Coumarin containing star shaped 4-arm Polyethylene glycol (PEG): Targeted Fluorescent Organic Nanoparticles for efficient Dual treatment of Photodynamic therapy (PDT) and Chemotherapy

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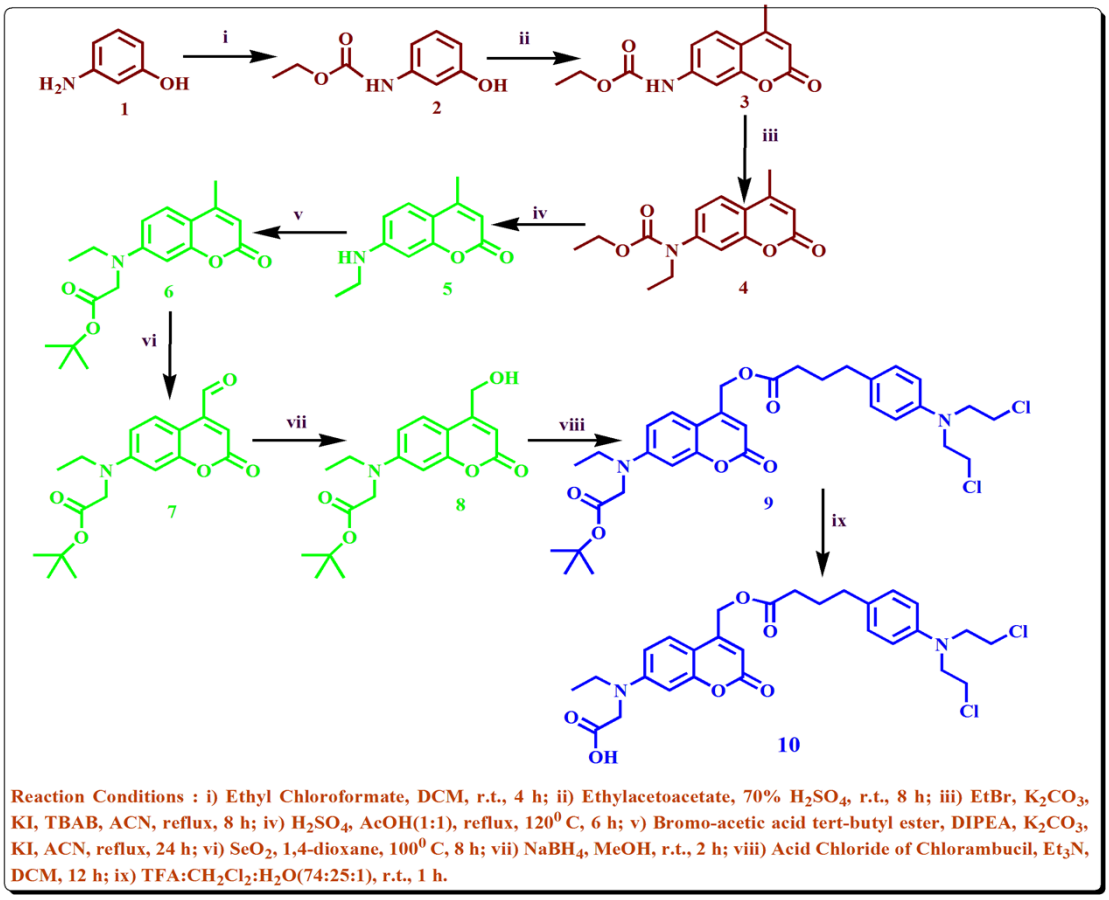
Supporting Information

1. General information

Materials: All reagents were purchased from Sigma Aldrich and used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2 before use. ^1H NMR spectra were recorded on a BRUKER-AC 200 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). ^{13}C NMR (50 MHz) spectra were recorded on a BRUKER-AC 200 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 77.0 ppm). UV/vis absorption spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer, fluorescence emission spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer, FT-IR spectra were recorded on a Perkin Elmer RXI spectrometer and HRMS spectra were recorded on a JEOL-AccuTOF JMS-T100L mass spectrometer. The morphology of NPs was measured through field emission scanning electron microscopy (SEM) with energy-dispersive X-ray (EDX) analysis (Hitachi S-4800) and transmission electron microscopy (TEM, JEOL, JEM-1200EX). Transmission Electron Microscopy (TEM) was measured on a FEI Tecnai G220S-Twin at 200 kV. The TEM sample was prepared by dispersing compounds in acetone and dropping on the surface of a copper grid. Photolysis of all the ester conjugates were carried out using 125 W medium pressure Hg lamp supplied by SAIC (India). Chromatographic purification was done with 60-120 mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) were used. RP-HPLC was taken using mobile phase acetonitrile, at a flow rate of 1mL / min (detection: UV 254 nm). Cell culture media and all the other materials required for culturing were obtained from Gibco, USA.

2. Synthesis and characterization of Coumarin-Chlorambucil conjugate (Cou-Cbl) (10)

Cou-Cbl **(10)** was synthesized following previous reports starting from *m*-Aminophenol (**1**) as shown in **scheme S1.** Brief synthetic procedures have been discussed herein.



Scheme S1: Synthesis of Cou-Cbl (**10**)

2.1 a) Synthesis of 7--[N-ethyl,N-carbethoxy]-4-methylcoumarin (4)

As presented in **scheme S1**, 7-carbethoxyamino-4-methylcoumarin (**3**) was initially synthesized from m-aminophenol and ethylacetoacetate following classical Pechmann condensation.[1] To a solution of 1g of 7-carbethoxyamino-4-methylcoumarin (1g, 2.379 mmol) in dry acetonitrile, 1.5 eqv of K_2CO_3 (0.492g, 3.56 mmol), 0.5 eqv of TBAB (0.383g, 1.1895 mmol) was added. The mixture was stirred for 15 min at 80ºC.After 15 min 0.5 eqv of KI (0.197g, 1.1895 mmol) and 20 eqv of ethyl bromide (3.57 mL, 47.58 mmol) were added to the reaction mixture and refluxed for 8h.[2] After the reaction was completed the reaction

mixture was cooled to room temperature and filtered. The filtrate was then concentrated under reduced pressure and the residue was washed with ethyl acetate and brine water. The organic layer was collected, dried over sodium sulphate and evaporated to give the product **4** in 90% yield. FTIR (KBr, cm^{-1}): 1790, 2375, 3200; UV-vis (EtOH): λ_{max} (log ε): 363 (1.29); ^1H NMR ($CDCl_3$, 200 MHz): δ = 7.607-7.561 (d, J= 9.2 Hz, 1H), 7.254-7.219 (d, J=8.8 Hz , 1H), 7.109 (s, 1H), 6.266 (s, 1H), 4.263-4.157 (q, J= 14.2 Hz 2H), 3.824-3.753 (q, J = 14.2 Hz, 2H), 2.444 (s, 3H), 1.301-1.246 (t, J = 11 Hz, 3H), 1.231-1.175 (t, J = 11.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 162.3, 155.3, 153.0, 151.2, 137.8, 126.8, 123.4, 117.1, 113.2, 109.7, 57.2, 44.2, 24.6, 13.3, 12.7. For $C_{15}H_{17}NO_4$ [MH+] = 277.1158, found 277.1156.

b) Synthesis of 7-ethylamino-4-methylcoumarin (5)[2]

Compound **4** was suspended in 1:1 mixture of conc. H_2SO_4 and glacial acetic acid. The mixture was refluxed at 120ºC for 6 h to give an orange solution. After completion of the reaction the reaction mixture was cooled to room temperature and poured into ice cold water and kept overnight for complete precipitation of an off-white solid. The suspension was brought to pH~7 with 50% NaOH solution.The precipitate was then filtered under suction to obtain the desired product **5** as a greenish powder in ~95% yield. The ^1H and ^{13}C NMRs matched with the literature.[2]

c) Synthesis of 7-[N-ethyl,N-*tert*-Butoxycarbonylmethyl]-4-methylcoumarin (6)[3]

7-ethylamino-4-methylcoumarin (**5**) (0.350 g, 1.72 mmol), bromoacetic acid tert-butyl ester (1.78 mL, 12.06 mmol), diisopropylethylamine (1.13 mL, 6.88 mmol), and KI (0.285 g, 1.72 mmol) in 20 mL CH_3CN were refluxed for 24 h. The mixture was cooled to room temperature, filtered, and the solvent evaporated in vacuo. The residue was dissolved in 250 mL EtOAc, washed with 350 mL brine, dried (Na_2SO_4), and concentrated in vacuo to afford a brown oil. The oil was separated and purified via flash chromatography (SiO_2, 15-45% EtOAc in hexane) to afford **6**(0.113 g, 32.28%). Brown liquid,; TLC R_f 0.2 (15% EtOAc in

pet ether); FTIR (KBr, cm^{-1}): 1715, 1619, 3200; UV-vis (EtOH): λ_{max} (log ε): 364 (1.39); ^1H NMR ($CDCl_3$, 200 MHz): δ = 7.424-7.380 (d, J= 8.8 Hz, 1H), 6.582-6.538 (d, J=8.8 Hz , 1H), 5.99 (s, 1H), 3.970 (s, 2H), 3.560-3.453 (q, J = 7.1 Hz, 2H), 2.341 (s, 3H), 1.456 (s, 9H), 1.280-1.210 (t, J= 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 173.6, 162.3, 155.3, 151.2, 145.3, 132.15, 129.3, 126.3, 114.3, 113.7, 109.7, 62.6, 53.6, 40.6, 33.9, 33.7, 26.8; For $C_{15}H_{17}NO_4$ [MH+] = 318.1627, found 318.1629.

