Naphthalimide-based peptide conjugate for concurrent imaging and apoptosis induction in cancer cells by utilizing endogenous hydrogen sulfide

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Material and methods

1.0 General information: Acetonitrile (CH_3CN), Dichloromethane (DCM) and Methanol (MeOH) were distilled following standard procedures before use. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl), 1-Hydroxybenzotriazole (HOBt), Trifluoroacetic acid (TFA), Piperidine, Beta-alanine and Hydroiodic acid (HI) were purchased from Avra synthesis Pvt. Ltd. (Hyderabad, India). 2, 4-Dinitrofluorobenzene and 4-Bromo-1,8-naphthalic anhhydride were purchased from TCI chemicals private limited (India). N, N'-Diisopropylcarbodiimide (DIC) and 4-Dimethylaminopyridine (DMAP) were obtained from Spectrochem (Mumbai, India). Triisopropylsilane (TIPS) and Sodium bicarbonate were obtained from Merck. Fmoc-Phe-OH and Wang resin were purchased from Novabiochem. Dry N, N-dimethylformamide (DMF) was purchased from Finar chemicals and used as such. Sodium carbonate was purchased from Rankem; potassium carbonate, potassium bicarbonate and hydrochloric acid were purchased from Thermo Fisher Scientific; tri-ethyl amine was purchased from S-D Fine Chem. Limited (Mumbai) and was used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on readymade TLC silica gel 60F254 plates (Merck, Darmstadt, Germany) and compounds were

visualized with UV light at 254 nm. 100−200 mesh silica gel (S. D. Fine-Chem Pvt. Ltd.) was used for chromatographic separation.

2.0 NMR spectroscopy: Samples were prepared by dissolving the compound in DMSO-d_6 and CD_3OD. ^1H and ^{13}C-NMR spectra were recorded at 25 °C on JEOL-JNM ECS 400 and 500 MHz JEOL ECX spectrometer. 1D spectrum was recorded at a peptide concentration of 10 mg/400 µL in DMSO-d_6 and CD_3OD at 298 K.

3.0 UV-vis studies: UV-vis absorption spectra were recorded on Lab India UV-VIS Spectrophotometer 3000+ with 10 mm quartz cell at 25±0.1 °C.

4.0 Fluorescence spectroscopy: Luminescence Cary eclipsed with 10 mm quartz cell at 25±0.1°C. All peptide samples were excited at λ_{ex} = 450 nm wavelength and the emission was recorded at λ_{em} = 550 nm for the **HSNPc**.

5.0 Scanning electron microscopy (SEM): Field emission scanning electron microscopy (FE-SEM) images were acquired on FEI QUANTA 200 microscope, equipped with a tungsten filament gun, operating at a working distance of 4.0 mm and 7.99 kV. A 10 µL aliquot of freshly prepared solution (in ethanol and dichloromethane) was placed on the appropriate surface(s) and allowed to dry at room temperature for overnight followed by drying under a high vacuum for another 30 minutes. The sample was gold-coated for 1 min and then imaged with FE-SEM.

6.0 Fluorescence optical microscopy: Hela cells and human mesenchymal stem cells (hMSCs) were treated with **HSNPc** and stained by DAPI. Next, treated cells were examined under a fluorescent optical microscope (Leica DM2500M). The samples were mounted on glass slides and analyzed under the fluorescent optical microscope using red, blue filters. **HSNPc** showed fluorescence in the red filter and DAPI showed fluorescence in the blue filter.

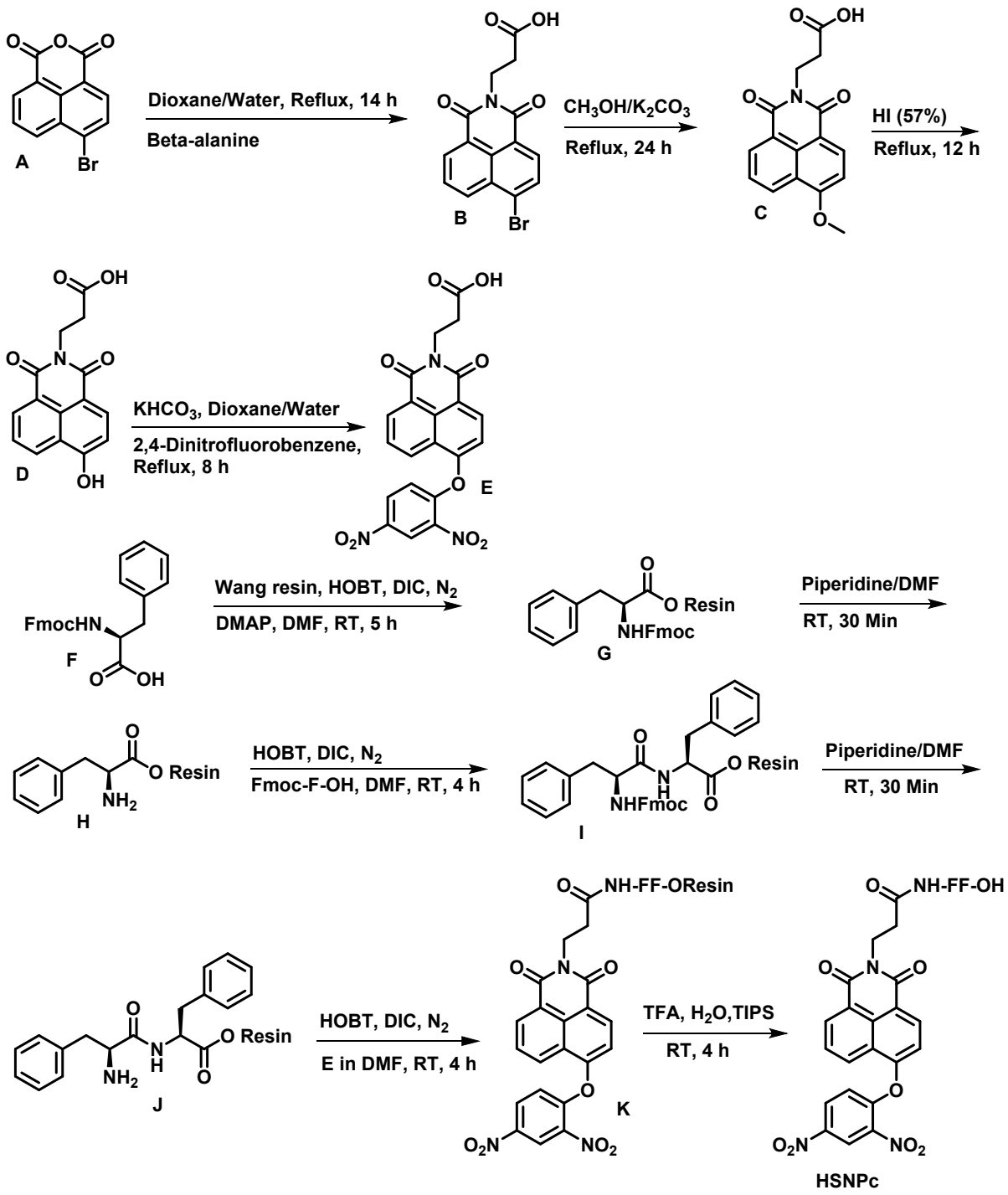
7.0 Mass spectrometry: HRMS mass spectra were recorded on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 kV.

