Electronic Supplementary Information

Targeted and Precise Drug Delivery Using a GSH-Responsive Ultra-Short Peptide-Based Injectable Hydrogel for Breast Cancer Cure

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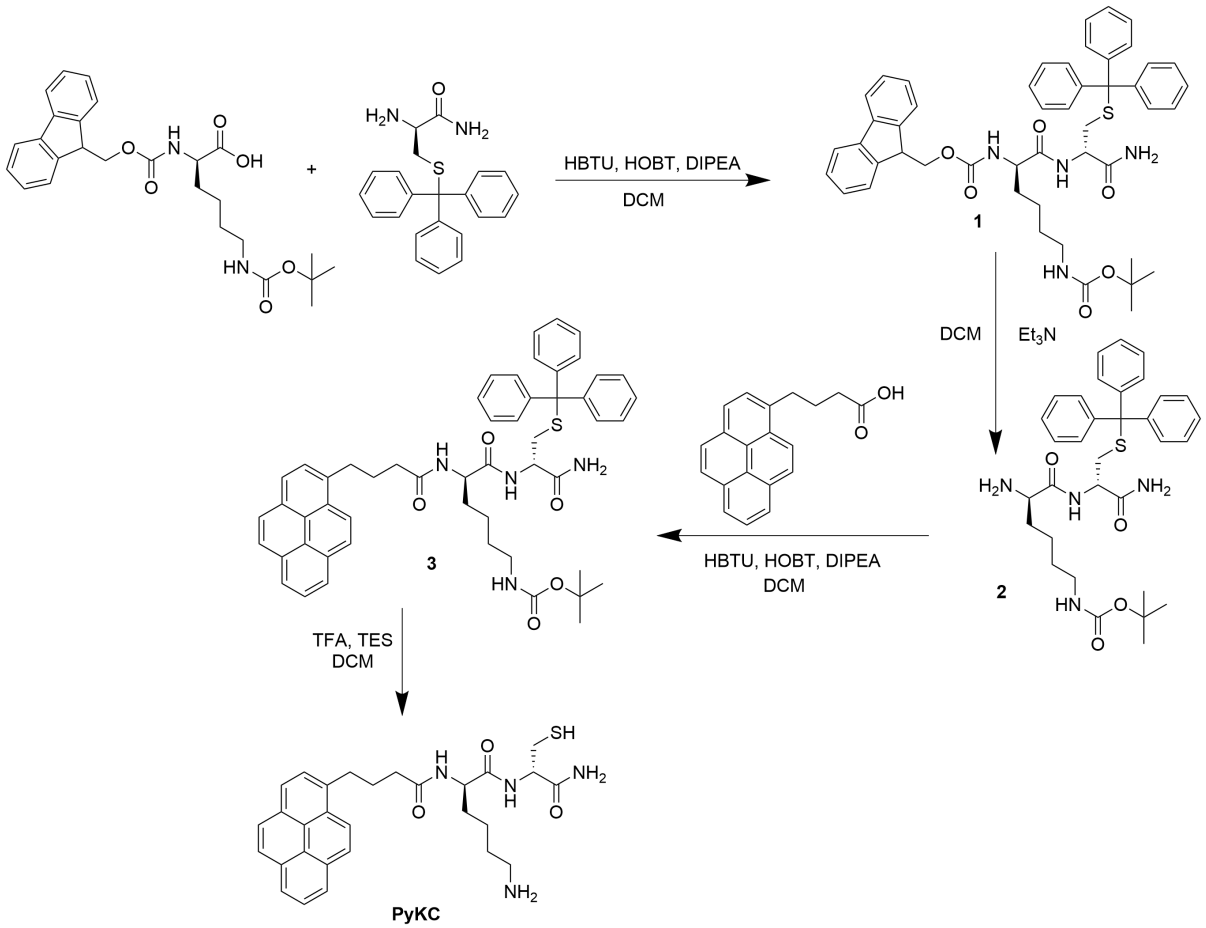
Materials and Methods:

Chemicals and reagents

Chemicals and reagents were obtained from Merck (India), Himedia (India), Invitrogen (India), SRL (India), and Sigma-Aldrich (USA). DCFDA (# D6883) was purchased from Sigma-Aldrich (India). Fetal bovine serum (#16000044) was obtained from Gibco, USA and MEM sodium pyruvate, MEM non-essential amino acids, L-glutamine and Gentamicin, were procured from Hi-Media, India.

Synthesis of PyKC

PyKC was synthesized following the synthetic route mentioned in Scheme S1.



Scheme S1. Synthetic route for PyKC.

Synthesis of 1

In a 100 ml round-bottom flask, Fmoc-Lys(Boc)-OH (646.58 mg, 1.38 mmol), HBTU (625.75 mg, 1.65 mmol), HOBT (223 mg, 1.65 mmol) and DIPEA (576.57 µL, 3.31 mmol) were combined with 30 ml of dry DCM and the solution was stirred in an ice bath for 30 minutes. Next, H-Cys(Trt)-NH_2 (500 mg, 1.38 mmol) dissolved in dry DCM was added to the above

solution. The reaction mixture was stirred overnight at room temperature. After completion of the reaction, as indicated by TLC, the reaction mixture was washed multiple times with brine. The organic phase was then separated, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The crude product so obtained was purified through column chromatography (70% ethyl acetate/hexane) to yield the pure **1** as a white solid. Yield = 750 mg (66.8%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) = 7.77 (dd, *J* = 7.6, 3.2 Hz, 2H), 7.58 (d, *J* = 7.3 Hz, 2H), 7.41 (d, *J* = 7.9 Hz, 8H), 7.35 – 7.26 (m, 8H), 7.20 (t, *J* = 7.3 Hz, 3H), 6.54 (s, 1H), 6.35 (s, 1H), 5.79 (s, 1H), 5.52 (s, 1H), 4.38 (dd, *J* = 7.2, 3.2 Hz, 2H), 4.15 (dq, *J* = 14.3, 7.1 Hz, 3H), 3.11 (d, *J* = 7.7 Hz, 2H), 2.87 – 2.76 (m, 1H), 2.57 (dd, *J* = 13.0, 5.2 Hz, 1H), 1.74 (s, 2H), 1.43 (s, 9H), 1.38 – 1.32 (m, 2H), 1.28 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm) = 172.48, 172.08, 171.57, 156.88, 144.61, 144.10, 141.64, 141.60, 129.84, 128.42, 128.13, 128.11, 127.47, 127.25, 125.39, 120.35, 79.73, 67.54, 60.77, 55.74, 52.19, 47.44, 39.71, 33.38, 30.04, 28.78, 22.46, 21.42. Mass (ESI-MS): m/z calcd. for $C_{48}H_{52}N_4O_6S$ + Na^+ $[M+Na]^+$: 835.350, found: 835.348.

Synthesis of 2.

The product from the first step (**1**) was transferred to a 100 ml round-bottom flask and mixed with 1 ml of Et_3N and 20 ml of dry DCM, then stirred at room temperature for 24 hours. The solvent was then removed under reduced pressure and the crude product was purified through column chromatography (5% DCM/MeOH), yielding the pure **2** as a white solid. Yield = 450 mg (82.5%). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) = 8.64 (s, 1H), 7.53 (s, 1H), 7.34 (t, *J* = 7.7 Hz, 6H), 7.31 – 7.23 (m, 9H), 6.77 (d, *J* = 5.7 Hz, 1H), 4.34 (d, *J* = 7.2 Hz, 1H), 3.63 (s, 2H), 2.84 (d, *J* = 6.8 Hz, 2H), 1.61 (p, *J* = 7.8 Hz, 2H), 1.36 (s, 9H), 1.34 – 1.29 (m, 2H), 1.26 (d, *J* = 7.8 Hz, 2H). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm) = 172.54, 171.12, 155.56, 144.21, 129.08, 128.14, 126.85, 77.44, 65.83, 52.45, 51.57, 45.42, 33.73, 31.47, 29.10, 28.30, 21.65. Mass (ESI-MS): m/z calcd. for $C_{33}H_{42}N_3O_5S^+$ $[M+H]^+$: 592.284, found: 592.298; $C_{66}H_{83}N_6O_{10}S_2^+$ $[2M+H]^+$: 1183.560, found: 1183.589.

Synthesis of 3.

In a 100 ml round-bottom flask, 1-Pyrenebutyric acid (200 mg, 0.7 mmol), HBTU (320 mg, 0.84 mmol), HOBT (113 mg, 1.65 mmol) and DIPEA (294 µL, 1.68 mmol) were combined with 20 ml of dry DCM and the solution was stirred in an ice bath for 30 minutes. Then, **2** (450 mg, 0.76 mmoL), dissolved in dry DCM, was added to the above solution and stirred overnight. Following the completion of the reaction, as confirmed by TLC, the reaction

mixture was washed multiple times with brine. The organic phase was then separated, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Purification of the crude product through column chromatography (75% ethyl acetate/hexane system) yielded the pure compound as an off-white solid. Yield = 510 mg (84.6%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 8.36 (d, *J* = 9.3 Hz, 1H), 8.25 (d, *J* = 7.6 Hz, 2H), 8.19 (dd, *J* = 8.5, 5.7 Hz, 2H), 8.13 – 8.01 (m, 3H), 7.92 (t, J = 8.5 Hz, 2H), 7.34 – 7.22 (m, 11H), 7.22 – 7.12 (m, 4H), 4.33 – 4.13 (m, 2H), 2.86 (d, *J* = 6.5 Hz, 2H), 2.43 – 2.22 (m, 4H), 2.00 (dd, *J* = 13.7, 6.0 Hz, 2H), 1.63 (d, *J* = 8.1 Hz, 2H), 1.52 (dd, *J* = 8.9, 5.0 Hz, 2H), 1.33 (s, 9H), 1.30 – 1.20 (m, 4H). ^{13}C NMR (151 MHz, DMSO) δ (ppm) 172.65, 171.77, 171.55, 155.59, 144.29, 136.60, 130.93, 130.48, 129.34, 129.06, 128.21, 128.02, 127.81, 127.62, 127.54, 127.49, 127.25, 126.70, 126.66, 126.52, 126.13, 124.96, 124.92, 124.79, 124.29, 124.22, 123.58, 79.21, 78.99, 78.77, 77.36, 65.71, 53.12, 51.43, 34.86, 33.74, 32.32, 31.31, 29.33, 28.29, 27.52, 22.83. Mass (ESI-MS): m/z calcd. for $C_{53}H_{56}N_4O_5S$ + Na^+ $[M+Na]^+$: 883.386, found: 883.377.

Synthesis of PyKC.