d) Synthesis of 7-[N-ethyl,N-*tert*-Butoxycarbonylmethyl]-4-formylcoumarin (7)[3]

6 (0.429 g, 1.35 mmol) was dissolved in 10 mL dioxane by heating, selenium dioxide (0.300 g, 2.705 mmol) was added, and the mixture was refluxed for 6 h with stirring. The mixture was filtered hot to remove black selenium, and the filtrate was concentrated under reduced pressure. The resulting precipitate gave 0.268 g (62.47 %) of the aldehyde (**7**) as orange-red crystals. FTIR (KBr, cm^{-1}): 1360, 1500, 3000; UV-vis (EtOH): λ_{max} (log ε): 364 (1.39); ^1H NMR ($CDCl_3$, 200 MHz): δ = 10.037 (s, 1H), 8.360-8.314 (d, J= 9.2 Hz, 1H), 6.605-6.559 (d, J=9.2 Hz , 1H), 6.532 (s, 1H), 3.981 (s, 2H), 3.567-3.461 (q, J = 7 Hz, 2H), 1.454 (s, 9H), 1.284-1.213 (t, J= 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 192.25, 168.84, 161.59, 156.90,151.3, 130.09, 129.08, 126.98, 126.3, 118.44, 109.63, 98.18, 82.35, 52.87, 46.69, 29.59, 27.94, 21.63, 12.18; For $C_{18}H_{21}NO_5$ [MH+] = 332.1420, found 332.1421.

e) Synthesis of 7-[N-ethyl,N-*tert*-Butoxycarbonylmethyl]-4-(hydroxymethyl)-coumarin (8)[3]

$NaBH_4$ (0.270 g, 0.815 mmol) was added to **7** (0.031 g, 0.815 mmol) in 40 mL methanol, and the mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with H_2O (40 mL), acidified (pH 5) with 0.1 N HCl and extracted three times with CH_2Cl_2 (30 mL each). Theorganic phase was washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo.The crude product was purified by flash chromatography (SiO_2, 30-50% EtOAc in hexane),and 0.110 g (40 %) of the hemihydrate of **8** was obtained as orange

solid. FTIR (KBr, cm^{-1}): 1360, 1650, 2800; UV-vis (EtOH): λ_{max} (log ε): 365 (1.5); ^1H NMR ($CDCl_3$, 200 MHz): δ = 7.456 (s, 1H), 7.339-7.296 (d, J= 8.6 Hz, 1H), 6.547-6.494 (d, J=10.6 Hz , 1H), 6.407-6.372 (d, J=7 Hz, 1H), 6.297 (s, 1H), 4.815 (s, 2H), 3.962 (s, 2H), 3.551-3.441 (q, J = 7.2 Hz, 2H), 1.457 (s, 9H), 1.284-1.213 (t, J= 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 169.47, 162.78, 155.45,150.67, 124.17, 108.75, 107.33, 105.69, 98.09, 82.33, 60.29, 52.77, 46.49, 29.56, 27.92, 12.18; For $C_{18}H_{21}NO_5$ $[MH+]_=$ 334.1576, found 334.1577.

f) Synthesis of 7-[N-ethyl, N-*tert*-Butoxycarbonylmethyl]-4-(hydroxymethyl)-coumarin-Chlorambucil caged ester (9)[3]

Chlorambucil (0.100g, 0.33 mmol) was dissolved in 1 mL oxalyl chloride and was stirred for 1 h at 60 °C. Then oxalyl chloride was removed under vacuum to afford the acid chloride of chlorambucil as brown oil. Then the acid chloride was dissolved in dry DCM (5 mL) and the esterification reaction was carried out without further purification. To the solution of the acid chloride (0.105 g, 0.33 mmol) in CH_2Cl_2 (5 mL) 7-[N-ethyl, N-tert-Butoxycarbonylmethyl]-4-(hydroxymethyl)-coumarin (0.110 g, 0.33 mmol) was added followed by triethylamine (71 μL, 0.51 mmol). The mixture was stirred at room temperature for 12 h, and then the solvent was removed under reduced pressure. The crude reaction mixture was purified by column chromatography using 40% EtOAc in pet ether to give the compound **9** (0.080 mg, 80%). FTIR (KBr, cm^{-1}): 1350, 1500, 3088; UV-vis (EtOH): λ_{max} (log ε): 365 (1.4); ^1H NMR ($CDCl_3$, 200 MHz): δ =7.323-7.279 (d, J= 8.8 Hz, 1H), 7.260-7.178 (d, J=6.4 Hz, 2H), 6.710-6.667 (d, J= 8.6 Hz, 2H), 6.627-6.575 (d, J=10.4 Hz, 1H), 6.563-6.518 (d, J=9 Hz , 1H), 6.164 (s, 1H), 5.205 (s, 2H), 3.971 (s, 2H), 3.750-3.675 (m, 10H), 2.628-2.554 (t, J=7.4 Hz, 2H), 2.448-2.366 (t, J=7.2 Hz, 2H), 2.044-1.881 (m, 2H), 1.459 (s, 9H), 1.284-1.213 (t, J= 7.7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 169.47, 162.78, 155.45,150.67, 124.17, 108.75, 107.33, 105.69, 98.09, 82.33, 60.29, 52.77, 46.49, 29.56, 27.92, 12.18; For $C_{18}H_{23}NO_5$ $[MH+]_=$ 619.2263, found 619.2265.

g) Synthesis of 7-[N-ethyl,N-carboxymethyl]-4-(hydroxymethyl)-coumarin-Chlorambucil caged ester (10)[4]

9 (0.100 g, 0.167 mmol) was stirred in a mixture (4 mL) of TFA/CH_2Cl_2/ H_2O (74:25:1) at room temperature for 1h. The solvents were evaporated, and the residue was coevaporated two times with ether to give 0.090 (90%) of the pure active ester **10**. Compound **10** was used directly for the attachment with 4-arm PEG without any further purification. FTIR (KBr, cm^{-1}): 1721, 3449; UV-vis (EtOH): λ_{max} (log ε): 365 (1.4); ^1H NMR ($CDCl_3$, 200 MHz): δ =7.353-7.308 (d, J= 9 Hz, 1H), 7.154-7.099 (d, J = 11 Hz, 6H), 6.703-6.638 (d, J= 13 Hz, 6H), 6.594-6.536 (d, J=11.6 Hz, 1H), 6.188 (s, 1H), 5.298 (s, 2H), 5.203 (s, 2H), 4.134-4.105 (q, 2H), 3.726-3.594 (m, 10H), 2.622-2.547 (t, J=7.4 Hz, 2H), 2.447-2.368 (t, J=7.2 Hz, 2H), 2.047-1.879 (m, 2H), 1.284-1.213 (t, J= 7.7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 174.32, 172.30, 160.58, 144.49, 129.68, 129.29, 124.33, 111.90, 105.67, 99.52, 79.16, 61.11, 52.21, 48.57, 33.32, 33.05, 32.70, 31.13, 29.80, 28.97, 26.56, 13; For $C_{18}H_{23}NO_5$ $[MH^+]$ = 562.1637, found 562.1636.