8.0 High-performance liquid chromatography (HPLC): HPLC analysis was performed with Agilent technologies 1260 infinity, HPLC system equipped with a quaternary pump

(G1311B), auto liquid sampler (G1329B), Diode array detector (G1315D) and analytical scale fraction collector (G1364C). Instrumental control, data acquisition, and processing were performed using ChemStation software (Agilent Technologies, Wokingham, UK). A ZORBAX Eclipse plus C18 (250 x 4.6 mm) column with 5 µm particle size at room temperature was used. Acetonitrile and water were used as mobile phase and the flow rate was 1 mL/min. Injection volume was 20 µL and effluent was measured at 220 nm and 254 nm. A 1 mM solution of **HSNPc** was treated with 10 mM of Na_2S. After Na_2S treatment, two peaks were observed in HPLC. These peaks belonged to 2, 4 dinitrobenzenethiol and naphthalimide moiety.

FT-IR Study- FT-IR spectra of HSNPc and Peptide linked 1,8-naphthalimide(control) were performed in the range of 4000 cm^{-1} to 500 cm^{-1} using Bruker Alfa II ATR, FTIR spectrometer. Spectra were processed; base line correction and smoothed using OPUS 7.0 software and removed unwanted noise.

FTIR data analysis: Savitsky–Golay method **was adopted for the** decomposition of FT-IR spectra. The second derivative spectra of the processed FT-IR spectra were obtained using origin software. Amides I bands of both compounds were deconvolated using multiple Gaussian peaks fitting using origin software. The second derivative spectrum was used to fit the number of initial peak values. The contribution of each component was calculated by measuring area of the curve of each component.



Scheme 1: Synthetic scheme of HSNPc

9.0 Synthesis: HSNPc was synthesized by using standard lab protocols[1–3] and similar to previously reported solid phase peptide synthetic methods[4] and solution phase synthetic methods followed by simple purification and characterization[5–8]

9.1 Synthesis of B: 4-Bromo-1, 8-naphthalic anhydride (5 g, 18.04 mmol) and β-Alanine (1.60 g, 18.04 mmol) were dissolved in 150 mL Dioxane:Water (80:20). The reaction mixture

was stirred and refluxed for 14 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The crude compound was purified through silica gel column chromatography to give pure B (6.0 g, yield: 95.54 %). ^{1}H NMR (400 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 8.46-8.33 (m, 2H), 8.20 (d, 1H), 8.10 (d, 1H), 7.89 (t, 1H), 4.18 (t, 2H), 2.55 (t, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 173.00, 163.22, 163.17, 133.10, 132.03, 131.81, 131.40, 130.19, 129.66, 129.25, 128.68, 123.14, 122.36, 36.40, 32.66.

9.2 Synthesis of C: A mixture of compound B (5 g, 14.36 mmol) and K_2CO_3 (15.87 g, 114.89 mmol) in 200 mL Methanol was refluxed for 24 h. After cooling to room temperature, pH value was adjusted to about 2 by adding concentrated HCl. The precipitate was filtered and washed with water (30 mL × 3) and dried to yield compound C as yellow solid (3.8 g, yield: 88.57 %). ^{1}H NMR (400 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 8.46-8.33 (m, 3H), 7.72 (t, 1H), 7.23 (d, 1H), 4.18 (t, 2H), 4.07 (s, 3H), 2.54 (t, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 173.02, 163.94, 163.28, 160.83, 133.76, 131.50, 129.00, 128.78, 126.85, 123.19, 122.29, 114.63, 106.74, 57.14, 36.10, 32.81.

9.3 Synthesis of D: A mixture of compound C (2 g, 6.68 mmol) and 50 mL concentrated HI (57%) was refluxed for 12 h. After cooling and adjusting pH to neutral, the precipitate was filtered. The crude compound was purified through silica gel column chromatography to give pure D (1.6 g, yield: 84.21 %). ^{1}H NMR (400 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 8.46 (d, 1H), 8.38 (d, 1H), 8.27 (d, 1H), 7.68 (t, 1H), 7.08 (d, 1H), 4.18 (t, 2H), 2.52 (t, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 173.05, 164.08, 163.37, 160.91, 134.08, 131.63, 129.70, 129.47, 126.06, 122.88, 122.21, 112.95, 110.46, 36.03, 32.84.

9.4 Synthesis of E: Compound D (1 g, 3.50 mmol) was dissolved in 50 mL Dioxane and $KHCO_3$ (7.0 g, 70 mmol) dissolved in 50 mL water and mixed together. After 5 min 1-Fluoro-2,4-dinitrobenzene (0.88 mL, 7 mmol) was added into the reaction mixture. The reaction mixture was then heated at 90˚C for 8 h under N_2 atmosphere. After cooling to room temperature, the reaction mixture was concentrated in vacuo and redissolved in ethyl acetate. The organic layer was then washed with 1N HCl (30 mL x 3). The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The crude compound was purified through silica gel column chromatography to isolate pure E

as a yellowish solid (1 g, yield: 66.66 %). ^{1}H NMR (400 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 8.97 (d, 1H), 8.55 (dd, 1H), 8.51 (m, 2H), 8.45 (d, 1H), 7.91 (t, 1H), 7.51 (d, 1H), 7.46 (d, 1H), 4.23 (t, 2H), 2.57 (t, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 173.0, 164.02, 163.32, 160.80, 152.36, 141.66, 139.72, 133.83, 131.57, 129.88, 129.05, 129.24, 126.46, 122.68, 122.39, 122.32, 120.44 114.57, 107.56, 36.15, 32.80.

9.5 Synthesis of Fmoc-Phe-Resin (G): Wang resin (263.15 mg, 0.3 mmol) was taken in reaction apparatus and washed by DMF (3 x 5 mL) after that left for swelling for 30 minutes in DCM (5 mL). The swelled resin was further washed by DMF (3 x 5 mL). A solution of Fmoc–Phe-OH (464 mg, 1.2 mmol), DMAP (18.3 mg, 0.15 mmol) and HOBt (162 mg, 1.2 mmol) in 5 mL of DMF was added to the resin and a continued flow of nitrogen gas purged to stir reaction mixture. Further DIC (233 µL, 1.5 mmol) was added to it and left reaction mixture to stir by purging nitrogen gas for 5 h.

After that, the amino acid bounded resin was washed by DMF (3 x 5 mL) and DCM (3 x 5 mL). Now that resin was dried under vacuum and weight of this amino acid bounded resin (Fmoc-Phe-resin) was found 380 mg.

9.6 Synthesis of Fmoc-Phe-Phe-Resin (I): Fmoc-Phe-resin (G) was taken in reaction apparatus and washed by DMF (3 x 5 mL) after that left for swelling in DCM (5 mL) for 30 minutes. Now that swelled Fmoc-Phe-resin was washed by DMF (3 x 5 mL). Then it was treated with 20% piperidine in DMF (5 mL) and stirred by purging nitrogen gas for 5 minutes and filtered. Again, it was treated with 20% piperidine in DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. Now, this Phe-resin was washed by DMF (3 x 5 mL).

A solution of Fmoc–Phe-OH (464 mg, 1.2 mmol) and HOBt (162 mg, 1.2 mmol) in 5 mL of DMF was added to the Phe-resin and a continued flow of nitrogen gas was purged to stir the reaction mixture. Further DIC (189 µL, 1.2 mmol) was added to it and left reaction mixture to stir by purging nitrogen gas for 4 h. After 4 h some beads of Fmoc-Phe-Phe-resin were tested for monitoring this coupling reaction by Kaiser's test. If the test was found positive, stirring was continued till Kaiser's test turn into negative. After complete reaction, Fmoc-Phe-Phe-resin was washed by DMF (3 x 5 mL).