Compound **3** was treated with a freshly prepared mixture of trifluoroacetic acid (TFA), tetraethylsilane (TES), and water (8.5:1:0.5 ratio) and stirred for 2 h. The reaction mixture was then concentrated on a rotary evaporator to a minimum volume. The cleaved peptide was precipitated from the cold, dry ether, centrifuged, and lyophilized to obtain the crude peptide. Purification of **PyKC** was performed using Dionex Ultimate 3000 HPLC with a Luna 5 µm (C18) column (Phenomenex) and acetonitrile/water (containing 0.1% TFA each) as the mobile phase. Yield = 270 mg (87.9%). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) = 8.39 (d, *J* = 9.2 Hz, 1H), 8.28 (dd, *J* = 8.2, 5.8 Hz, 2H), 8.23 (dd, *J* = 8.3, 6.4 Hz, 2H), 8.13 (dd, *J* = 11.2, 6.0 Hz, 3H), 8.07 (t, *J* = 7.6 Hz, 1H), 8.00 – 7.90 (m, 2H), 7.65 (s, 3H), 7.40 (s, 1H), 7.19 (d, *J* = 22.0 Hz, 1H), 4.35 – 4.24 (m, 2H), 2.88 – 2.69 (m, 4H), 2.39 – 2.22 (m, 3H), 2.03 (p, *J* = 7.1 Hz, 2H), 1.67 (d, *J* = 12.5 Hz, 1H), 1.59 – 1.46 (m, 3H), 1.41 – 1.30 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) = 173.13, 172.97, 172.38, 172.32, 172.19, 171.89, 137.06, 137.00, 131.36, 130.89, 129.78, 128.63, 128.06, 127.93, 127.91, 127.70, 127.00, 126.64, 125.42, 125.40, 125.27, 124.70, 124.61, 124.03, 123.98, 55.06, 53.18, 52.31, 40.82, 40.50, 39.18, 35.28, 32.71, 31.45, 28.01, 27.91, 27.10, 27.08, 26.57, 22.82. Mass (ESI-MS): m/z calcd. for $C_{29}H_{35}N_4O_3S^+$ $[M+H]^+$: 519.242, found: 519.243; $C_{58}H_{69}N_8O_6S_2^+$ $[2M+H]^+$: 1037.477, found: 1037.478.

Preparation of PyKC hydrogel

To prepare the hydrogel, an appropriate amount of PyKC was added in the required volume of 20 mM PPBS buffer, pH 7.4 (to attain a concentration of 1 wt%) and shaken to completely dissolve the solid. The solution was kept undisturbed at room temperature for 12 h to get the self-supporting hydrogel. Unless otherwise mentioned, all the studies were performed with 1 wt% hydrogel at room temperature.

For DOX-loaded PyKC-hydrogel preparation, a stock solution of DOX was prepared in 20 mM PPBS buffer, pH 7.4. The required amount of the DOX stock solution was taken and mixed with an appropriate volume of freshly prepared 20 mM PPBS buffer, pH 7.4. This mixture was added to the required amount of PyKC (to attain a concentration of 1 wt%) and shaken to completely dissolve the solid. The solution was kept undisturbed at room temperature for 12 h to get the self-supporting hydrogel.

Except for in-vitro drug release/dissolution studies, all other in-vitro studies were performed with 100 μL of the DOX-loaded hydrogels prepared using the above-mentioned method.

Rheology

The viscoelastic properties of the hydrogel were analyzed using an Anton Paar MCR 102 rheometer equipped with a 20 mm parallel plate (with 0.3 mm zero gaps) measuring system at 25 °C. Appropriate amounts of peptides were dissolved in Tris buffer of pH 8 to make 1 wt% gel and kept undisturbed at room temperature for 12 h to get the hydrogels. Appropriate amounts of hydrogels are taken very carefully with the help of a spatula to avoid any damage to the hydrogel samples and placed on the lower plate of the rheometer. The measuring system is then lowered until it reaches the position where the gap between the two plates is 0.3 mm. Then the respective rheological measurements were performed. All the experiments were performed in triplicate. Strain sweep tests were performed to identify the linear viscoelastic region (LVR) over a range from 0.01 to 1000 % strain at a fixed oscillatory frequency of 1 rad/s. The LVR can be defined as the range where strain has no impact upon G′ and G″. Frequency sweep tests were carried out under an appropriate strain (γ = 0.1 %) selected from the LVR with the frequency ranging from 0.1 to 100 rad/s at 25 °C. To investigate the thixotropic property of the gels (1 wt%), cyclic dynamic strain sweep experiment was performed at a constant angular frequency of 1 rad/s by altering the applied strain from 0.1 to 100 %. In this experiment, a higher strain (γ = 100 %) and a lower strain

(γ = 0.1 %) are applied on the gel alternatively over a period of 2300 s and four successive cycles.

FETEM

5 μL of the samples were cast on carbon-coated copper grid (300 mesh Cu grid with thick carbon film from Pacific Grid Tech, USA) and allowed to air dry for 10 minutes, and then the excess sample was bloated with a tissue paper. The grid was then allowed to air dry for 1 day. The FETEM images were taken using JEOL JEM-2100F microscope.

Dissolution and in-vitro DOX release study

The dissolution and DOX release studies were performed following our previous report.[1] Typically, 500 μL of the hydrogel (1 wt%) samples (with or without DOX) were added to 10 mL of buffers (for pH-dependent dissolution and release) or GSH solutions (in cell culture medium, for GSH-mediated release), and the samples were shaken at room temperature at 100 rpm. For the pH-dependent dissolution and release studies, after 7 days of incubation, the supernatants were collected and analyzed using UV-Visible spectroscopy. The absorbance values obtained at 352 nm (for PyKC dissolution) or 496 nm (for DOX release) were plotted against the calibration curves to get the % dissolution or release. For GSH-mediated release, aliquots were taken at different intervals and replaced by equal amounts of GSH solutions. The samples were analyzed using UV-Visible spectroscopy to get the cumulative release.

Cell culture and maintenance

Human triple negative breast cancer cell line MDA-MB 231 was procured from the central cell repository of National Centre for Cell Science (NCCS), Pune. Mice breast cancer cell line 4T1 was obtained from Bose Institute, Kolkata. All the cell lines were cultured in a T25 flask with DMEM, supplemented with10% Fetal bovine serum, non-essential amino acids, 1mMsodium pyruvate, 2mM L-glutamine, 100 mg/L streptomycin, 100 units/L penicillin, and 50 mg/L gentamycin in a 37 °C humidified incubator containing 5% CO_2.

MTT cell proliferation assay

MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was performed to determine the rate of cell growth and, conversely, the decline in cell viability when

threatened with PyKC-hydrogel, DOX (5µM) and DOX (5µM)-hydrogel.[2-3] For this assay, cells were seeded on 96-well plates at a density of 0.5 $\times 10^5$ cells/well. The cells were treated for different time periods: 12 h, 24 h, 48 h, and 72 h. After the incubation period, MTT solutions (100 µl, 0.5 mg/ml) were added to each well and incubated in a humidified incubator containing 5% CO_2 at 37 °C for 4 h. After discarding the supernatant, the purple-colored formazan crystals formed in the wells were dissolved with 100 µl DMSO per well and the absorbance was measured at 490 nm using a microplate reader. Cell viability was expressed as a percentage of the control experimental setup.

Cell cycle profiling assay by propidium iodide staining

Briefly MDA-MB 231 and 4T1 cells were seeded at a density of 1×10^6 cells /ml in each petri dish for 24 hours. Thereafter the cells were treated with PyKC-hydrogel, DOX (5µM), and DOX (5µM)-hydrogel for 24 h. DOX-loaded hydrogel was placed directly on the cell culture media containing the seeded cells. Post-treatment, cells were harvested into a single-cell suspension and fixed with 75% ethanol for 24 h at -20 °C. After centrifugation, the cell pellets were resuspended in 1× PBS followed by RNaseA (20 µm) treatment for 2 h at 37 °C. Finally, propidium iodide was added and incubated at room temperature for 20 min. Subsequently the samples were analyzed using BD FACS Verse flow cytometer (BD Biosciences, San Jose, CA) and analyzed using BD FACS DIVA software. CellQuest statistics was employed to quantitate the data at different phases of cell cycle and histogram display of counts (*y* axis) versus DNA content(*x* axis, PI fluorescence) has been displayed.[4]

Annexin V-FITC/PI staining for apoptosis assay

Induction of apoptosis was quantified via flow cytometric analyses of control, PyKC-hydrogel, DOX (5µM), and DOX (5µM)-hydrogel treated cells that were stained with annexin V-FITC/PI, using the Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol (BD Bioscience).[5] Briefly, post-treatment MDA-MB231 and 4T1 cells were harvested with 1X Trypsin and washed in ice-cold 1x PBS, followed by being re-suspended in 100 µl of 1X binding buffer solution supplied within the kit. Finally, cells were incubated with 5 µl of annexin V-FITC and 5 µl of PI for 15 min at room temperature in the dark before acquiring data using BD FACS Verse flow cytometer (BD Biosciences, San Jose, CA). Annexin V/FITC

positive cells were regarded as apoptotic cells analyzed using Cell Quest Software (BD Biosciences).

Measurement of cellular ROS using DCFDA

To estimate the intercellular reactive oxygen species (ROS) production due to PyKC-hydrogel, DOX (5μM), and DOX (5μM)-hydrogel treatment, the DCFDA method was used.[6] MDA-MB 231 and 4T1 cells were seeded in a 6-well plate and treated with PyKC-hydrogel, DOX (5μM), and DOX (5μM)-hydrogel for 24 h. Post treatment, the media was discarded and incubated with 10 μM H_2DCFDA for 30 min at 37 °C. For fluorescent imaging, H_2DCFDA incubated cells were washed, resuspended in 1x PBS, and directly imaged under a fluorescent microscope (Leica). For flow cytometric analyses, cells were then trypsinized, washed with 1x PBS, and collected in centrifuge tubes. DCF fluorescence was then measured using BD FACS Verse flow cytometer (BD Biosciences) and analyzed using Cell Quest Software (BD Biosciences).

Detection of Mitochondrial Membrane Potential by JC1 staining

The changes in mitochondrial membrane permeability were determined by JC1 (Molecular probes).[4] Briefly, MDA-MB 231 and 4T1 cells were treated with PyKC-hydrogel, DOX (5μM), and DOX (5μM)-hydrogel for indicated time period, harvested, washed twice in 1x PBS, re-suspended in PBS supplemented with JC-1 dye (3 μM final concentration), incubated for 15 min in the dark at 37 °C and flow cytometric analyses were immediately performed using a FACS-Verse instrument (BD) or images were captured with a fluorescence microscope (Leica).