2.2 Characterization of Cou-Cbl (10)

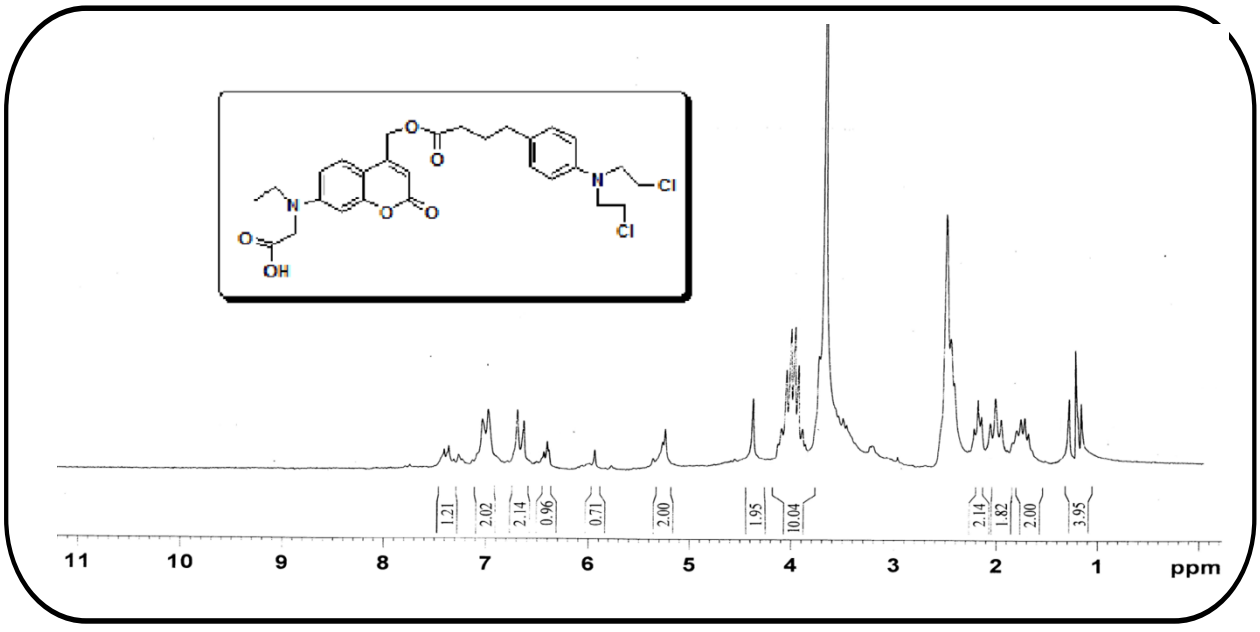


Figure S1: ^1H NMR spectrum of Coumarin-chlorambucil conjugates (Cou-Cbl)

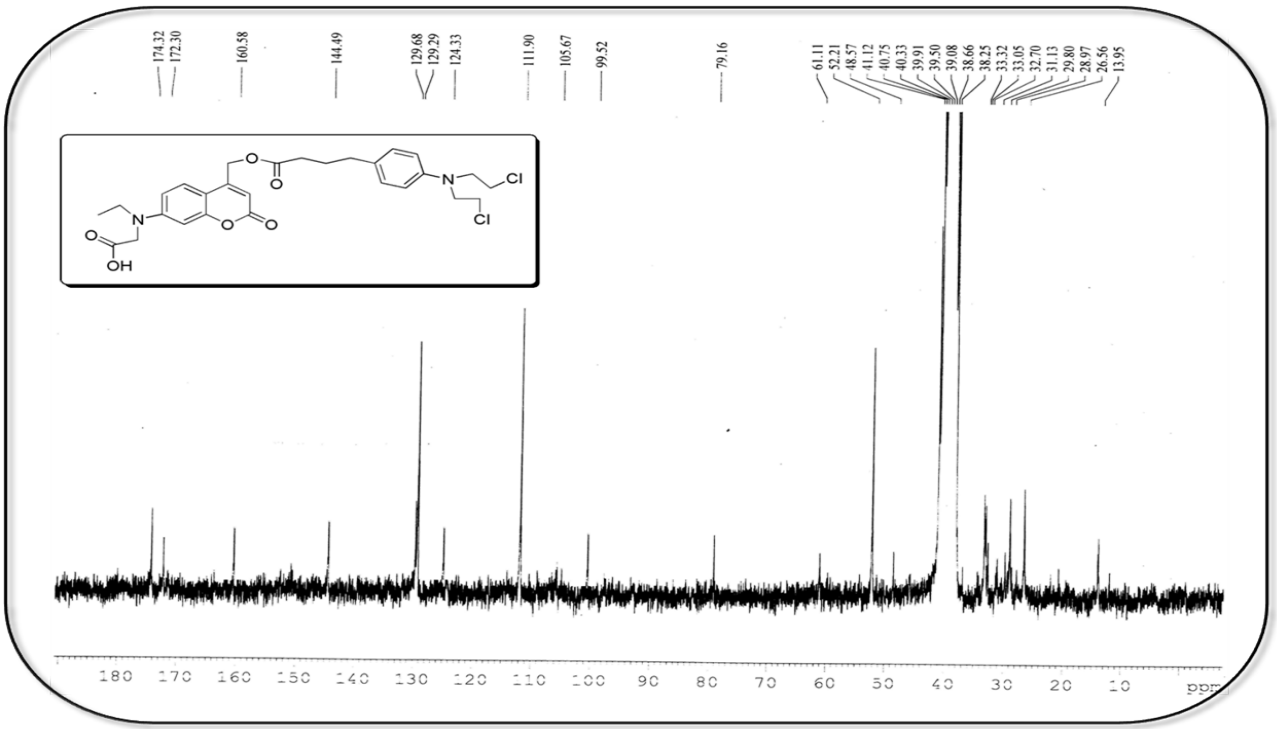
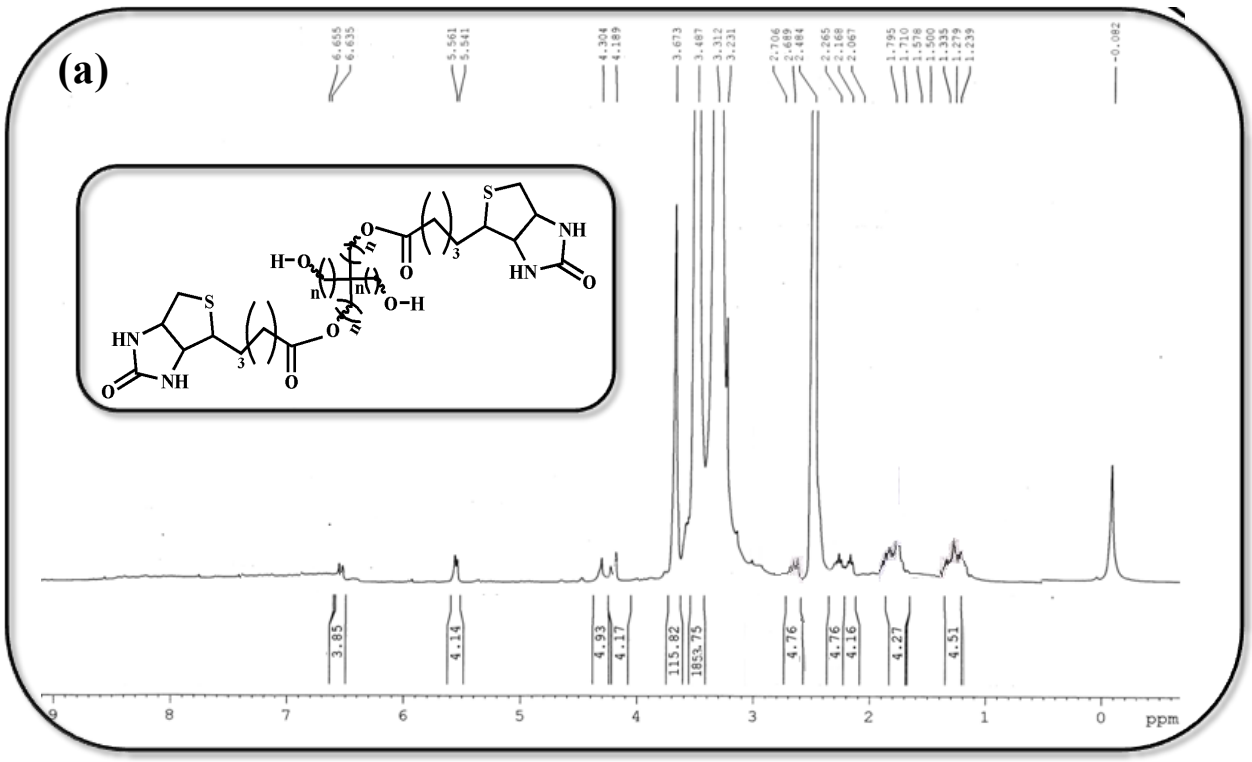


Figure S2: ^{13}C NMR spectrum of Coumarin-chlorambucil conjugates (Cou-Cbl)