9.7 Synthesis of K: Fmoc-Phe-Phe-resin (I) (415 mg, 0.3 mmol) was treated with 20% piperidine in DMF (5 mL) and stirred by purging nitrogen gas for 5 minutes and filtered. Again, it was treated with 20% piperidine in DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. Now, this Phe-Phe-resin was washed by DMF (3 x 5 mL).

A solution of (E) (541 mg, 1.2 mmol) and HOBt (162 mg, 1.2 mmol) in 5 mL of DMF was added to the Phe-Phe-resin and a continued flow of nitrogen gas purged to stir the reaction mixture. Further DIC (189 µL, 1.2 mmol) was added to it and left reaction mixture to stir by purging nitrogen gas for 4 h. After 4 h some beads of (K) were tested for monitoring this coupling reaction by Kaiser's test. If the test was found positive, stirring was continued till Kaiser's test turn into negative. After complete reaction, (K) was washed by DMF (3 x 5 mL). Then it was dried under vacuum.

9.8 Synthesis of HSNPc: (K) was treated with 10 mL mixture of TFA:TIPS:water (95:2.5:2.5 %) to cleave resin from **HSNPc** conjugate. Reaction was completed in 4 h. The resin was removed by filtration and the filtrate was evaporated under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. Now it was dried under vacuum to give crude **HSNPc** which was purified by silica gel chromatography using 5% MeOH/DCM to give yellow colour solid (yield: 85%). ^{1}H NMR (400 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 12.77 (s, 1H), 8.97 (d, 1H), 8.61-8.45 (m, 4H), 8.19 (dd, 2H), 7.91 (t, 1H), 7.48 (dd, 2H), 7.28-7.12 (m, 10H), 4.55-4.34 (m, 2H), 4.11(s, 1H), 3.10-2.82 (m, 4H), 2.49 (d, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6, 25 ˚C) δ(ppm) = δ 173.25, 171.78, 170.03, 163.68, 163.06, 155.91, 153.56, 143.67, 140.86, 138.46, 137.96, 132.58, 132.23, 130.63, 129.92, 129.68, 128.91, 128.73, 128.60, 128.45, 126.99, 126.94, 126.67, 124.08, 122.85, 122.70, 119.77, 115.29, 54.03, 40.94, 37.15, 36.85, 33.58.; HRMS $[M+Na]^+$ for $C_{39}H_{31}N_5O_{11}$; 768.1923 (calcd.), 768.1935 (anal).

Figures

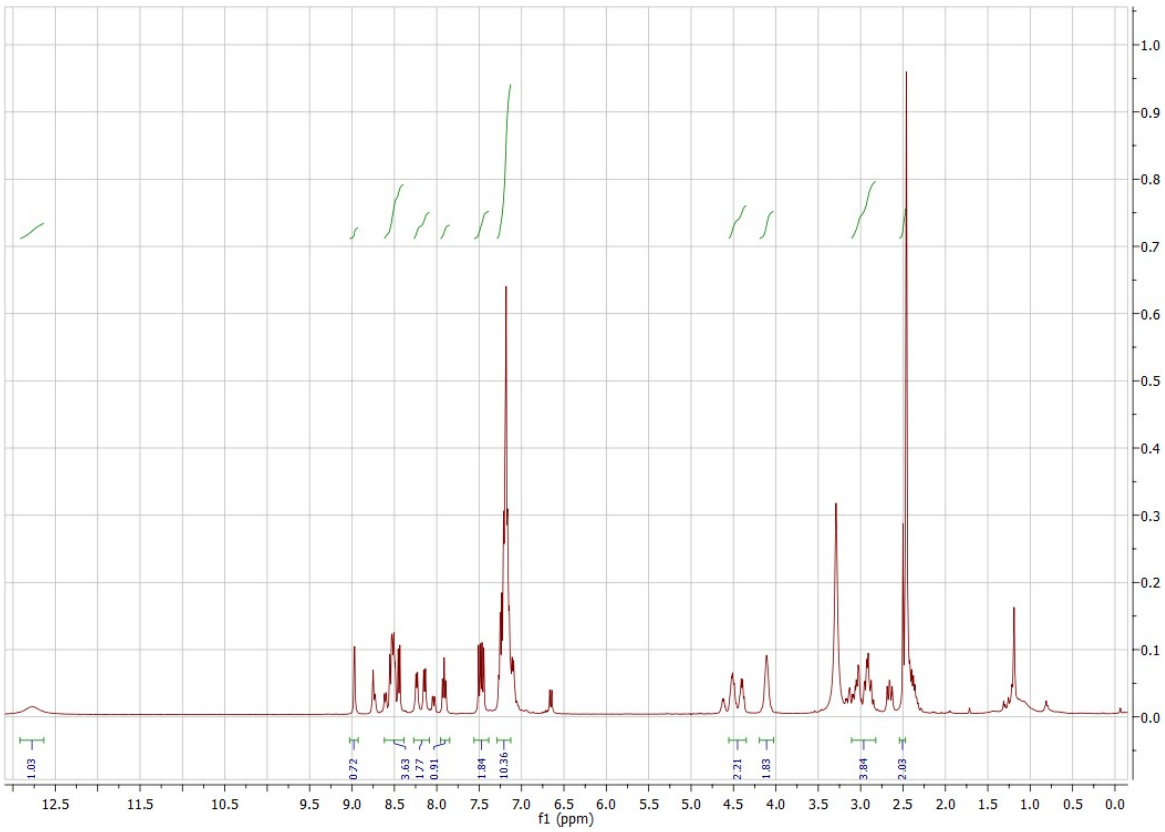


Figure S1.[1]H-NMR spectrum of **HSNPc**.

