, W. Huang,D. Wang, X. Huang, X. Zhu and D. Yan, *Biomacromolecules*, 2013, **14**, 2601.
9. S. Karthik, B. N. P. Kumar, M. Gangopadhyay, M. Mandal and N. D. P. Singh, *J. Mater. Chem. B*, 2016, **4**, 521-528.
10. M. Gangopadhyay, S. K. Mukhopadhyay, S. Gayathri, S. Biswas, S. Barman, S. Dey and N. D. P. Singh, *J. Mater. Chem. B,* 2016, **4**, 1862-1868.
11. S. Barman, S. K. Mukhopadhyay, M. Gangopadhyay, S. Biswas, S. Dey and N. D. P. Singh, *J. Mater. Chem. B*, 2015, 3, 3490-3497.
12. J. T. C. Wojtyk, A. Wasey, N. N. Xiao, P. M. Kazmaier, S. Hoz, C. Yu, R. P. Lemieux and E. Buncel, *J. Phys. Chem. A*, 2007, **111**, 2511-2516.
13. S. R. Keum and M. J. Lee, *Bull. Korean Chem. Soc.,* 1999, **20**, 1465.
14. Dr. E. Verploegen, Dr. J. Soulages, M. Kozberg, T. Zhang, G. McKinley and P. Hammond, *Angew. Chemie Inter. Edi.*, 2009, 48, 3494-3498.
15. M. Moniruzzaman, C. Sabey and G. Fernando, Polymer, 2007, **48**, 255-263.
16. M. Alonso, V. Reboto, L. Guiscardo, A. S. Martin and J. R. Cabello, *Macromolecules*, 2000, **26**, 9480-9482.
17. S. C. Wang and C. T. Lee, *The Journal of Physical Chemistry B*, 2006, **32**, 16117-16123.
18. D.Roy, J. N. Cambre and B. S. Sumerlin, *Progress in Polymer Science*, 2010, **1**, 278-301.
19. S. Son, E. Shin and B. S. Kim, *Biomacromolecules*, 2014, **15**, 628−634.
20. S. Karthik, N. Puvvada, B. N. P. Kumar, S. Rajput, A. Pathak, M. Mandal and N. D. P. Singh, *ACS Appl. Mater. Interfaces*, 2013, **5**, 5232−5238.
21. S. Biswas, M. Gangopadhyay, S. Barman, J. Sarkar, N. D. P. Singh, *Sensors and Actuators B: Chemical*, 2015, **222**, 823-828.
22. S. Atta, M. Ikbal, A. Kumar, N.D. P. Singh, *J. of Photochem. Photobio. B*, 2012, **111**, 39–49.
23. J. T. C. Wojtyk , A. Wasey , N. N. Xiao , P. M. Kazmaier , S. Hoz , C. Yu, R. P. Lemieux and E. Buncel, *J. Phys. Chem. A*, 2007, **111**, 2511–2516.

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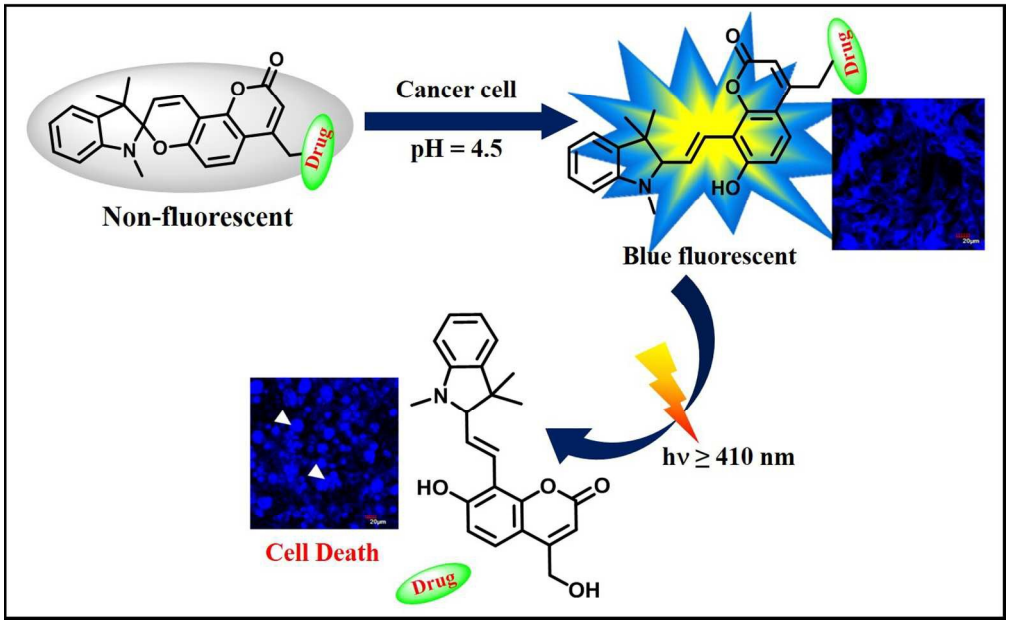

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