3. Characterization of PEG-Bio and PEG-Bio-Cou-Cbl

a) ^{1}H NMR spectra



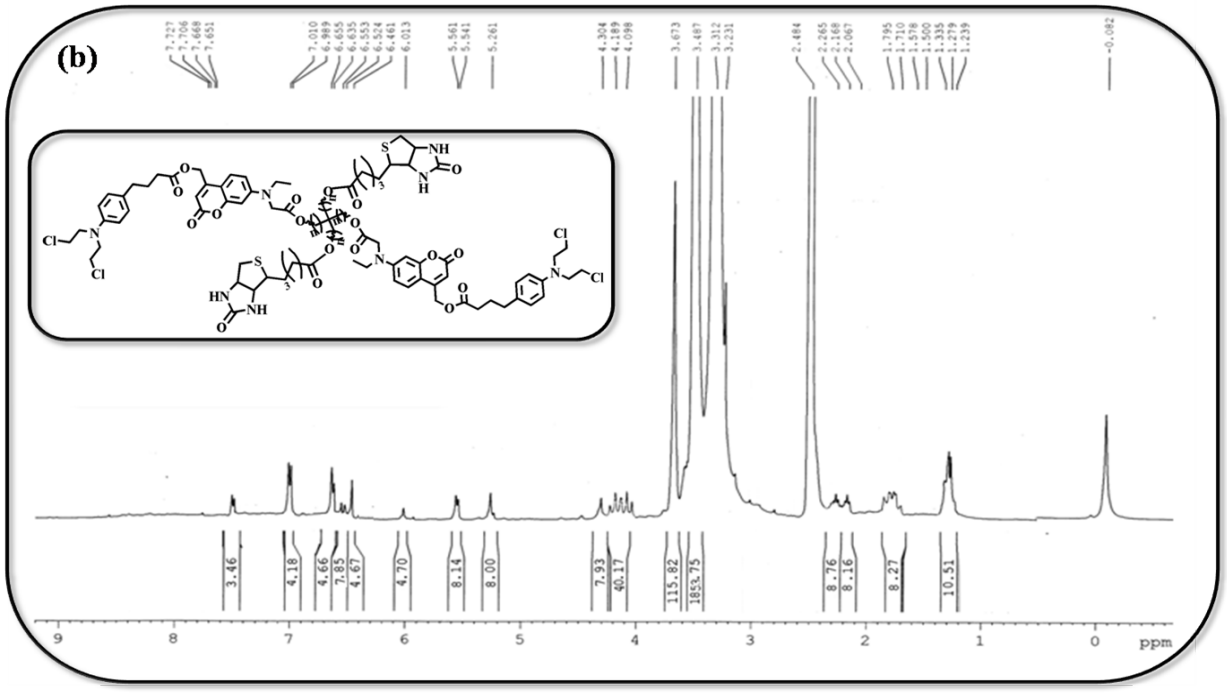
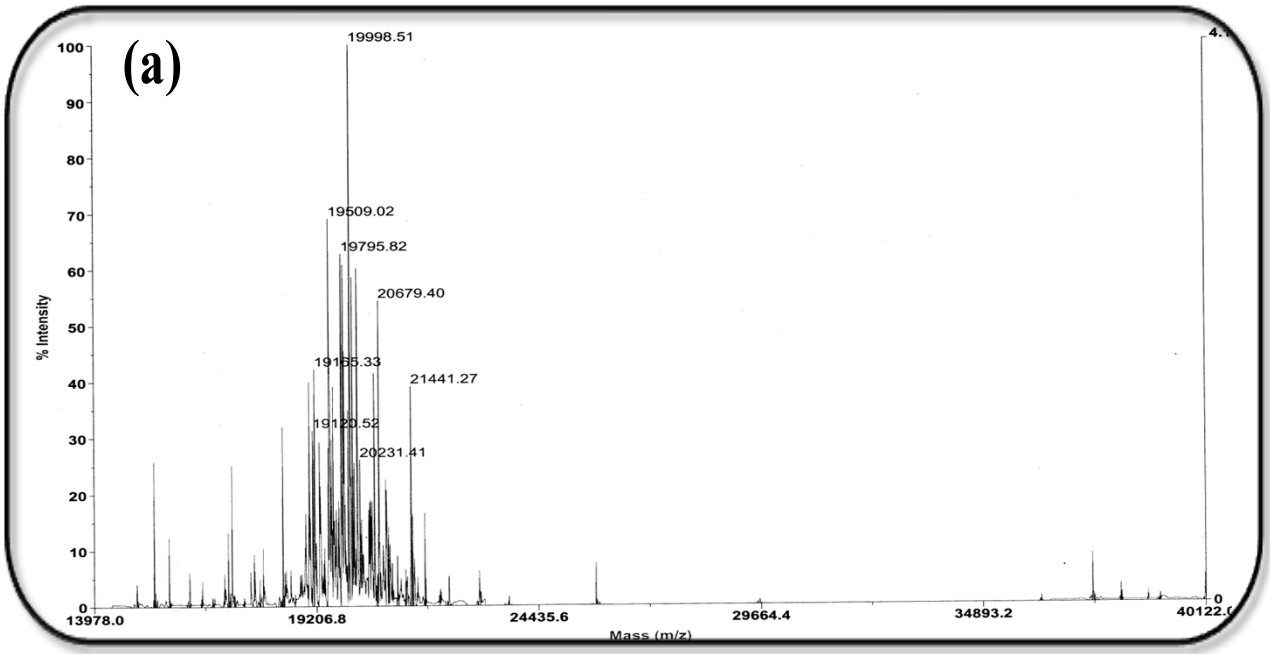


Figure S3: ^{1}H NMR spectra of **(a)** PEG-Bio and **(b)** PEG-Bio-Cou-Cbl

b) MALDI-TOF analysis



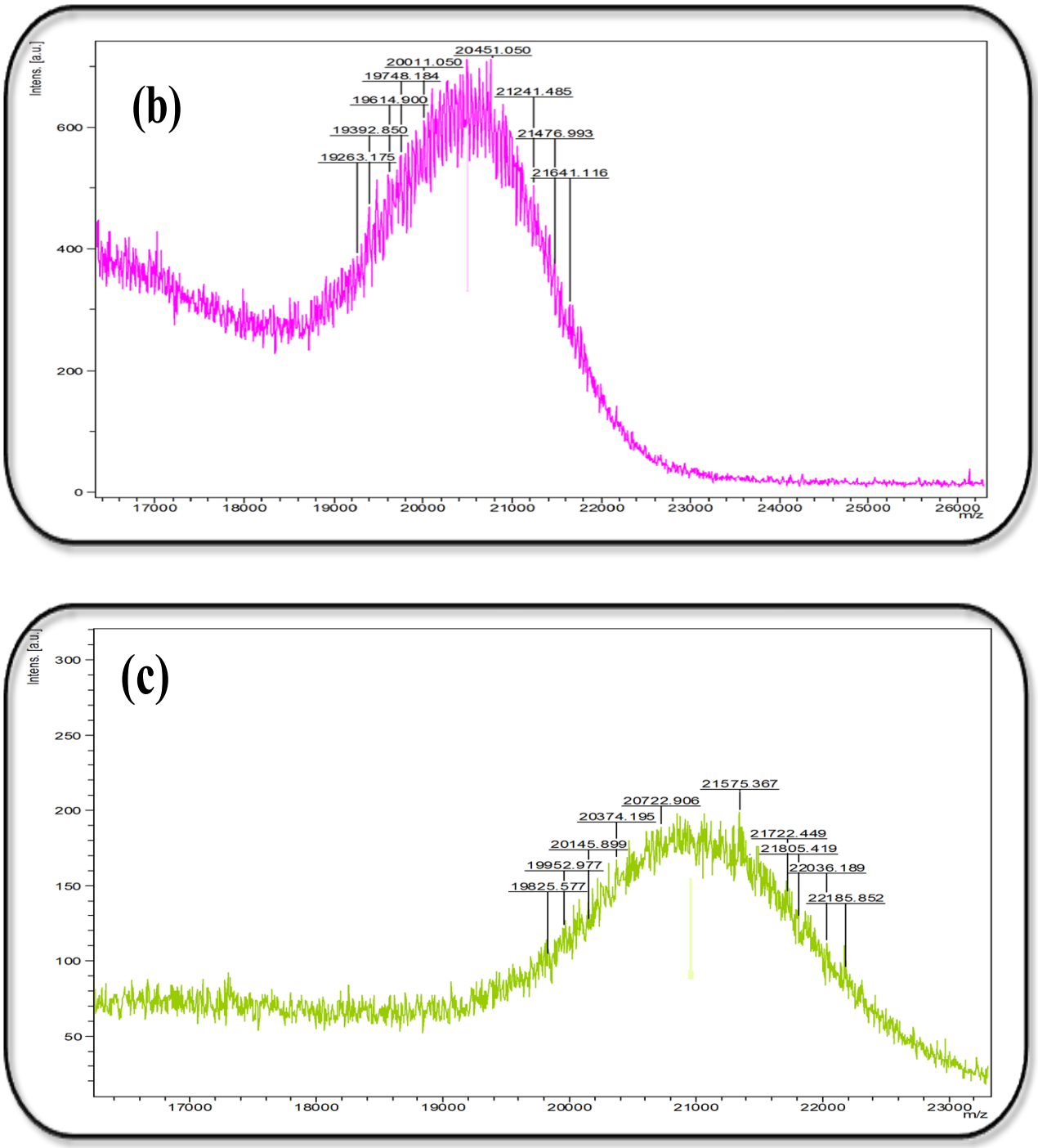


Figure S4 : MALDI-TOF spectra of **(a)** 4-arm PEG (MW= 19,998.51), **(b)** PEG-Bio (MW= 20,451.050), **(c)** PEG-Bio-Cou-Cbl (MW= 21,575.367)