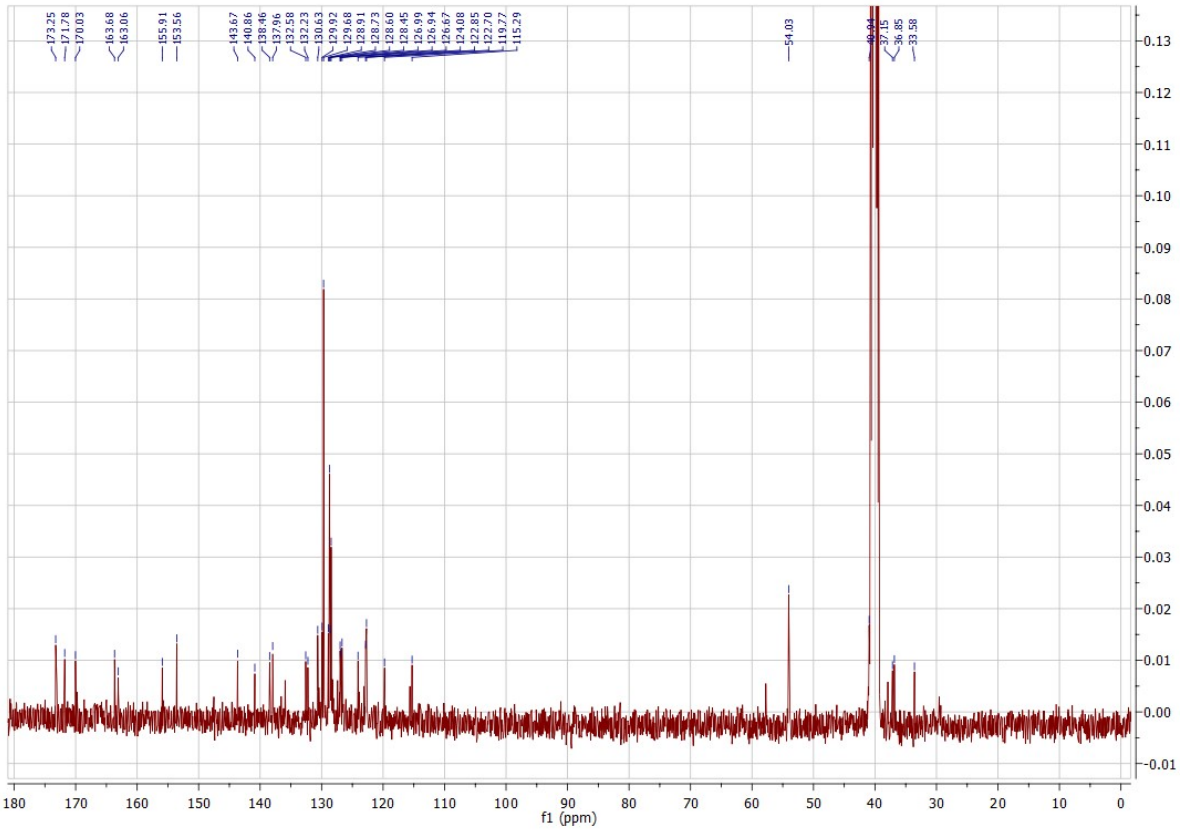


Figure S2. ^{13}C-NMR spectrum of **HSNPc**.

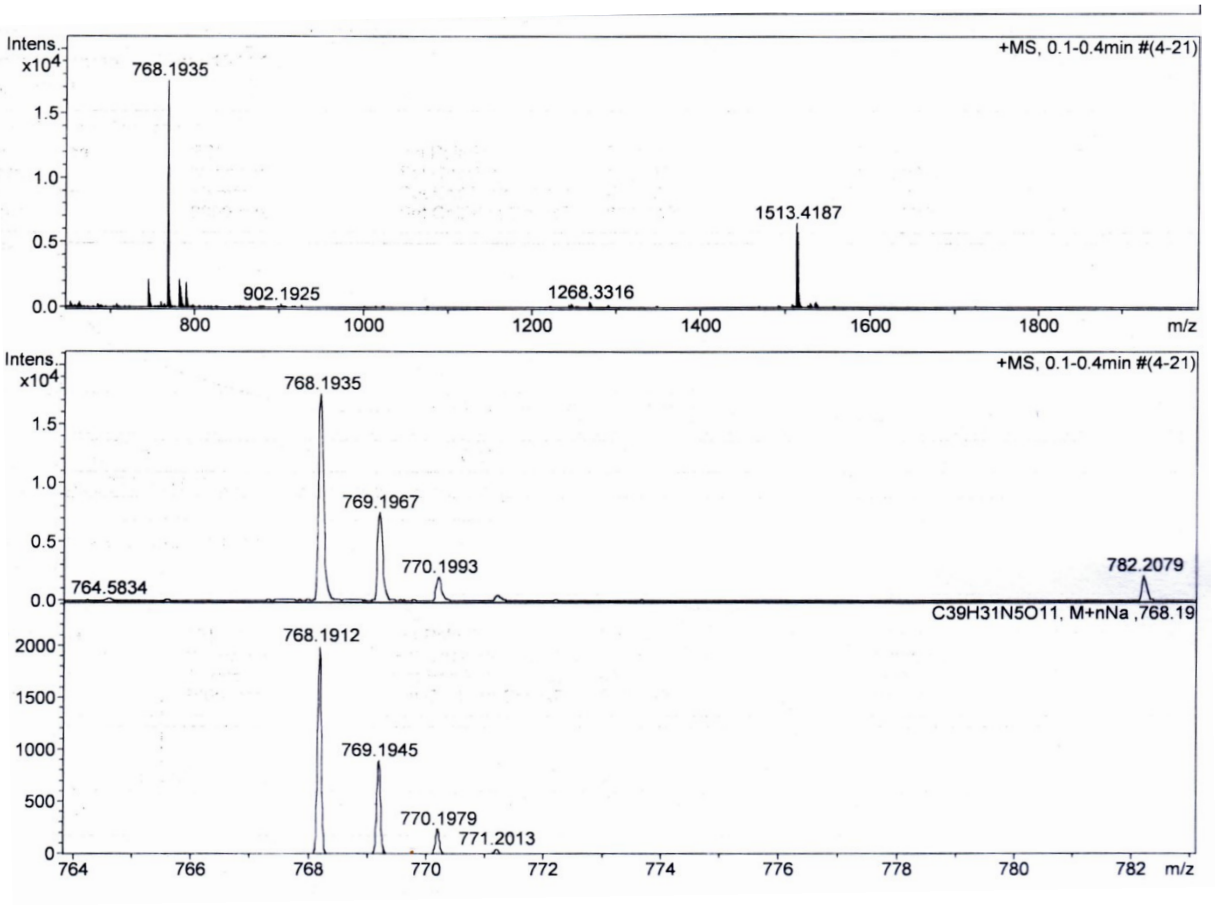


Figure S3. ESI-HRMS spectrum of **HSNPc**.

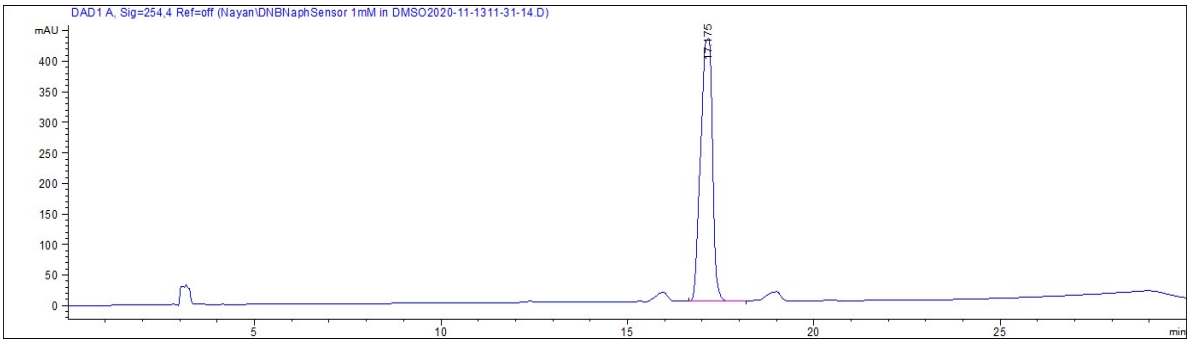


Figure S4. Analytical HPLC spectrum of pure **HSNPc** (1 mM).