Apoptotic Nuclear Morphology Study by DAPI Staining

DAPI staining was performed to detect any morphological changes within the nucleus of 4T1 and MDA-MB 231 cells after treatment with PyKC-hydrogel, DOX (5μM), and DOX (5μM)-hydrogel (5μM) for the indicated time period. After treatment, cells were cleaned with 1x PBS and stained with DAPI. Images were captured with a fluorescence microscope (Leica).

Cellular uptake studies

To better understand the intracellular uptake of DOX, MDA-MB-231 and 4T1 cells were grown on six-well culture plates and treated with PyKC-hydrogel, DOX (5μM), and DOX (5μM)-hydrogel for the time period of 2 h, 4 h, and 6 h and the intensity of DOX fluorescence

on cellular uptake was measured by flow cytometric analysis and compared with native doxorubicin using histograms by FACS-Verse instrument (BD).

SEM analysis for the morphological study of MDA-MB-231 cell

For SEM analysis of MDA-MB-231 cells, the cells are typically cultured on coverslips and treated with PyKC-hydrogel, DOX (5 μM), and DOX(5 μM)-hydrogel for the indicated time period. Following treatment, the cells are gently washed with phosphate-buffered saline (PBS) to remove any residual media or debris. The cells are then fixed using a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for approximately 1-2 hours at room temperature or overnight at 4 °C. Following post-fixation, the samples are dehydrated through a series of ethanol washes (e.g., 30%, 50%, 70%, 90%, and 100% ethanol). Subsequently, the dried samples are mounted onto SEM stubs using conductive adhesive coated with a thin layer of conductive material gold, using a sputter coater. The prepared samples are then imaged using a scanning electron microscope at appropriate magnifications and operating conditions to visualize cellular morphology and ultrastructure.

Detection of Mitochondrial ROS by Mitosox staining

The Mitosox assay, a widely used technique for assessing mitochondrial reactive oxygen species (ROS) levels, involves the use of Mitosox Red, a fluorogenic dye that selectively targets mitochondria and reacts with superoxide. To perform the assay, MDA-MB 231 and 4T1 cells were treated with PyKC-hydrogel, DOX (5 μM), and DOX(5 μM)-hydrogel for the indicated time period, harvested, washed twice in 1 x PBS, re-suspended in PBS supplemented with Mitosox (3 μM final concentration), incubated for 15 min in the dark at 37 °C and excess Mitosox Red is removed by washing with PBS, and the samples are analyzed using fluorescence microscopy appropriate excitation and emission filters. The red fluorescence signal emitted by Mitosox Red upon reaction with superoxide is quantified to assess mitochondrial ROS levels.

4T1 breast tumor model

BALB/C female mice (6–8 weeks old, healthy) were obtained from West Bengal Livestock Development Corporation Limited, Kolkata, India, and were maintained according to the guiding principle of the Institutional Animal Ethics Committee (IAEC) using the CPCSEA

approved protocol. All animal experiments complied with the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978, U.S.A.). All animal experiments complied with ARRIVE guidelines (Animal Research: Reporting of *in vivo* Experiments; https://arriveguidelines.org/arrive-guidelines). To create a solid tumour for the tumorigenic assay in vivo, 4T1 cells (1.5×10^6 cells/animal) were subcutaneously injected into the mammary fat pad of BALB/c mice. Animals with solid tumour were randomly assigned to six groups, with four animals in each group. The vehicle control group was treated with normal saline, whereas the other five groups were administered an intraperitoneal injection of PyKC-hydrogel, DOX (2.5 mg/kg), DOX (2.5 mg/kg)-hydrogel, DOX (5 mg/kg), and DOX (5 mg/kg)-hydrogel starting 12 days after tumor development (7 doses, at 48 h interval). Every third day, Vernier calipers were used to measure the tumor's degree in order to monitor the progression of the disease. The tumor size calculation was done using the rule V = 0.5×a×b2, where "a" and "b" indicate major and minor diameter, respectively. Finally, the tumors were excised, fixed in 10% formalin for 48 h at 4 °C, and transferred to 1×PBS for paraffin-embedded blocks.

Hepatoxicity study

Following PyKC-hydrogel, DOX (2.5 mg/kg), DOX (2.5 mg/kg)-hydrogel, DOX (5 mg/kg), and DOX (5 mg/kg)-hydrogel treatment, each with three days intervals and 100 µl blood samples were manually taken into heparinized capillary tubes by perforating the saphenous vein with a needle, and finally into 0.5 ml microcentrifuge tubes. Within 30 minutes of collection, the blood samples were centrifuged at 1640 rpm for 5 minutes at 4 °C to separate the plasma. Individual kits were used to measure the serum levels of ALT, AST, ALP, creatinine, and urea nitrogen in accordance with the manufacturer's instructions.

Table S1. Table representing a dose-dependent increase in the proportion of MDA-MB-231 cell and 4T1 cell in G2/M phase along with a decrease in G1 phase in response to PyKC-hydrogel, DOX (5µM) and DOX(5µM)-hydrogel.

	MDA-MB-231				**4T1**			
	Sub-G0	G0/G1	S	G2/M	Sub-G0	G0/G1	S	G2/M
Control	9.05 ± 1.33	62.13 ±	12.36 ±	16.46 ±	10.14 ±	65.28 ±	12.36 ±	12.22 ±

		2.49	2.26	1.46	2.03	3.16	2.26	1.86
PyKC-	10.52 ±	57.45 ±	13.22 ±	18.81 ±	12.14 ±	55.12 ±	14.65 ±	18.09 ±
hydrogel	1.05	1.69	2.56	1.49	2.11	2.68	2.56	2.25
DOX (5 μM)	25.67 ±	30.32 ±	20.34 ±	23.67 ±	20.71 ±	30.32 ±	17.69 ±	31.28 ±
	1.37	1.59	1.55	2.55	1.79	1.59	2.87	3.22
DOX (5 μM)	13.05 ±	23.29 ±	12.06 ±	51.60 ±	14.49 ±	27.05	15.28 ±	43.18 ±
-hydrogel	1.58	1.93	1.77	1.27	2.44	±1.63	1.99	3.65

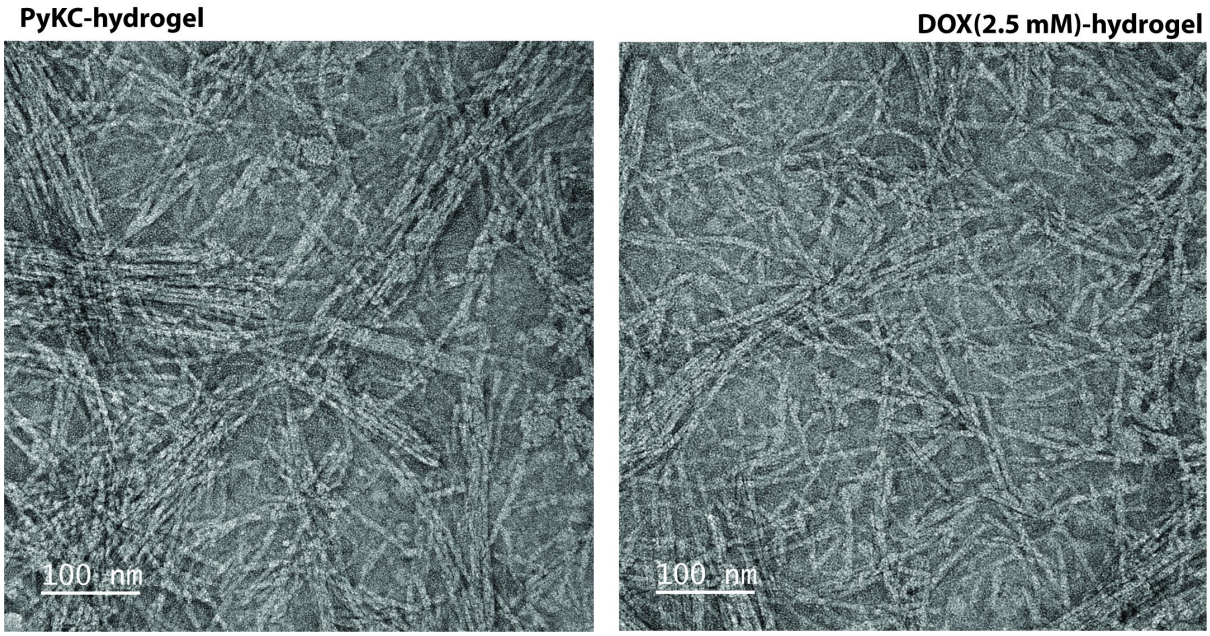


Fig. S1. Morphology. FETEM images of PyKC hydrogel without and with DOX.

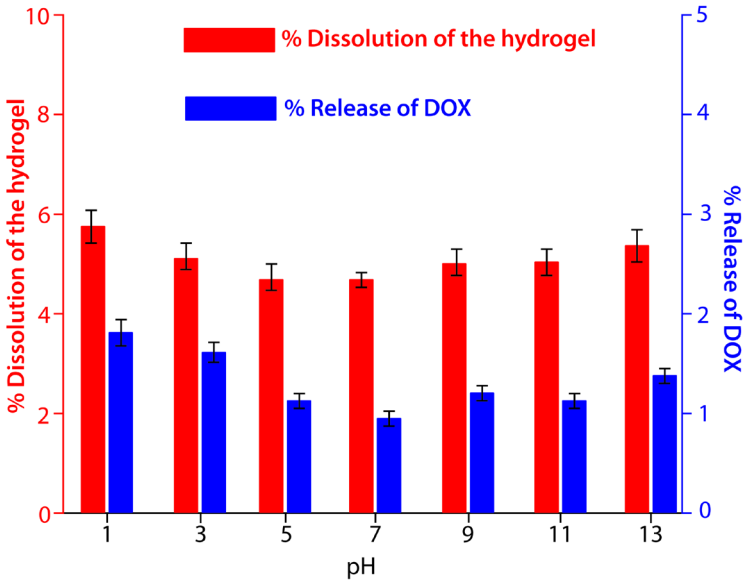


Fig. S2. Dissolution and release. Dissolution of the hydrogel and % release of the DOX from the DOX-hydrogels when incubated in bulk buffer solutions of different pHs for 7 days.