c) Thermogravimetric and GPC analysis

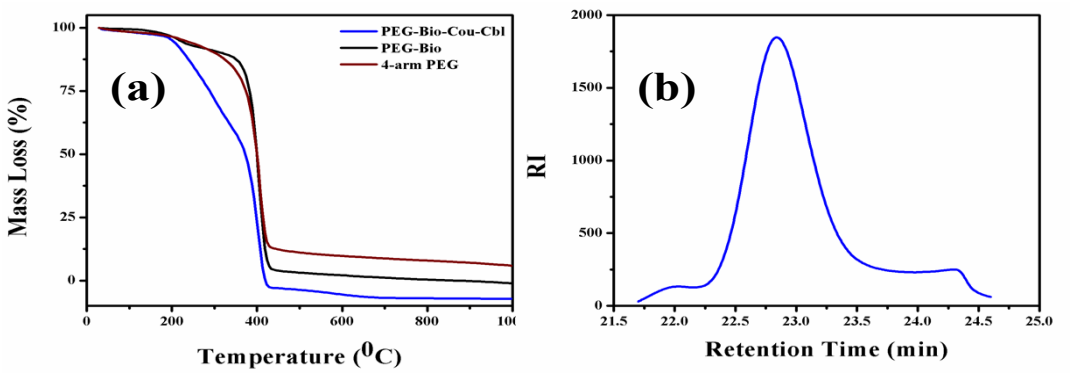


Figure S5 : (a) TGA curves for PEG-Bio-Cou-Cbl, PEG-Bio and 4-arm PEG under N_2 environment; (b) GPC data for PEG-Bio-Cou-Cbl (M_n = 22150, PDI = 1.105)

d) CMC calculation for PEG-Bio-Cou-Cbl NPs

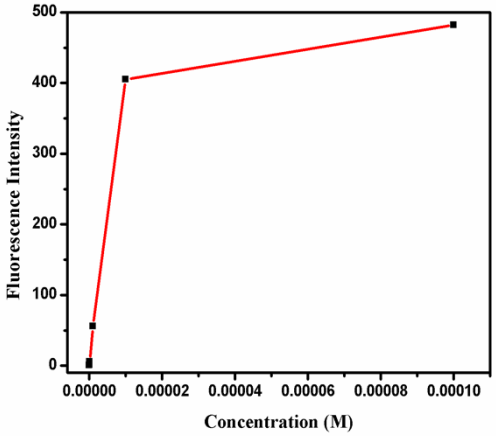


Figure S6 : The CMC of PEG-Bio-Cou-Cbl NPs was found to be 1.013 x 10^{-5} (M) from the fluorescence curves of PEG-Bio-Cou-Cbl solutions of different standard concentrations.

4. 1. Photophysical studies of the NPs

(i) Measurement of fluorescence quantum yields[5]

The quantum yield of PEG-Bio-Cou-Cbl were determined by reference point method.[5] Quinine sulfate in 0.1 M H_2SO_4 (literature quantum yield: 0.54) was used as a standard sample to calculate the QY of PEG-Bio-Cou-Cbl, which were dissolved in ultra pure water. The absorbance values of the solutions at the excitation wavelength were measured with UV–Vis spectrophotometer. Photoluminescence (PL) emission spectra of all the sample solutions were recorded by Hitachi F-7000 fluorescence spectrophotometer at an excitation wavelength of 360 nm.

$$\frac{\phi_S}{\phi_R} = \frac{A_S}{A_R}\frac{(Abs)_R}{(Abs)_S}\frac{\eta_S^2}{\eta_R^2}$$

Where **Φ** represents quantum yield, **Abs** represents absorbance, **A** represents area under the fluorescence curve, and **η** is refractive index of the medium. The subscripts **S** and **R** denote the corresponding parameters for the sample and reference, respectively.

Table S1: Quantum yield of the fluorescent PEG-Bio-Cou-Cbl

Sample	Intergrated emission intensity (I)	Abs. At 360 nm (A)	Refractive index of solvent (η)	Quantum yield at 360nm (Q)
Quinine sulfate	55337.365	0.0717	1.33	0.54 (known)
PEG-Bio-Cou-Cbl	3777.416	0.042	1	0.063

Fluorescence quantum yield (excitation wavelength 360 nm, error limit within ± 5%).

(ii) Photodegaradation of DPBF: Singlet oxygen generation study

We carried out the singlet oxygen generation of PEG-Bio-Cou nanoparticle in citrate buffer solution containing 10% acetonitrile. The results are attached herein. The quantum yield for PEG-Bio-Cou NPs were found to be 0.34 taking rose bengal as reference (rose bengal in ACN Φ_Δ= 0.83).

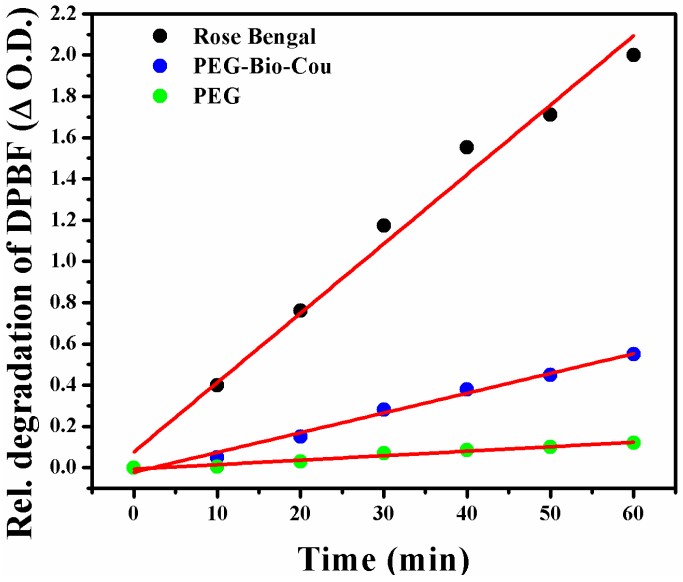


Figure S7 Photodegradation rate of DPBF in the presence of PEG, PEG-Bio-Cou NPs and rose bengal at 425 nm

(iii)Triplet State lifetime measurement[6]

A nanosecond flash photolysis set-up (Applied Photophysics) containing an Nd:YAG (Lab series, Model Lab 150, Spectra Physics) laser was used for the measurement of transient absorption spectra. The sample was excited at 355 nm (FWHM=8 ns) laser light. Transients were monitored through absorption of light from a pulsed xenon lamp (250 W). The photomultiplier (1P28) output was fed into a Tektronix oscilloscope (TDS 3054B, 500 MHz, 5 Gs s^{-1}) and the data were transferred to a computer using TEKVISA software. The software ORIGIN 7.5 was used for curve fitting. The solid curves have been obtained by connecting the points using B-spline option. The samples were de-aerated by passing pure argon gas for 20 min prior to each experiment. No degradation of the samples was observed during the experiment.

5. Synthesis and Characterization of PEG-Bio-Cou NPs (2')

5.1. Synthesis of PEG-Bio-Cou (2')

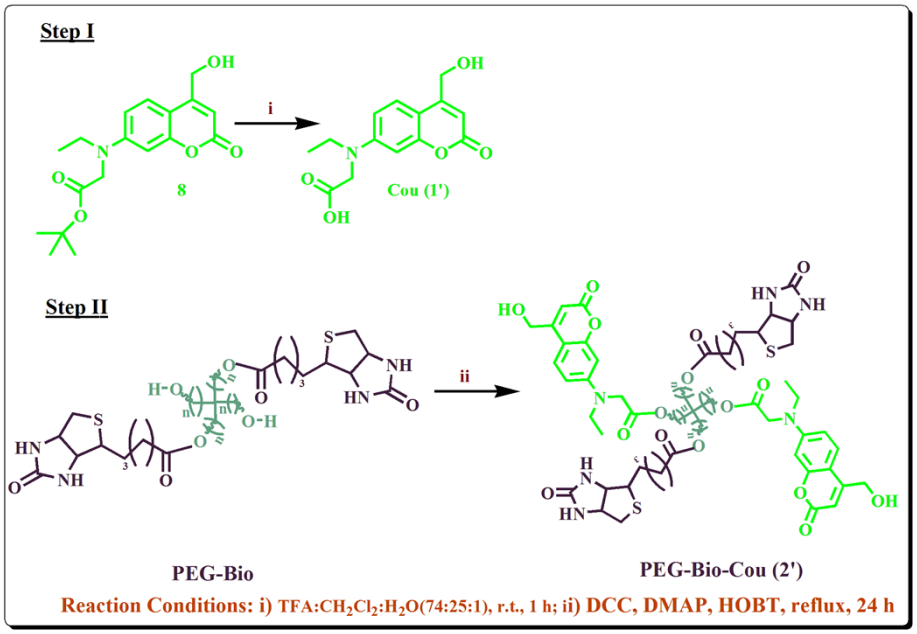
i) Synthesis of 7-[N-ethyl,N- carboxymethyl methyl]-4-(hydroxymethyl)-coumarin (Cou)