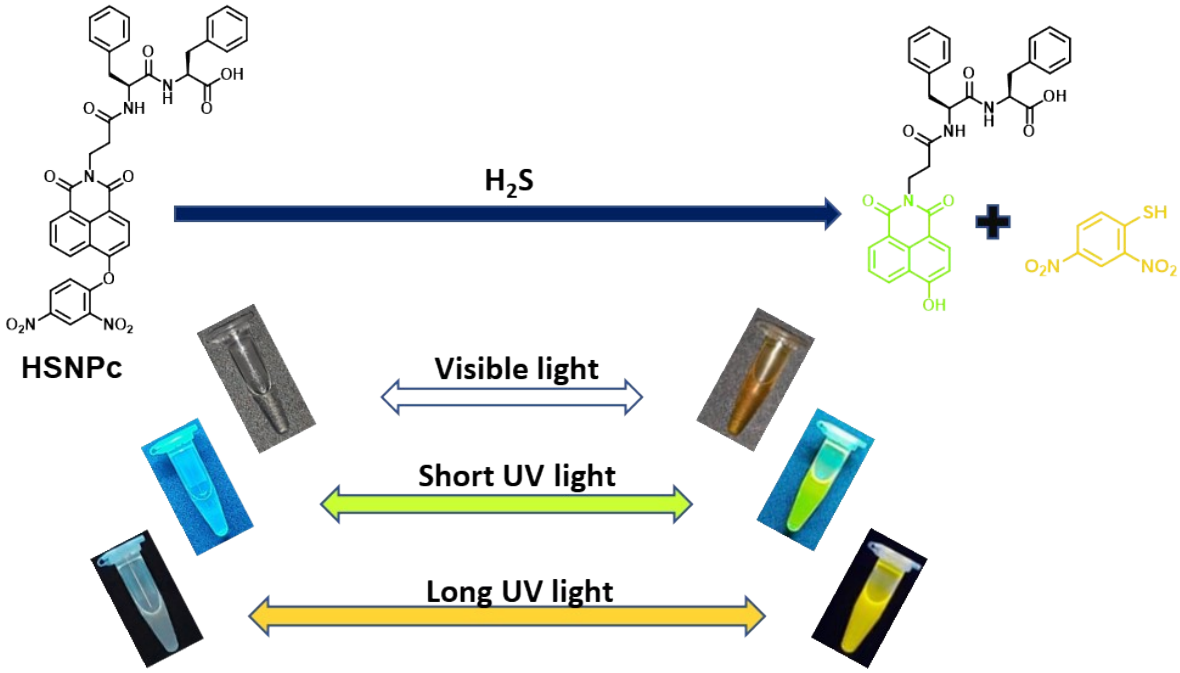


Figure S5. Colour change after the addition of H_2S solution in the solution of **HSNPc**.

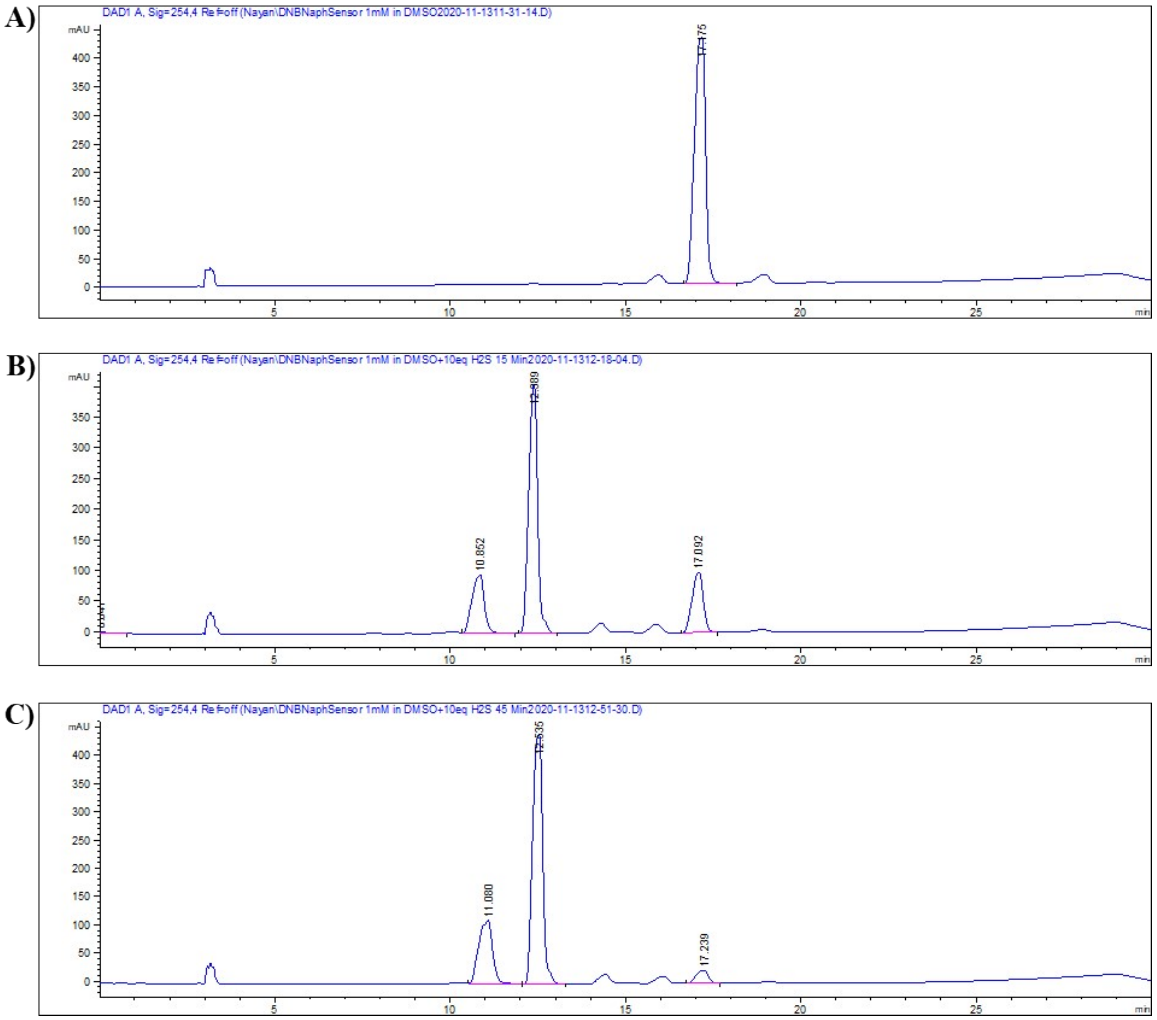


Figure S6. Analytical HPLC spectra of A**)** pure **HSNPc** (1 mM), B) 1 mM **HSNPc** treated with 10 mM Na_2S, reaction time 15 min, C) 1 mM **HSNPc** treated with 10 mM Na_2S, reaction time 45 min.