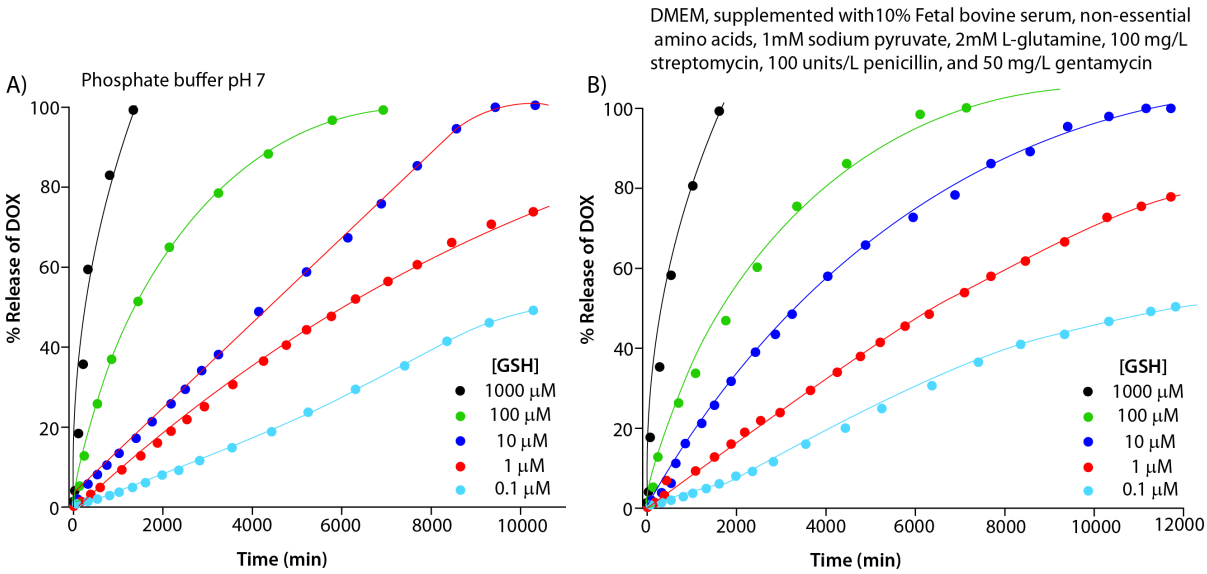


Fig. S3. Release in response to GSH. DOX release profile from the DOX-hydrogel when incubated at varying concentrations of GSH. [PyKC] = 1 wt%, [DOX] = 2.5 mM, all studies performed at RT.

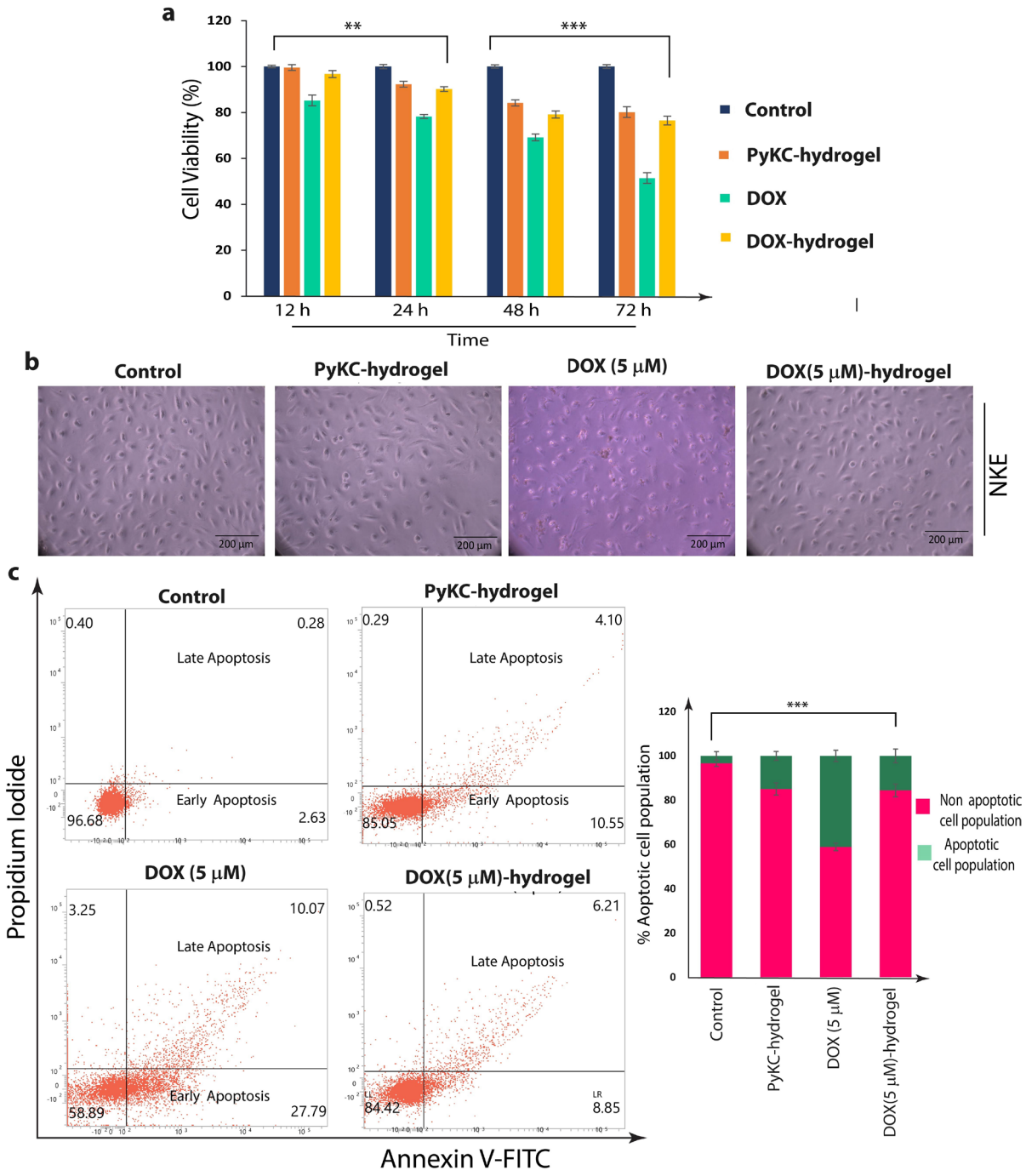


Fig. S4. Cytotoxicity. Cytotoxic activity of PyKC-hydrogel, DOX (5µM) and DOX(5µM)-hydrogel treated **a**, NKE cells using the MTT assay. B, Photomicrographs showing the dose-dependent anti-proliferative effect of PyKC-hydrogel, DOX (5µM) and DOX(5µM)-hydrogel treated NKE cells, and **c,** Flow cytometric analysis of Hydrogel, DOX (5µM) and DOX (5 µM)-hydrogel treated NKE cells that were stained with annexin V-FITC/PI. All the data represents Mean ± SEM of a minimum of three independent experiments (*p<0.05, **p< 0.01, ***p<0.001).

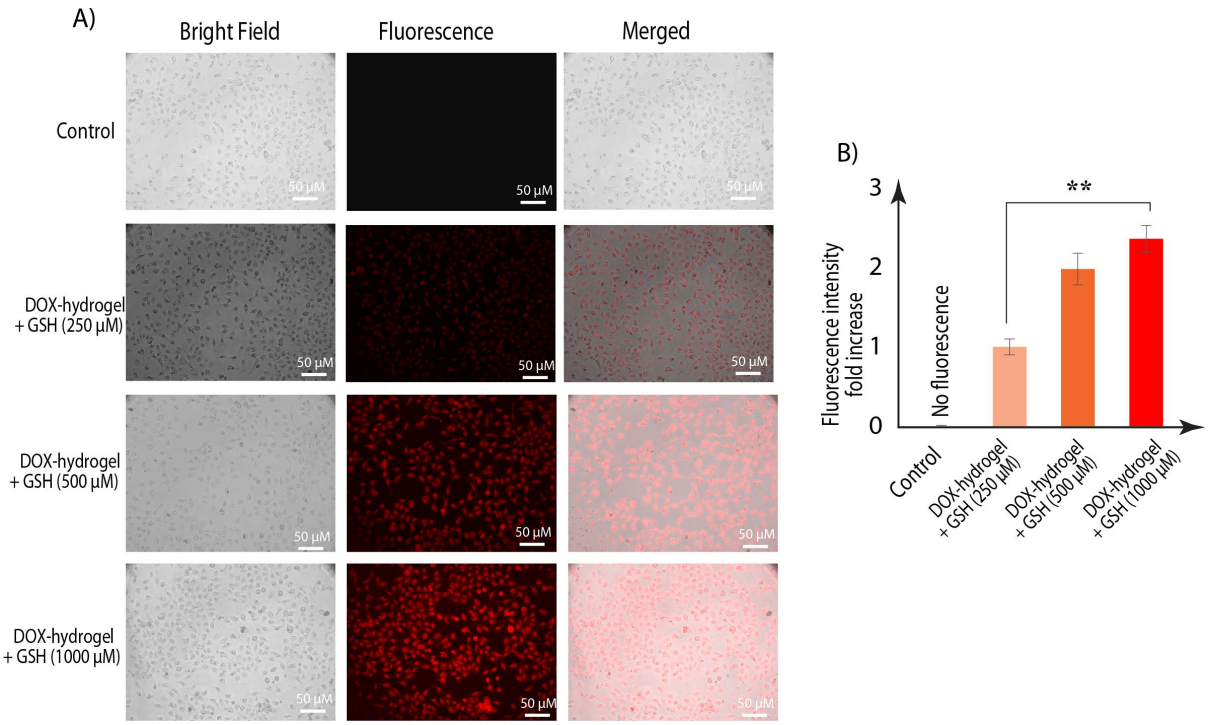


Fig. S5. MDA-MB-231 cells pre-treated with varying concentrations of GSH (250 μM, 500 μM, and 1000 μM). The cells were incubated with the DOX-loaded hydrogel for 6 hours, washed with PBS, and then analyzed using fluorescence microscopy to assess DOX uptake. A) fluorescence microscopic analyses, and B) quantitative analyses of the data in terms of enhanced fluorescence.