In order to synthesize PEG-Bio-Cou, 7-[N-ethyl,N- carboxymethyl methyl]-4-(hydroxymethyl)-coumarin (**Cou**) was initially prepared by deprotecting BOC protecting group in 7-[N-ethyl,N-*tert*-Butoxycarbonylmethyl]-4-(hydroxymethyl)-coumarin (**compound 8 in Scheme S1**) following the reported procedure. **8** (0.100 g, 0.167 mmol) was stirred in a mixture (4 mL) of TFA/CH_2Cl_2/ H_2O (74:25:1) at room temperature for 1h. The solvents were evaporated, and the residue was coevaporated two times with ether to give 80% of Cou.[4] Cou was used directly for the attachment with 4-arm PEG without any further purification.

ii) Attachment of Cou with PEG-Bio[7]

Cou **(1')** was successfully attached with star shaped 4-arm PEG attached with biotin following the literature procedure in 85% yield as shown in **Scheme S2.** To a CH_2Cl_2

solution of PEG-Bio (0.100 g, 0.005 mmol), 5 eqv of Cou **(1')** (0.016 g, 0.028 mmol), catalytic amount of DMAP, HOBT (0.004 g, 0.025 mmol) and DCC (0.008 g, 0.042 mmol in about 10 ml of CH_2Cl_2) was added and the mixture was refluxed for 24 h under nitrogen atmosphere. The mixture was then concentrated in vacuo and the residual syrup was dissolved in toluene (~1 ml) and filtered. The toluene was removed in vacuo at about 45°C and the residue was treated with 0.25 ml of CH_2Cl_2 and triturated with 2-propanol (1.5 ml). The resulting precipitate was collected by vacuum filtration and dried in vacuo to obtain a pure water-soluble product (0.080 g, 85 % yield). It was characterised by IR, UV/vis, fluorescence spectra and MALDI-TOF analysis.



Scheme S2 : Synthesis of PEG-Bio-Cou (**2'**)

5.2. Characterization of PEG-Bio-Cou

i) NMR spectra

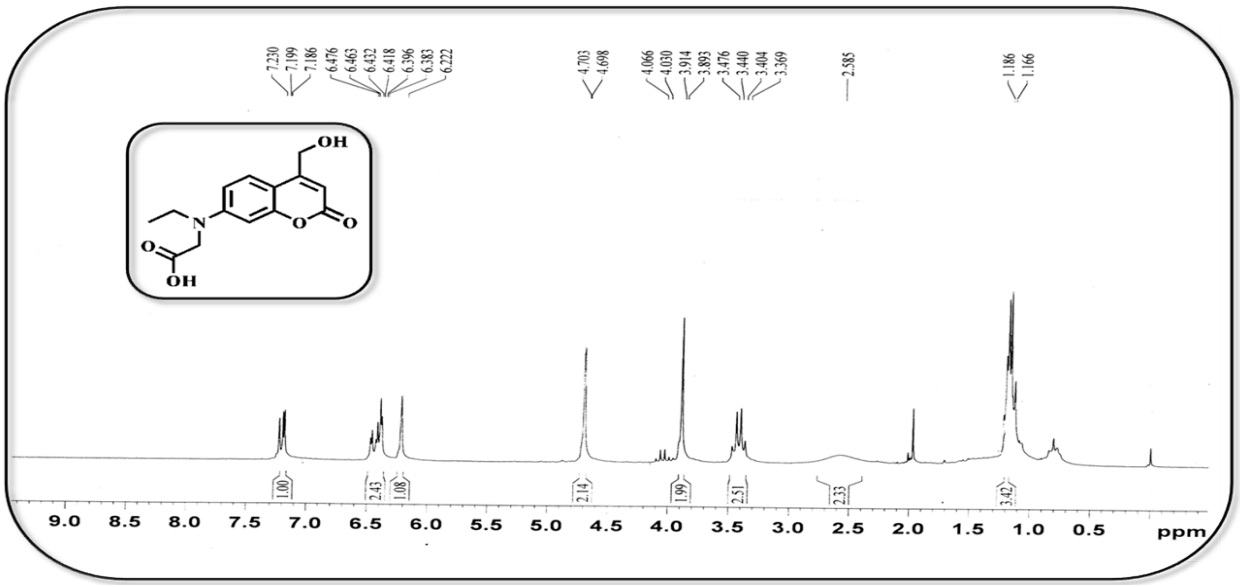


Figure S8: ^{1}H NMR spectra of 7-[N-ethyl,N- carboxymethyl methyl]-4-(hydroxymethyl)-coumarin (Cou)

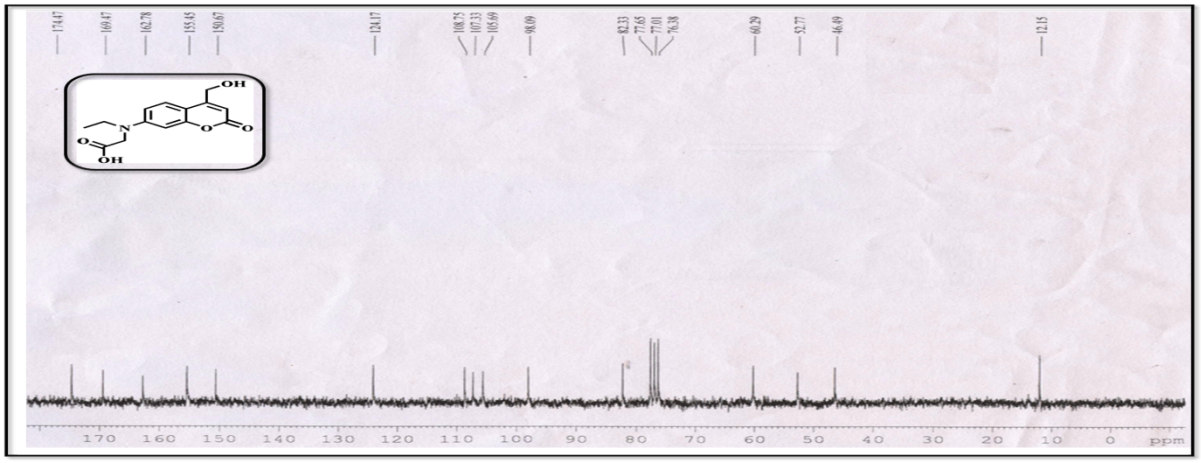


Figure S9 : ^{13}C NMR spectrum of 7-[N-ethyl,N- carboxymethyl methyl]-4-(hydroxymethyl)-coumarin (Cou)