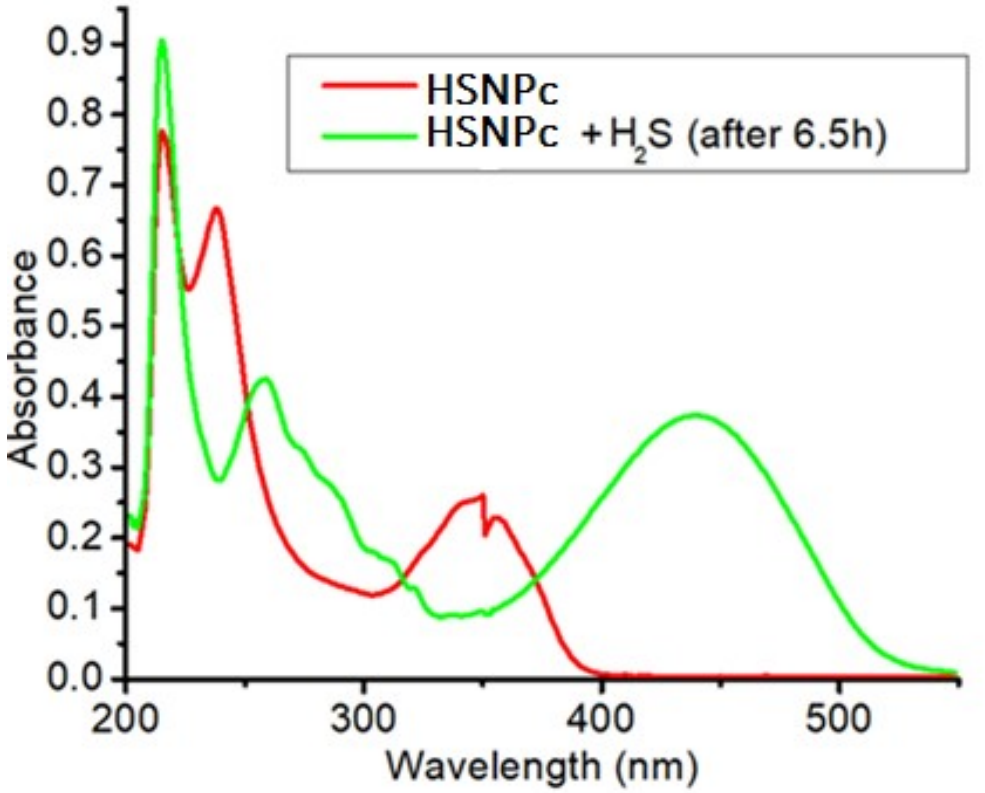


Figure S7. UV-vis spectrum of **HSNPc** (red) & **HSNPc** with H_2S (green).

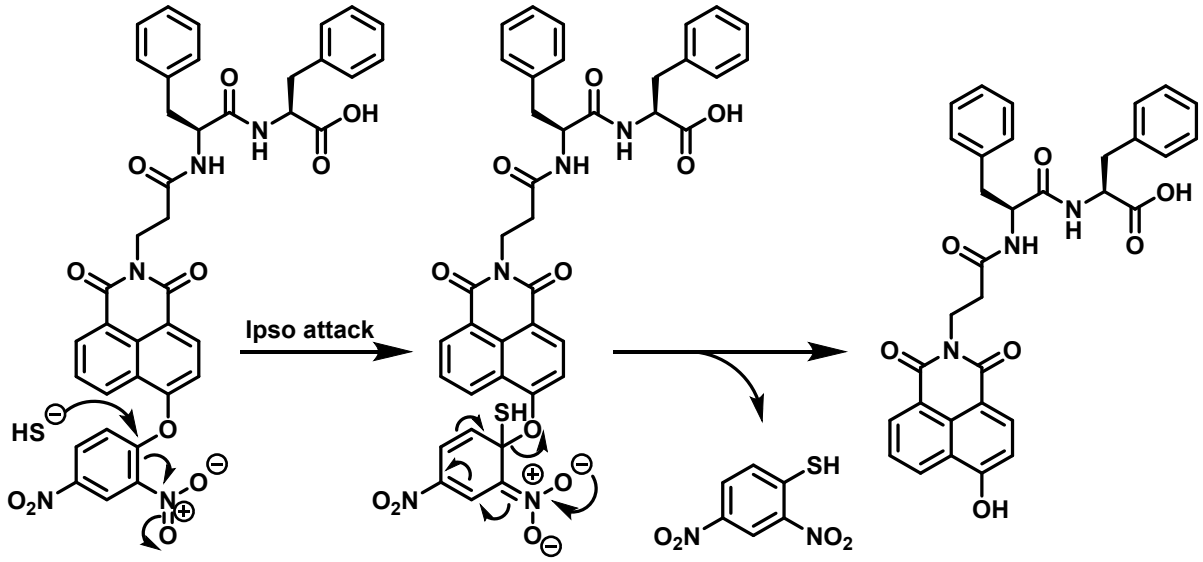


Figure S8. Nucleophile (HS^-) attack and cleavage of H_2S responsive group.

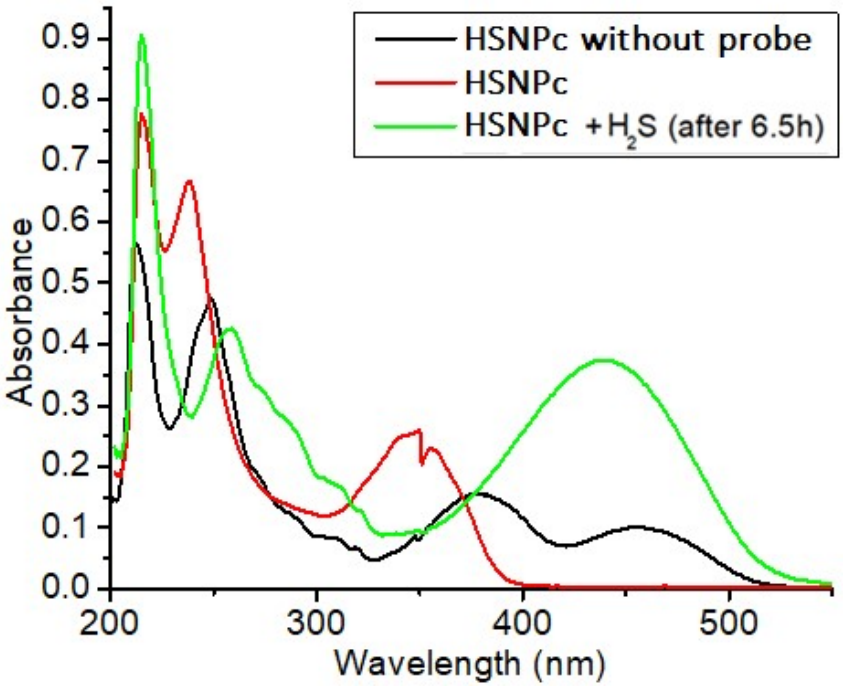


Figure S9. UV-vis spectrum of **HSNPc** without probe (black), **HSNPc** (red) & **HSNPc** with H_2S (green).

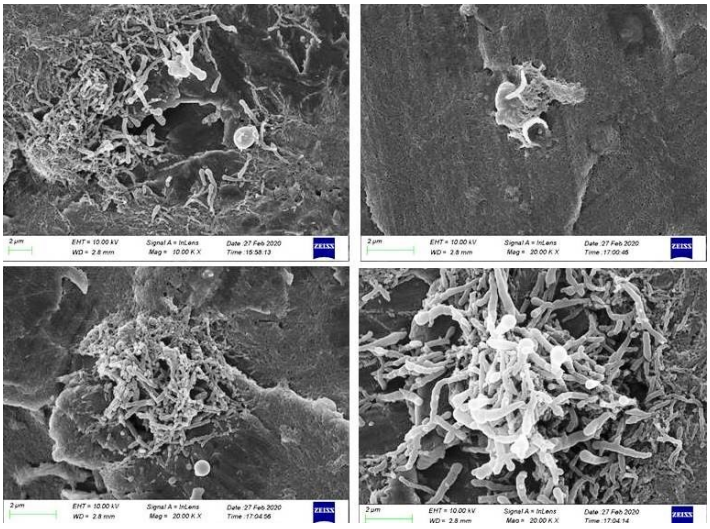


Figure S10. FE-SEM images of **HSNPc** (500 µM solution in Ethanol & Dichloromethane).