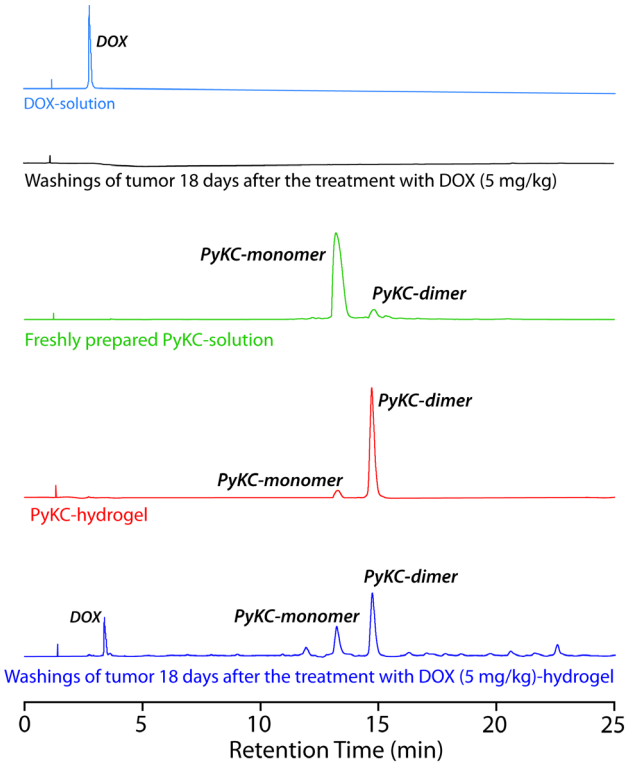


Fig. S6. HPLC chromatograms of the washings of the tumors after 18 days of treatment with single injections of DOX (5mg/kg) and DOX (5 mg/kg)-hydrogel.

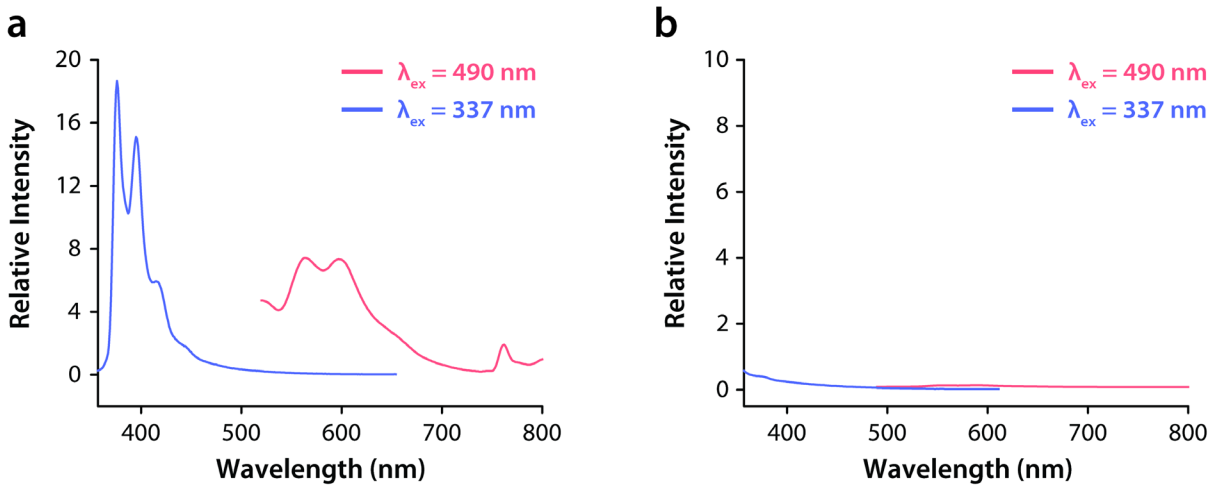


Fig. S7. Emission spectra of the washings of the tumors after 18 days of treatment with single injections of **a**) DOX (5 mg/kg)-hydrogel, and **b**) DOX (5mg/kg). In both cases, the samples were separately excited at 337 nm (absorption maxima of PyKC) and 490 nm (absorption maxima of DOX) to evaluate the presence of both compounds in these samples.

Fig. S8. Photograph showing the injectable behavior of the hydrogel.

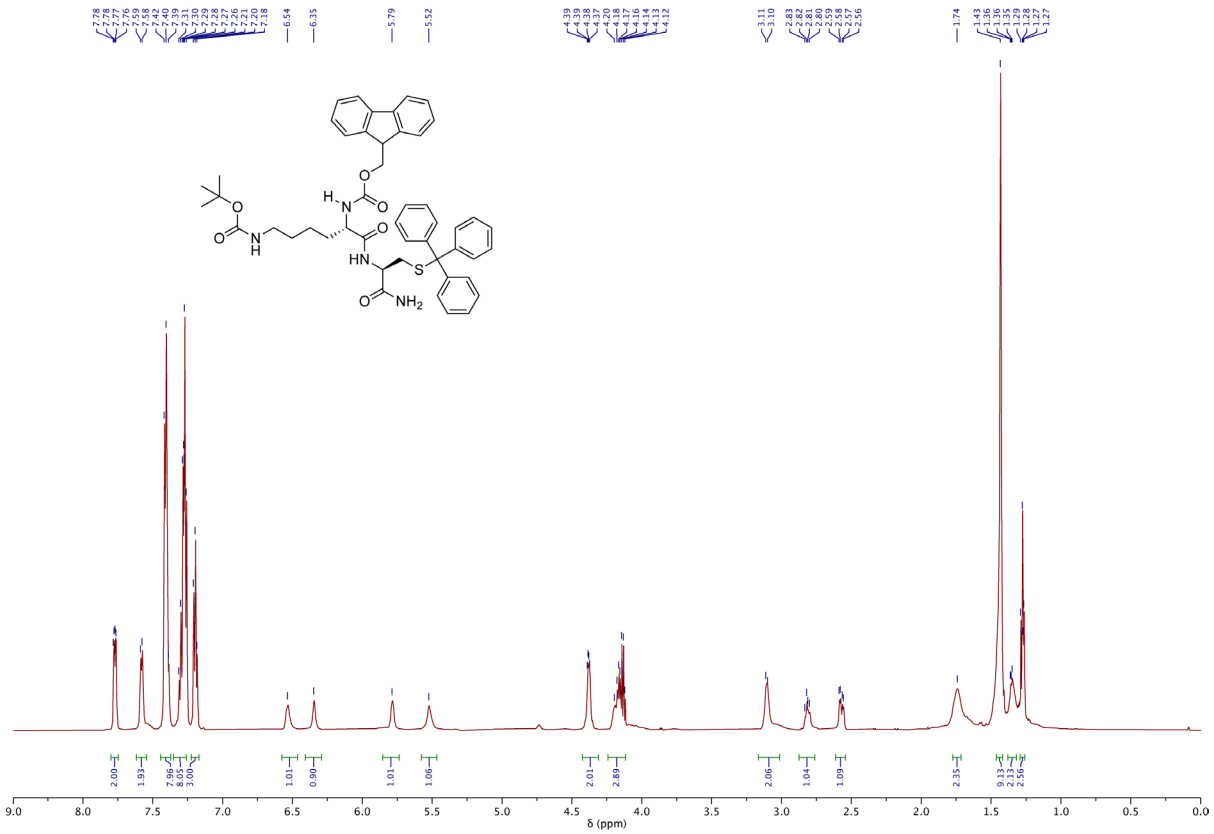


Fig. S9. ^{1}H NMR of **1** in $CDCl_3$.

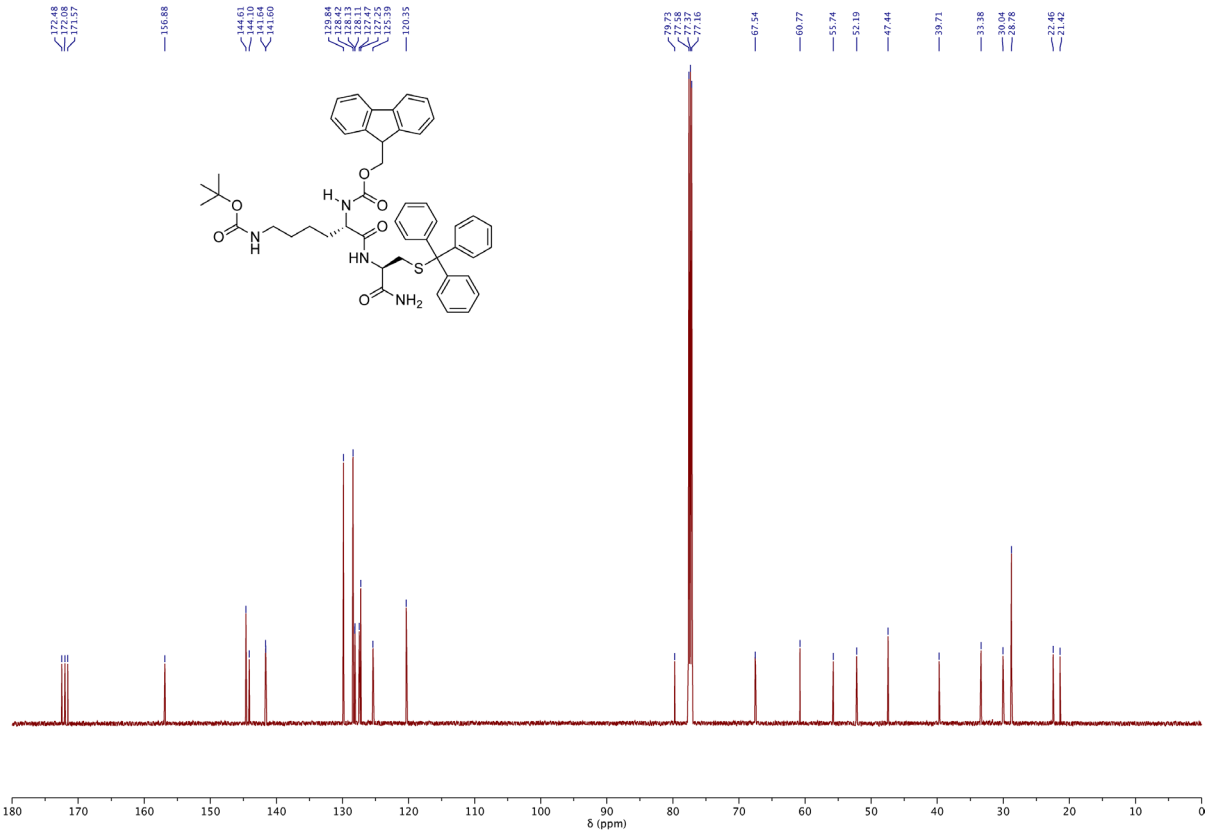


Fig. S10. ^{13}C NMR of **1** in $CDCl_3$.