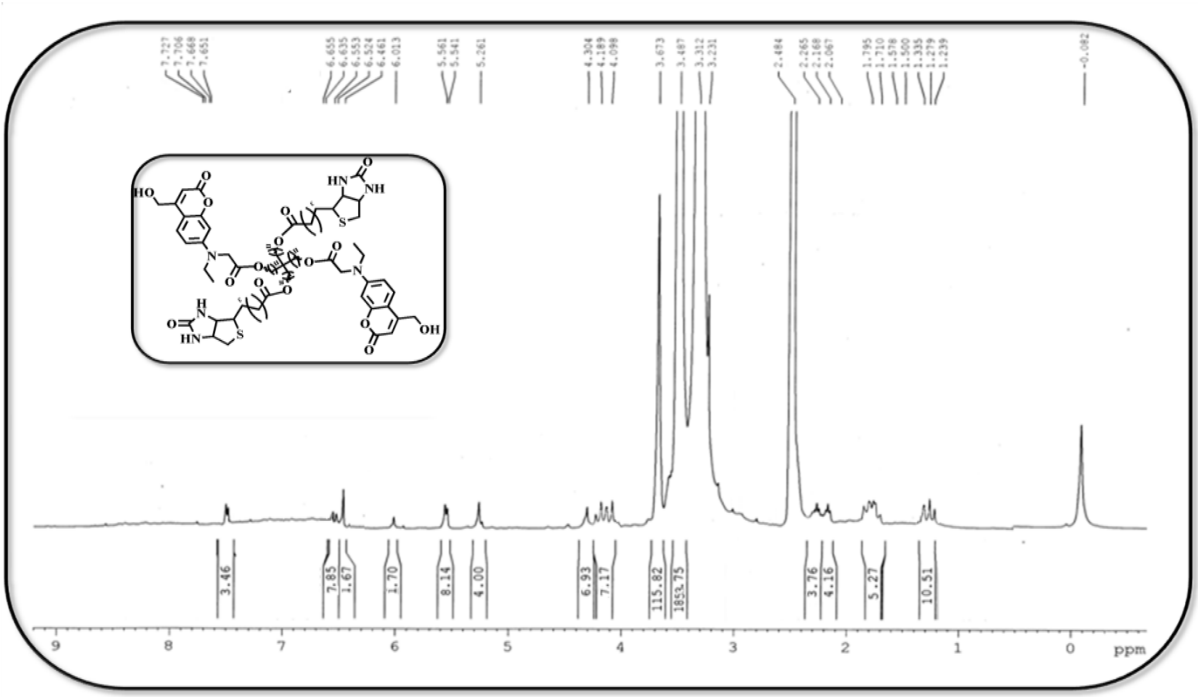


Figure S10 : ^{1}H NMR spectrum of PEG-Bio-Cou

ii) MALDI-TOF analysis

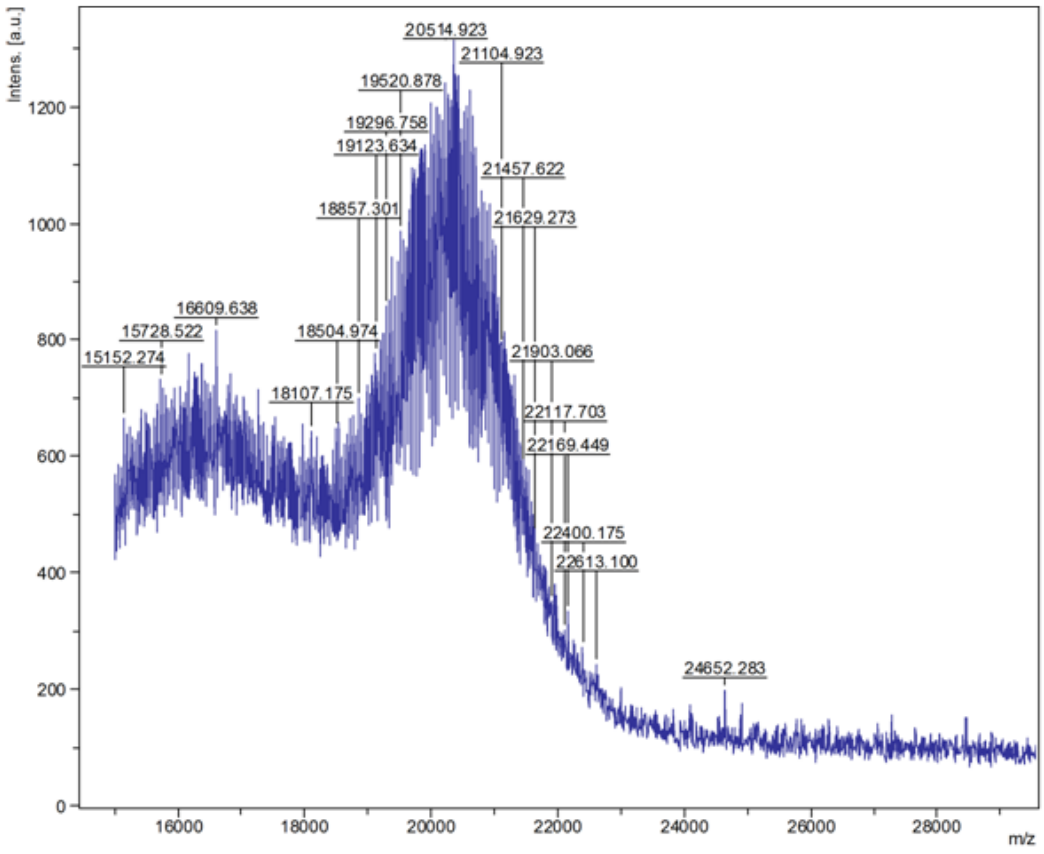


Figure S11 : MALDI spectrum of PEG-Bio-Cou (MW= 20514.73)

ii) Thermogravimetric and GPC analysis

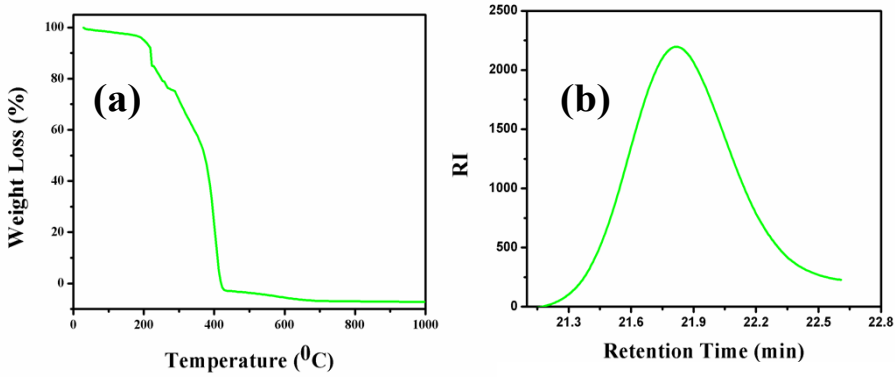


Figure S12 : (a) TGA curve under N_2 environment and (b) GPC plot for PEG-Bio-Cou (Mn = 20589; PDI = 1.02)

iii) TEM, DLS, Zeta Potential values of PEG-Bio-Cou NPs

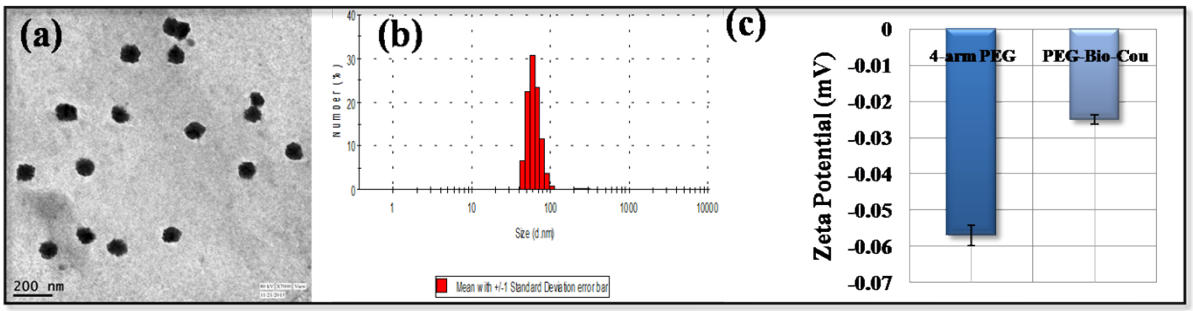


Figure S13 : (a) TEM, **(b)** DLS and **(c)** Zeta potential measurements of PEG-Bio-Cou NPs in H_2O

6. Photoinduced Anticancer Drug release by PEG-Bio-Cou-Cbl NPs

Determination of photochemical quantum yield of* PEG-Bio-Cou-Cbl NPs *(Φp) [8]

These experiments were carried out using a previously described method. 1 mg of PEG-Bio-Cou-Cbl NPs was dissolved in 1 mL water in quartz cuvette. It was then irradiated under UV light by 125 W medium pressure Hg vapor lamp using a suitable filter 1 M $CuSO_4$ in water, (the transmittance for the above filter = 350 to 410 nm). At regular interval of time, 20 µL of the aliquots was taken and analyzed by RP-HPLC using mobile phases 95 : 5 trifluoro acetic acid (TFA) : H_2O solution and 96 : 4 TFA in acetonitrile, at a flow rate of 1 mL/min (detection: UV 254 nm). Peak areas were determined by RP-HPLC, which indicated gradual decrease of the caged compound with time, and the average of three runs. The reaction was followed until the consumption of the caged compound is less than 5% of the initial area. Based on

HPLC data for each caged compounds, we plotted normalized [A] (HPLC peak area) versus irradiation time. We observed an exponential correlation for the disappearance of the caged compounds, which suggested a first order reaction. Further, the quantum yield for the photolysis of caged compounds was calculated using below equation.

$$\phi_p = \frac{(k_p)_{CP}}{I_0 (F_{CP})}$$

where, the subscript 'CP' denotes caged compound. Φp is the photolysis quantum yield, kp is the photolysis rate constant, and I_0 is the incident photon flux and F is the fraction of light absorbed. Potassium ferrioxalate was used as an actinometer.

Table S2 : Photochemical Quantum Yield of PEG-Bio-Cou-Cbl

Cage compound	Quantum yield (Φp)
Potassium ferrioxalate	1.21
PEG-Bio-Cou-Cbl	0.044

Photochemical quantum yield (error limit within ± 5%).