To check the reactivity of **HSNPc** with other sulfur-containing molecules like glutathione (GSH) and cysteine, we treated **HSNPc** with the 10 equivalents of glutathione and cysteine and did not observe any change in **HSNPc** peak after 24 h while in the case of sodium sulfide treated **HSNPc**, we observed a new peak at ~550 nm in fluorescence spectroscopy (Figure S11 A and B) and this happened because of peptide conjugated

naphthalimide moiety which was produced after HSNPc reaction with HS^- ion generated from aqueous sodium sulfide. The same results were found with UV-Vis spectroscopy (Figure S11 C) where we observed a new peak at ~440 nm in the case of sodium sulfide treated **HSNPc** whereas, no change in **HSNPc** peak was found in the case of glutathione and cysteine treatment. Previous literature also supports the selectivity and sensitivity of such molecules over other sulfur-containing molecules.[9]

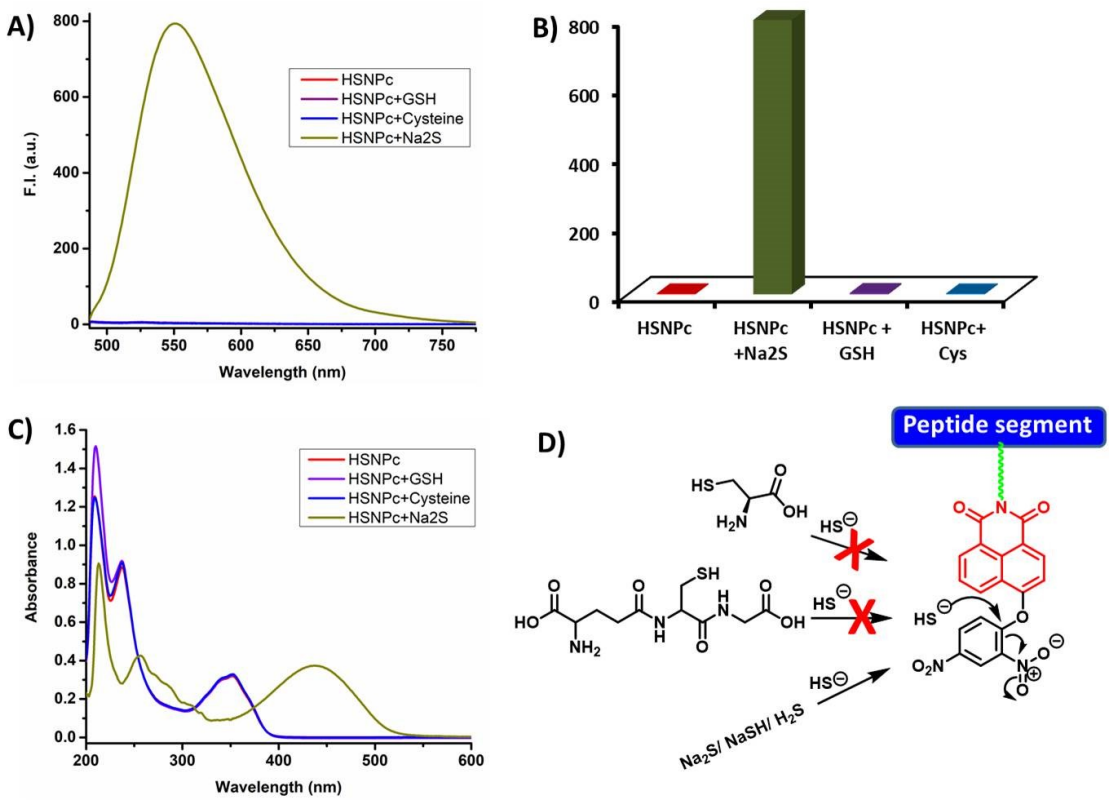


Figure S11. Control experiments depict the selectivity of HSNPc towards endogenous/exogenous H_2S only. A) Fluorescence Spectra of GSH, Cysteine, and Na_2S treated HSNPc after 24 h and B) corresponding bar graph clearly represent the HSNPc is not active in the presence of GSH and L-cysteine, however it is active in the presence of H_2S only, C) further the UV-Vis spectra of HSNPc in the presence of GSH and L-cysteine and H_2S clearly revealed that the HSNPs conjugate only active in the presence of H_2S and D) the proposed mechanism.

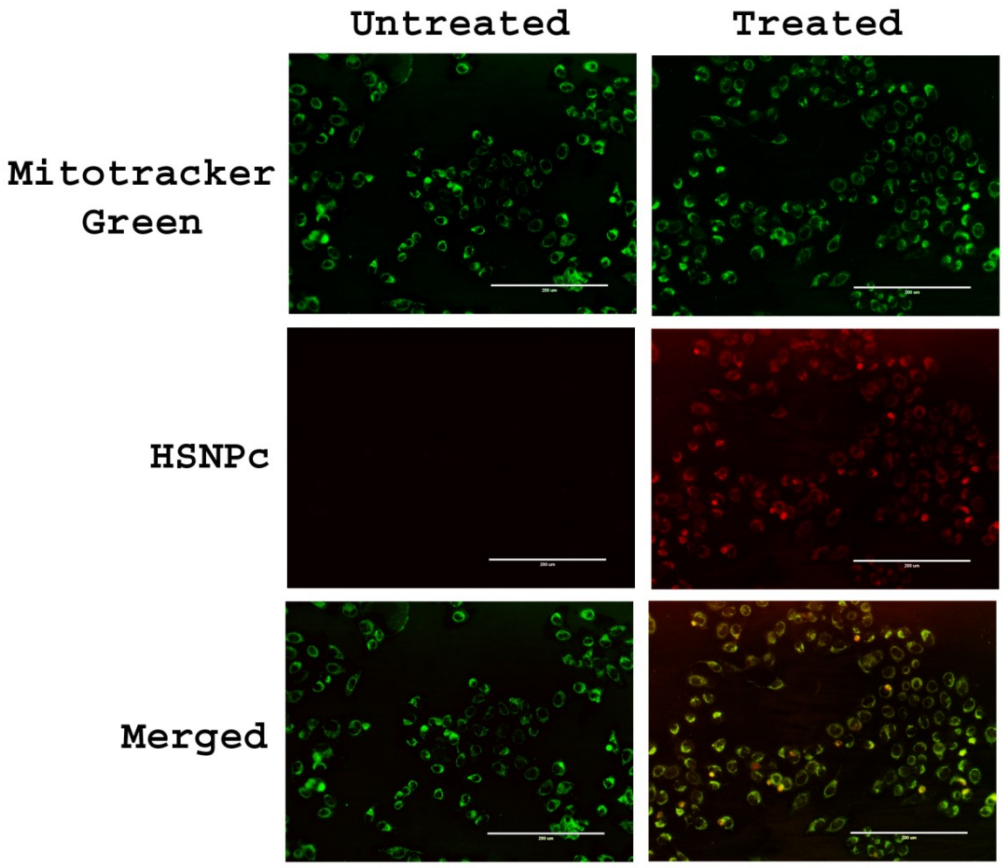


Figure S12. **HSNPc** colocalization with mitotracker green.