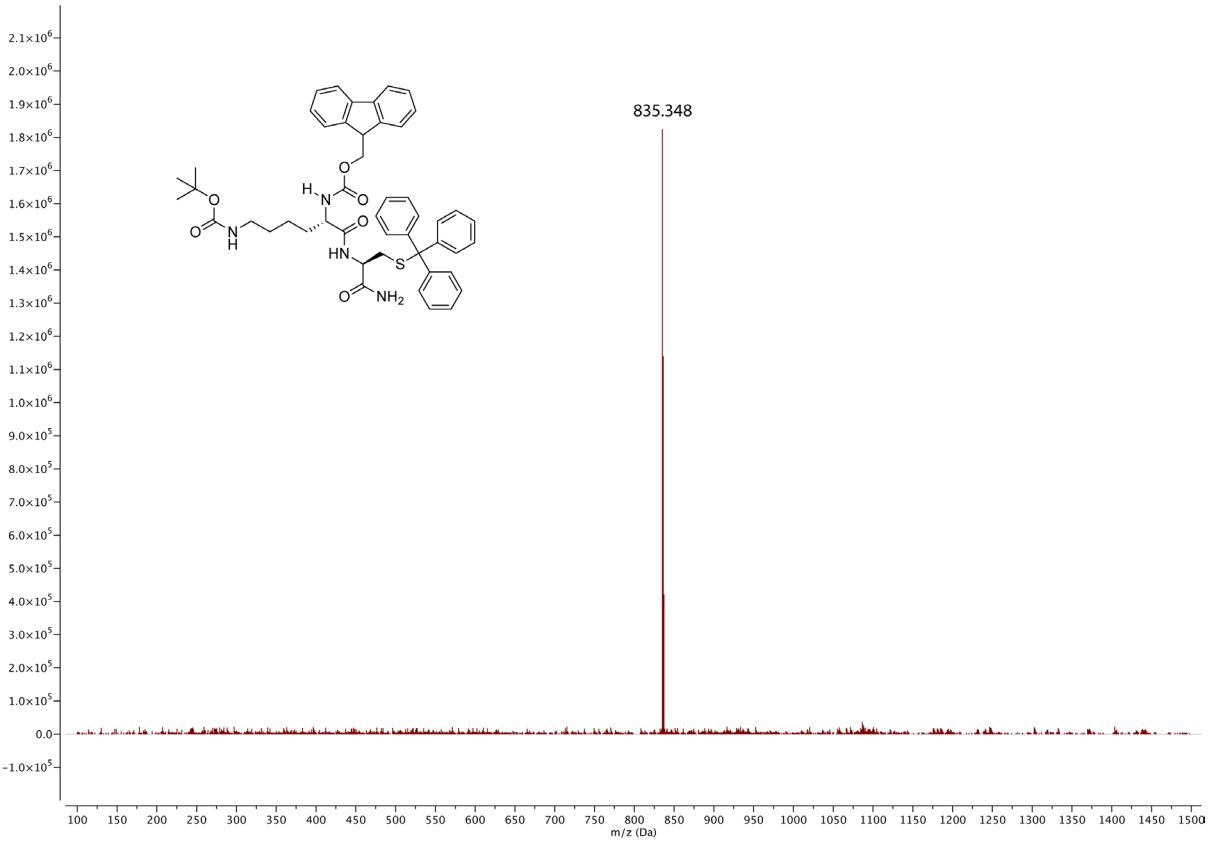


Fig. S11. ESI-MS spectrum of **1**.

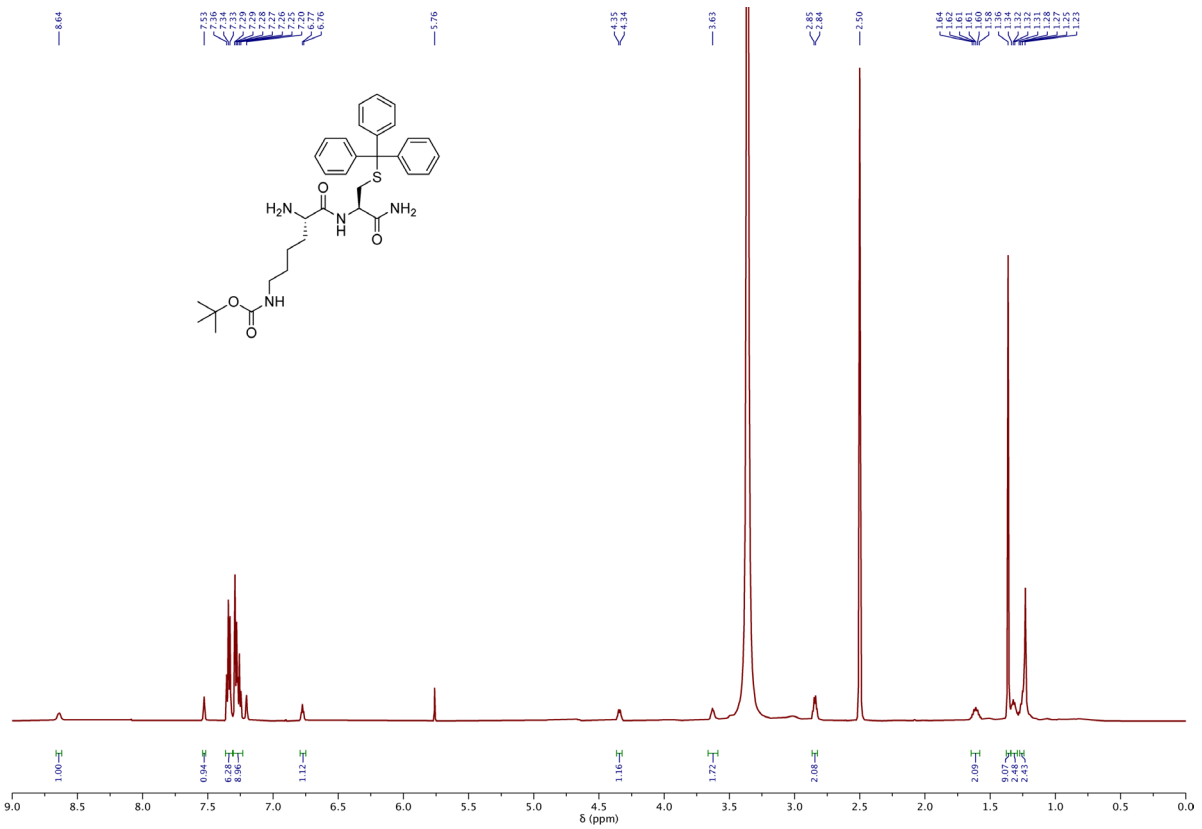


Fig. S12. ^{1}H NMR of **2** in DMSO-d_6.

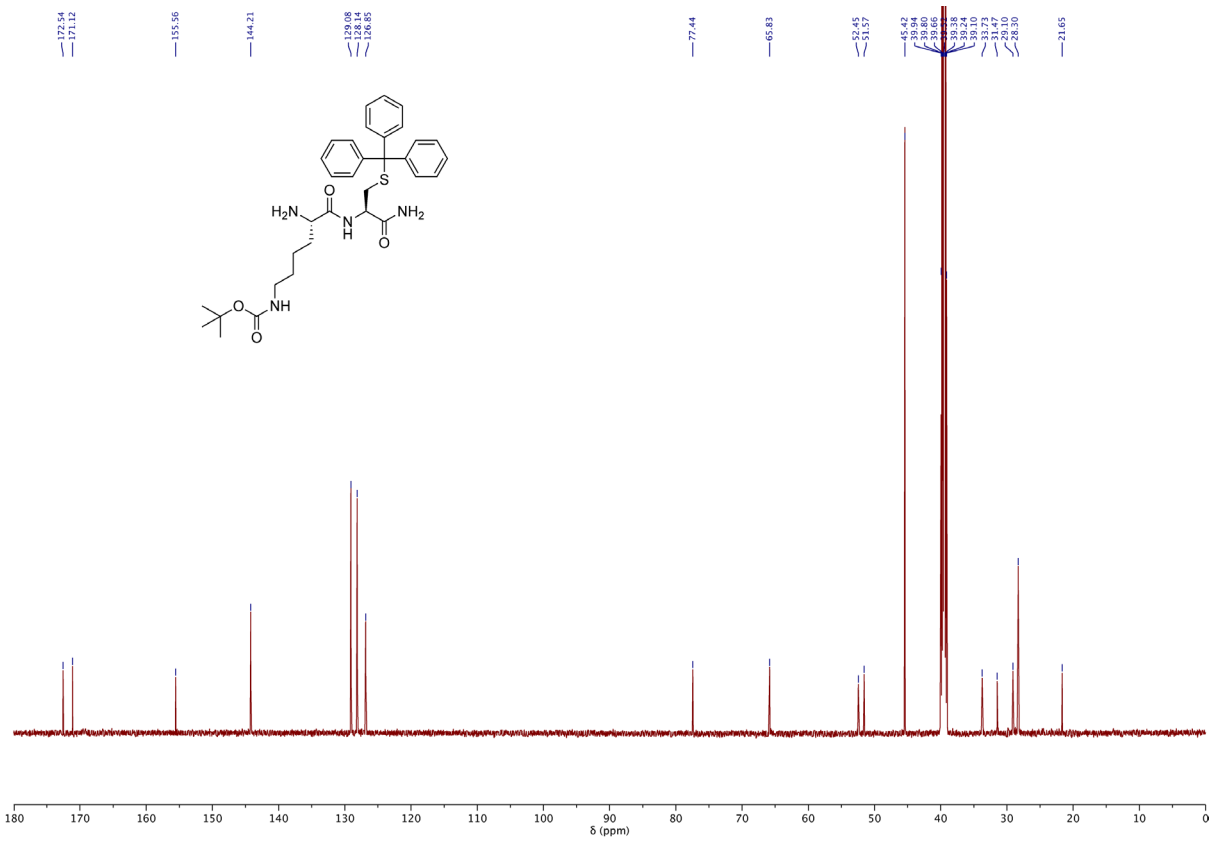


Fig. S13. ^{13}C NMR of **2** in DMSO-d_6.