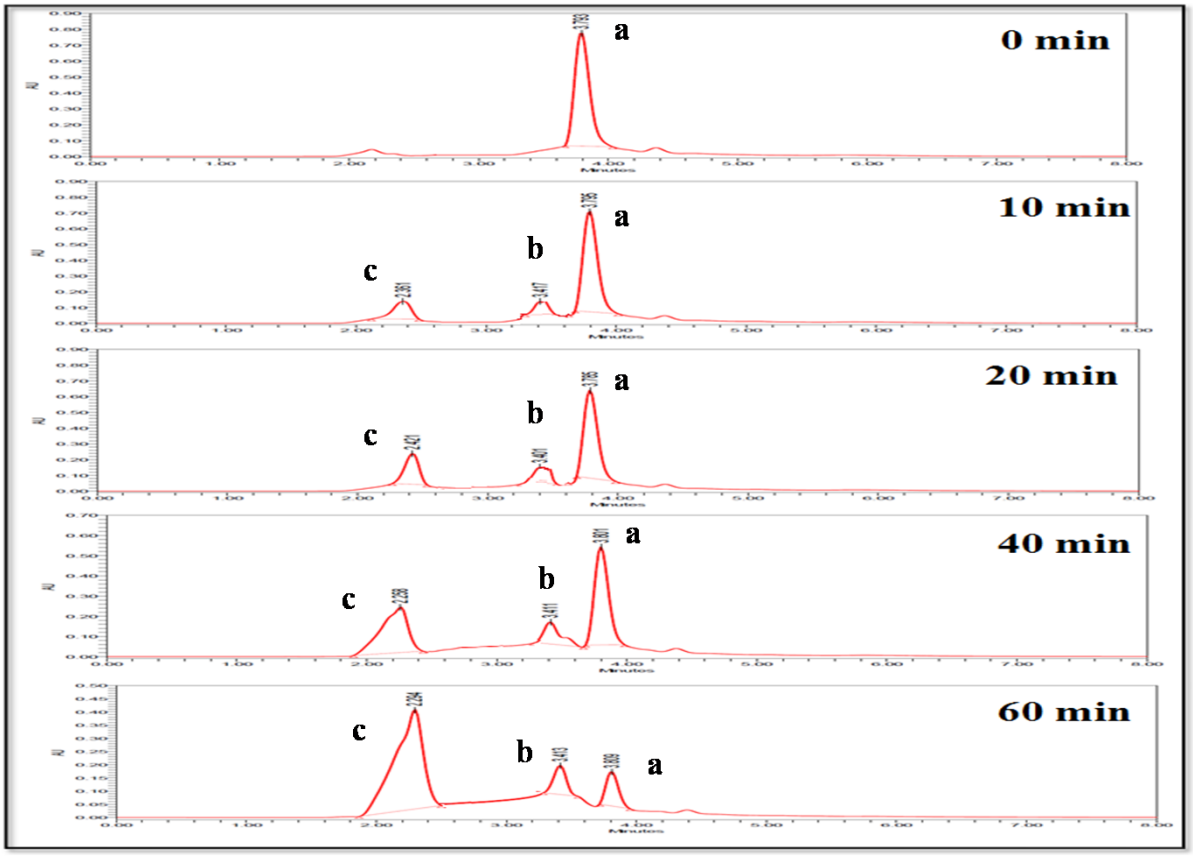


Figure S14: RP HPLC profile of photolysis of PEG-Bio-Cou-Cbl at different time intervals. a = PEG-Bio-Cou-Cbl, b = Chlorambucil (Cbl), c = PEG-Bio-Cou

7. Cellular localization of Non-targeted PEG-Cou-Cbl NPs :

To establish the tumor cell targeting property of the organic polymeric NPs PEG-Bio-Cou-Cbl, we carried out cellular internalization study with non-biotinylated polymeric NPs viz. PEG-Cou-Cbl. Due to over-expression of biotin receptors in tumor cells, the biotinyalted NPs i.e. PEG-Bio-Cou-Cbl could selectively internalize and accumulated inside the Hela cells whereas PEG-Cou-Cbl NPs did not contain any biotin moiety to facilitate internalization into the Hela cells. Hence, from the confocal images (**figure S15)** and the comparative fluorescence intensity plot **(figure S16)** we concluded that non-targeted NPs internalized into the tumor cells relatively in a lesser extent compared to the targeted NPs.

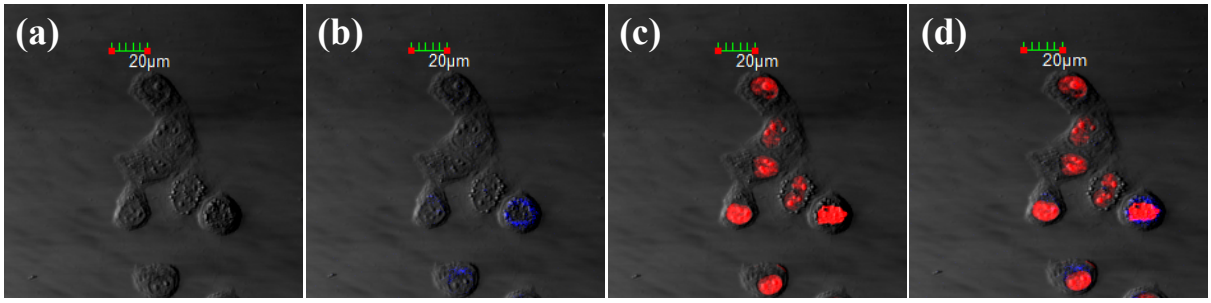


Figure S15 Cellular internalization study of PEG-Cou-Cbl NPs with PI using confocal microscopy; (a) bright field images, (b-c) fluorescence images (b) in the 625 nm (PI) and (c) 461nm (PEG-Cou-Cbl NPs) emission channels and (d) overlays of the bright-field images and the fluorescence images in Hela cell line.

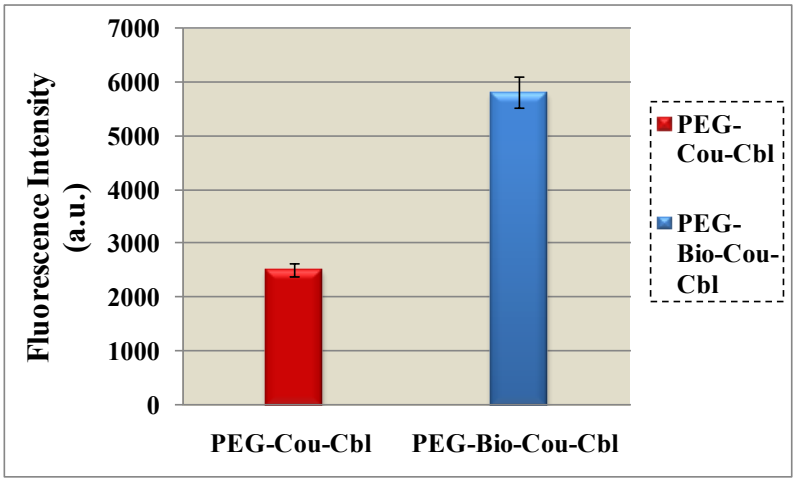


Figure S16 Comparative fluorescent intensity measurements inside Hela Cells for PEG-Cou-Cbl and PEG-Bio-Cou-Cbl NPs

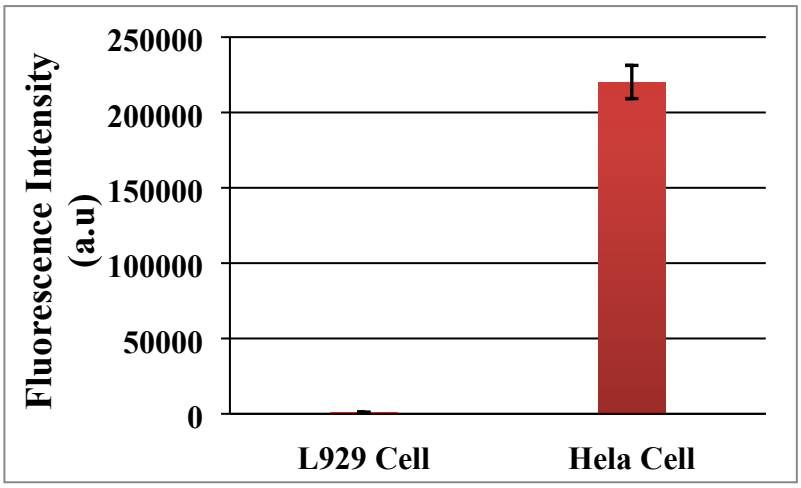


Figure S17 Relative fluorescence intensities of PEG-Bio-Cou-Cbl NPs in L929 and Hela cells.

8. In vitro anticancer drug release from PEG-Bio-Cou-Cbl NPs:[9]

A dialysis bag (MWCO = 1000 Da) containing 6 mL of the NPs was sealed and immersed in a phosphate buffer solution (250 mL, 0.01 M, pH 7.4) at 37 ºC in a thermostatically controlled bath. At designated time intervals, 3 mL of dialysate was removed from sample and stored at – 20 °C for later analysis. Dialysate volume was reconstituted by adding 3 mL of fresh PBS to each sample. After the experiment the dialysate samples were analyzed. The amount of chlorambucil released from PEG-Bio-Cou-Cbl NPs was determined by UV spectrometer at 300 nm.

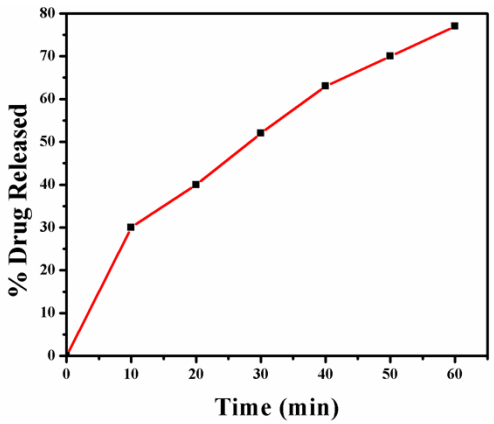


Figure S18 In vitro release profile of chlorambucil from PEG-Bio-Cou-Cbl NPs

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