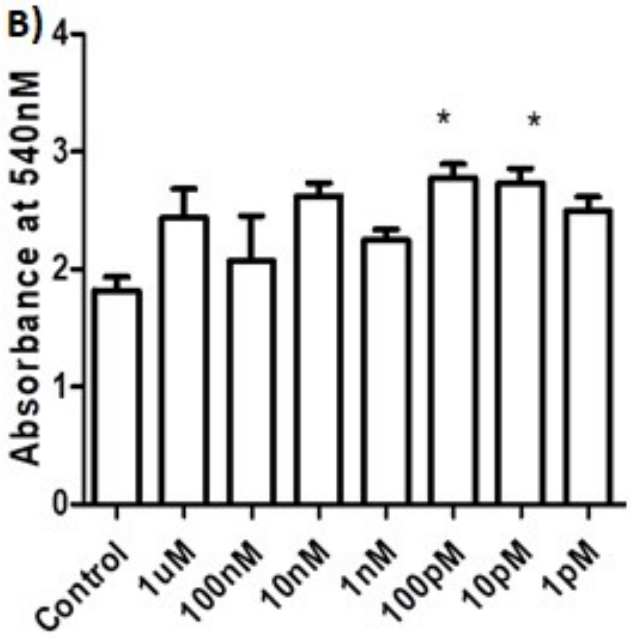


Figure S13. hMSC were exposed to vehicle (DMSO) that served as control, and **HSNPc** at indicated concentrations for 48 h to determine cellular toxicity by MTT assay. Data represents mean ± SEM of three independent experiments in triplicate; *p < 0.05 **p < 0.01, ***p < 0.001 versus control (receiving vehicle).

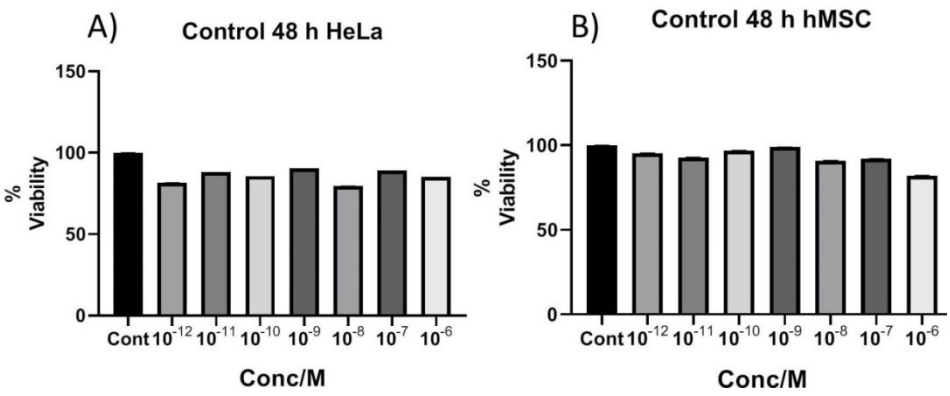


Figure S14: A) 1,8-naphthalimide linked peptide (Control) treated HeLa cells and B) 1,8-naphthalimide linked peptide (Control) treated hMSC cells.

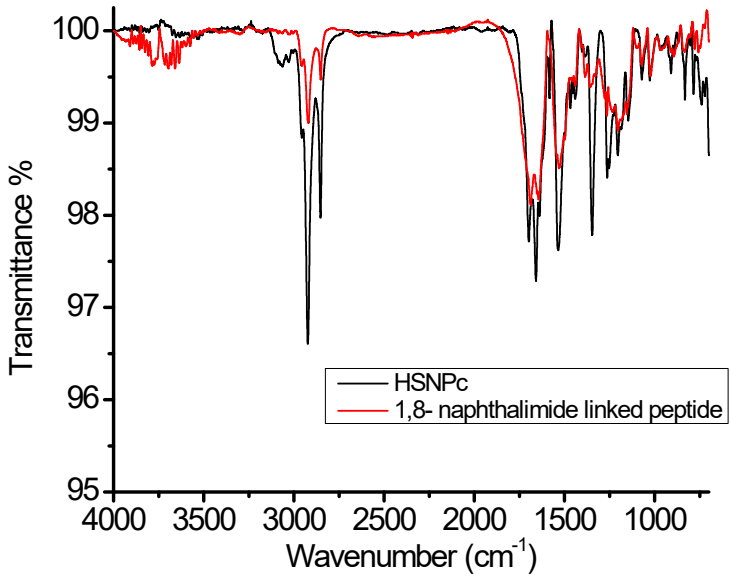


Figure S15. FT-IR Spectra of HSNPc and peptide linked 1,8-naphthalimide.

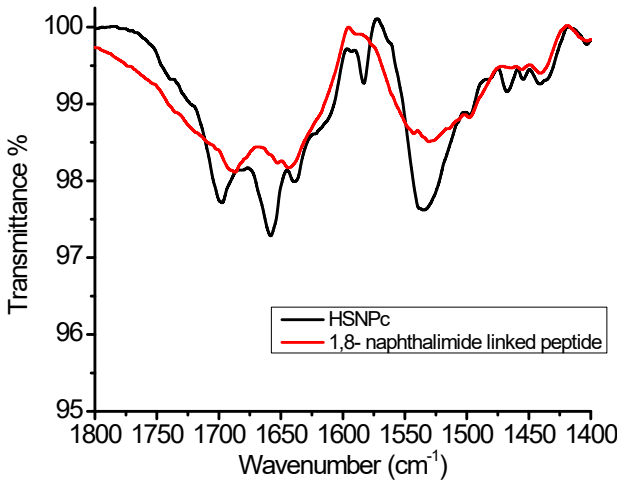


Figure S15. FT-IR Spectra of HSNPc and peptide linked 1,8-naphthalimide from 1400-1800 cm^{-1} highligting the amide I region.

Table S1. The percentage contribution of β-sheet, α-helix, antiparallel β-sheet and random coil pattern of secondary structure of HSNPc.

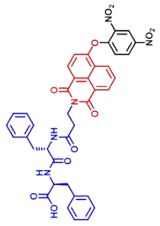
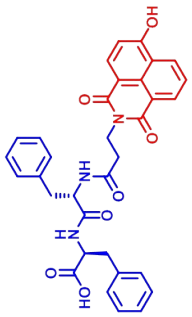
HSNPc	Structure	Wavenumber	Peak	Percent
	β-Sheet	1610-1640 cm^{-1}	1619 cm^{-1}, 1637 cm^{-1}	23.02%
	Random Coil	1640-1650 cm^{-1}	---	0%
	α-Helix	1650-1660 cm^{-1}	1657 cm^{-1}	32.29%
	Antiparallel β-Sheet	1660-1695 cm^{-1}	1695 cm^{-1}	44.05%

Table S2. The percentage contribution of β-sheet, α-helix, antiparallel β-sheet and random coil pattern of secondary structure of control molecule.

Peptide linked 1,8-naphthalimide (control)	Structure	Wavenumber	Peak	Percent
	β-Sheet	1610-1640 cm^{-1}	1617 cm^{-1}, 1639 cm^{-1}	15.17%
	Random Coil	1640-1650 cm^{-1}	---	0%
	α-Helix	1650-1660 cm^{-1}	--	0%
	Antiparallel β-Sheet	1660-1695 cm^{-1}	1689 cm^{-1}	84.83%

References:

1 N. Singh, R. Singh, M. Shukla, G. Kaul, S. Chopra, K. B. Joshi and S. Verma, *ACS Infect. Dis.*, 2020, **6**, 2441–2450.

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3 N. Singh, R. Singh, K. B. Joshi and S. Verma, *Chempluschem*, 2020, **85**, 2001–2009.

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