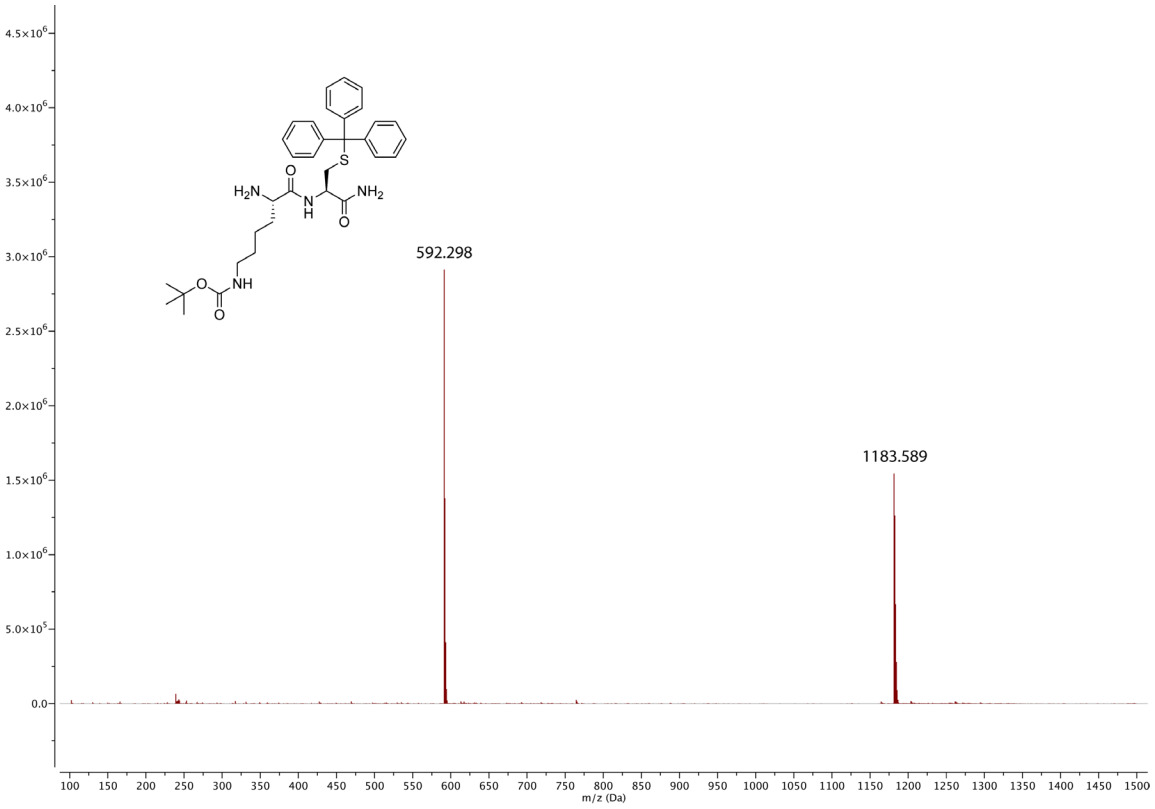


Fig. S14. ESI-MS spectrum of **2**.

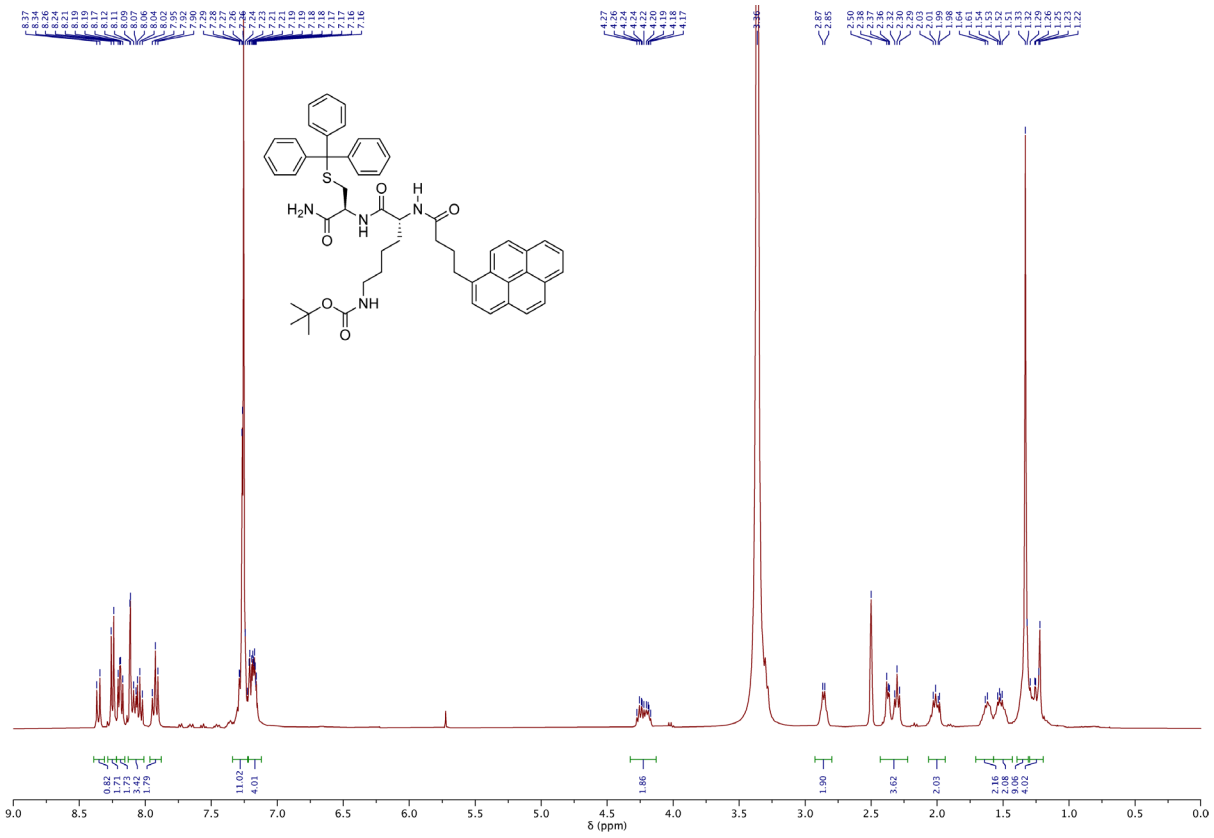


Fig. S15. ^{1}H NMR of **3** in DMSO-d_6.

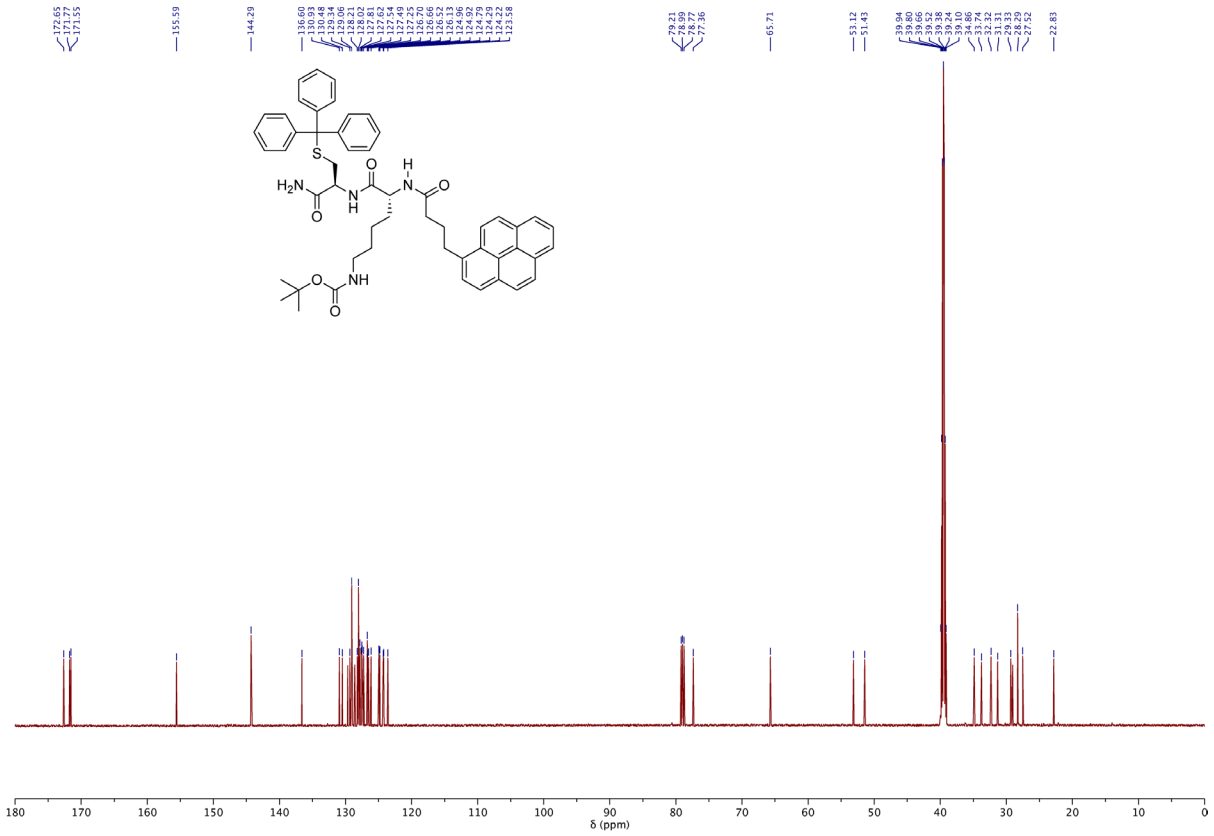


Fig. S16. ^{13}C NMR of **3** in DMSO-d_6.

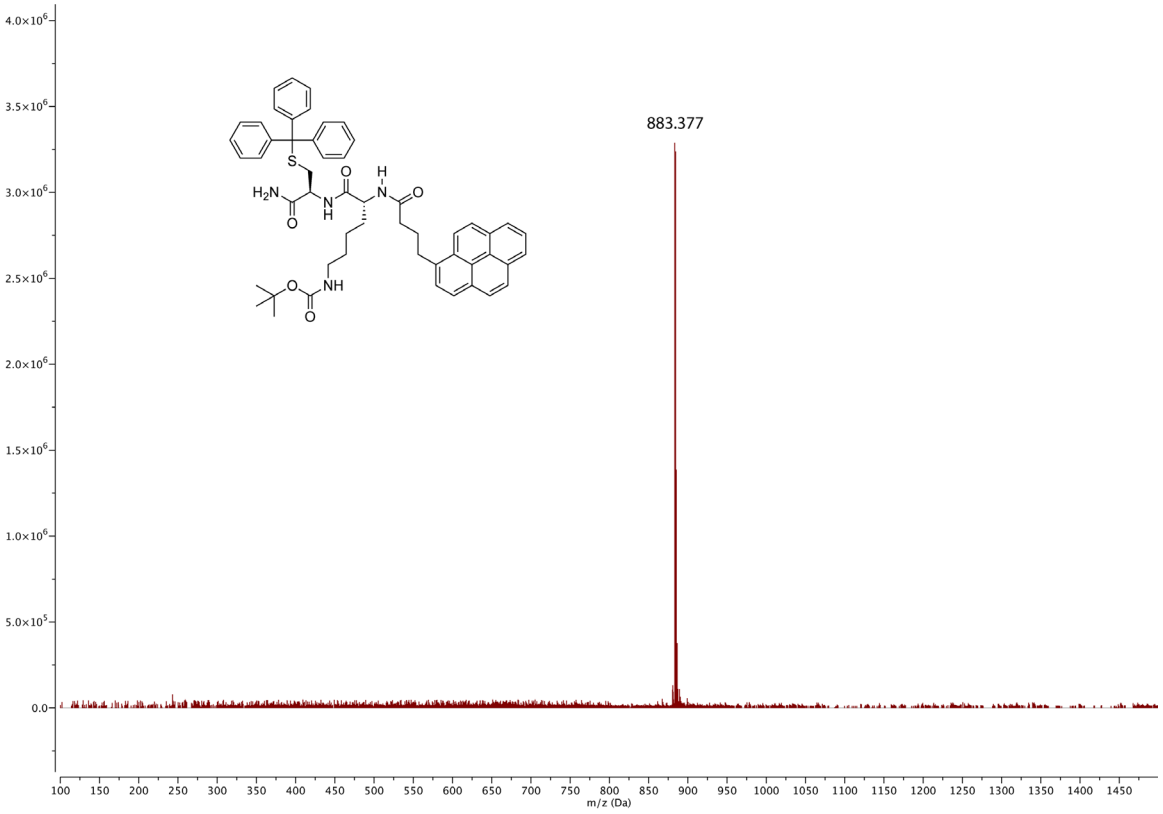


Fig. S17. ESI-MS spectrum of **3**.

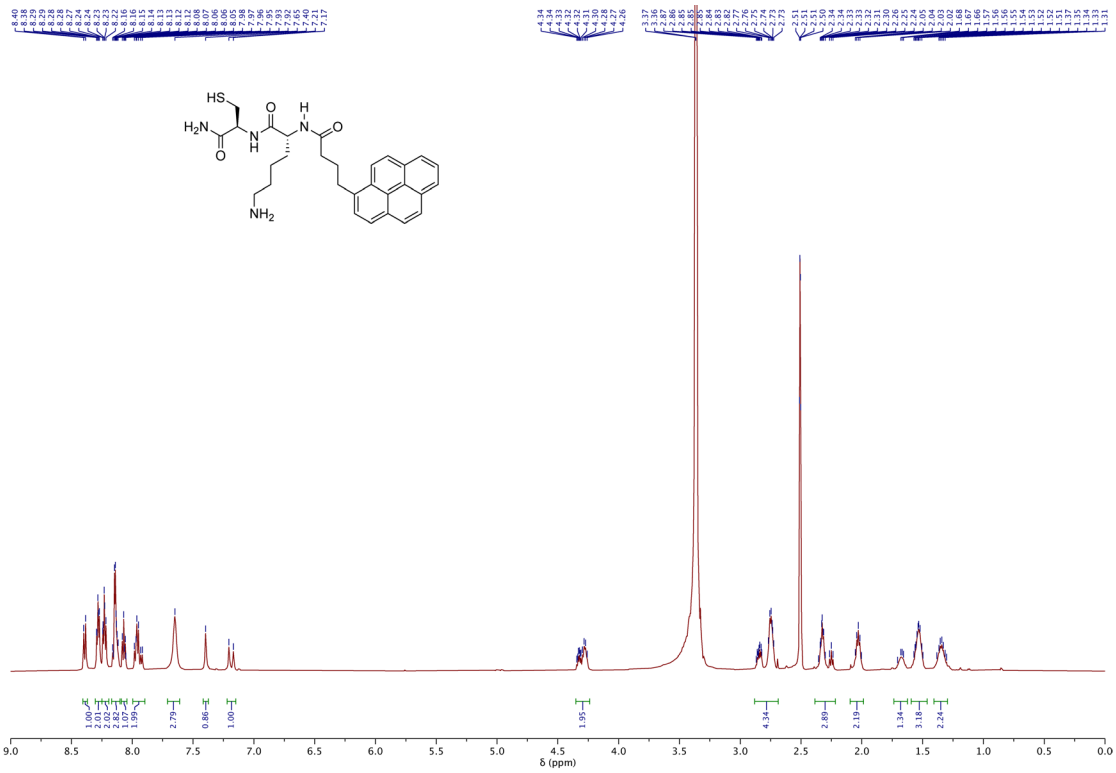


Fig. S18. ^{1}H NMR of **PyKC** in DMSO-d_6.

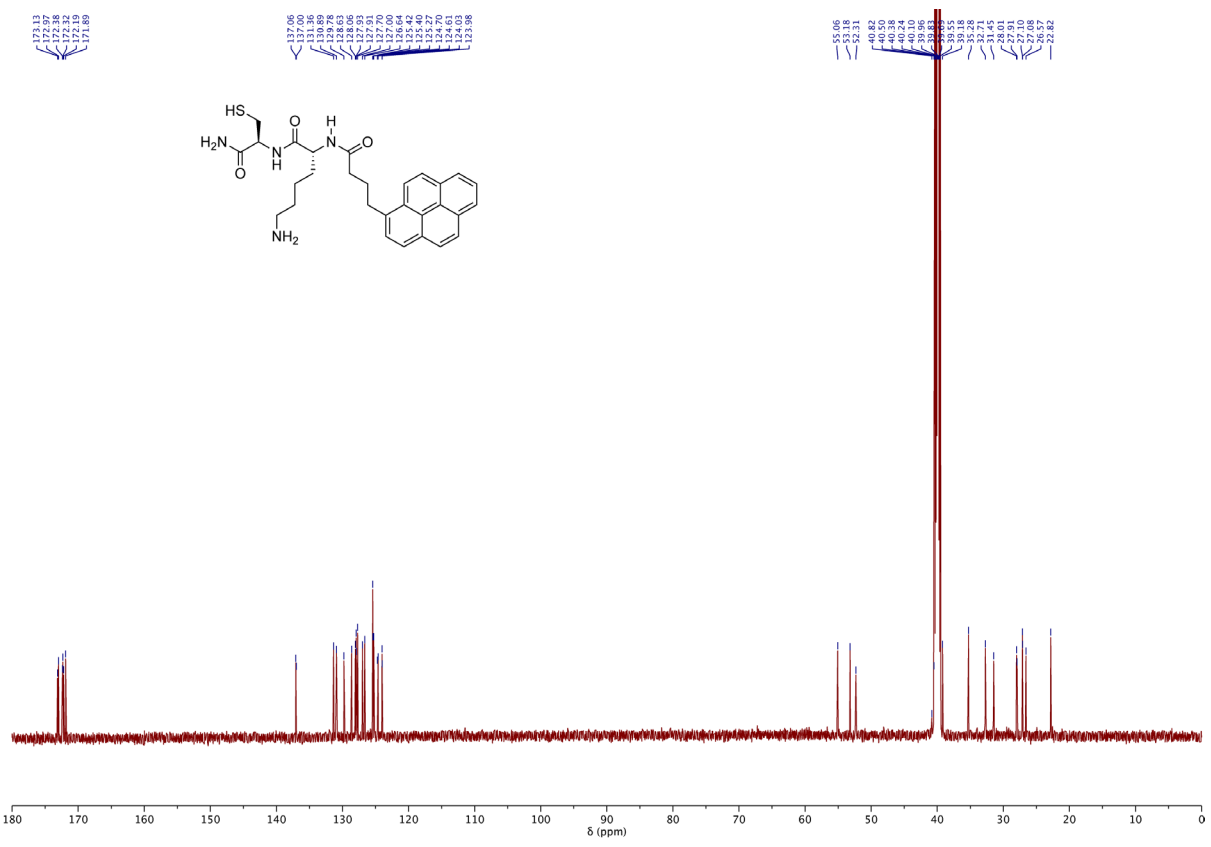


Fig. S19. ^{13}C NMR of **PyKC** in DMSO-d_6.

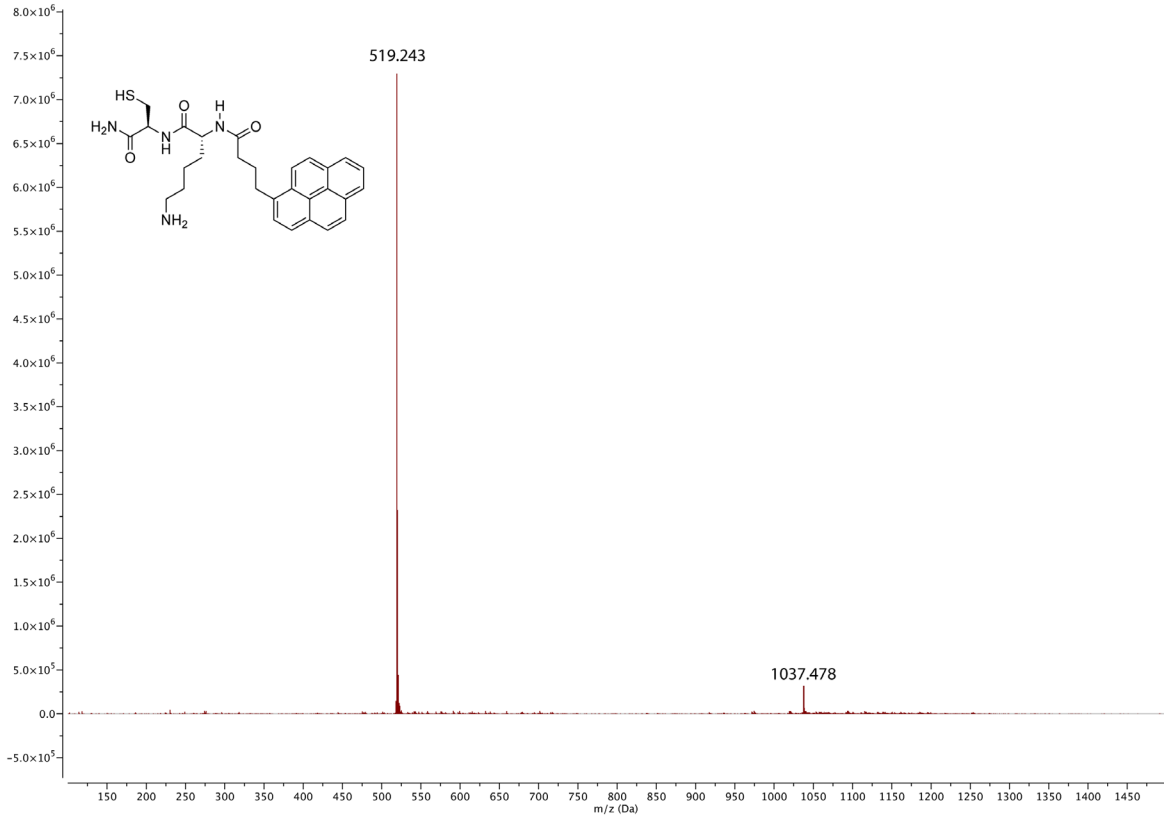


Fig. S20. ESI-MS spectrum of **PyKC**.

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