Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Self-assembled π-conjugated system as an anti-proliferative agent in prostate cancer cells and probe for intra-cellular imaging

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Experimental Section

Materials and Methods All commercial chemicals used for the synthesis of coumarin derivatives(**3a-c**) and fluorescent probes (**5a-c**) were purchased from Sigma Aldrich, Merck, Alfa aesar and Avra chemicals. Solvents used for gelation studies are of AR grade. For purification of compounds we have used LR grade solvents. Solvents were distilled before use, if required. Column chromatography was performed on Silica Gel (100-200 mesh) purchased from Avra synthesis, INDIA. L929 cell line was purchased from Sigma Aldrich and PC3 cells were obtained from National Centre For Cell Science(*NCCS*), *Pune*, India.

Purification of Cardanol:

The key constituent of CNSL being cardanol **1b**, a bio based non isoprene lipid, consisting of a rich mixture of phenolic lipids: 5% of 3-*n*-pentadecylphenol (3-PDP), 50% of 3-(8*Z*-pentadecenyl) phenol, 16% of 3-(8*Z*,11*Z*-pentadecadienyl) phenol and 29% of 3-(8*Z*,11*Z*,14-pentadecatrienyl) phenol. CNSL was distilled at a temperature between 210 and 280°C, under a pressure from 2 to 8 mm Hg to get cardanol. Cardanol is obtained as pale yellow liquid which darkens during storage. After a second distillation, mixture of cardanol mono-, di- and tri-ene was obtained.

Characterisation: ^{1}H, ^{13}C NMR spectras were recorded on a Bruker DRX 300 MHz instrument in either $CDCl_3$ or $CDCl_3$ with few drops of DMSO-d_6. Chemical shifts (δ) are reported in parts per million (ppm) from the standard TMS and coupling constants (*J*) in Hz. Proton multiplicity is assigned using the following abbreviations: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m). High resolution MS analyses were performed on an Agilent 6520 Q-TOF instrument by dissolving the solid sample in methanol.

Gelation method

The gelation test was carried out as follows: the gelator was mixed in a capped test tube with appropriate amount of solvent, and the mixture was heated to 90-120 ºC until the solid was dissolved. By this procedure the solvent boiling point becomes higher than that under standard atmospheric pressure. The resulting solution was slowly allowed to cool to room temperature, and gelation was visually observed. A gel sample was obtained that exhibited no gravitational

flow in inverted tube. All gels obtained are thermally reversible. Above their gelation temperature, the gels dissolved in the solvent, but could be returned to their original gel state upon cooling.

Gel-sol melting temperature (Tg).

Gel melting temperature was determined by typical tube inversion method. In a 5 mL vial gel was prepared as described above; the vial was immersed in the oil-bath 'upside down' and slowly heated. The temperature at which the viscous gel melted down was recorded as T_{gel}.

Morphological analysis

Morphological analysis of gel formed by gelator at higher concentration was studied using optical microscopy A glass slide containing a small portion of gel was mounted on Phase Contrast Microscope and the morphology of gel was identified.

Morphological studies of the self-assembled structure by gelator at lower concentration was studied using HRTEM analysis.

Molecular modeling studies

MM2 energy minimised diagram was performed using ChemBio 3D Ultra 13. Red colour dotted line shows the possible hydrogen bond formation.

X-Ray diffraction studies.

A small portion of a wet gel sample formed by gelator in decanol was transferred in a sample holder and coated like a thin film.. The XRD measurement was performed on XPert-PRO Diffractometer system.

Raw Data Origin	XRD measurement (*.XRDML)
Scan Axis	Gonio
Start Position [°2Th.]	10.0251
End Position [°2Th.]	79.9251
Step Size [°2Th.]	0.0500
Scan Step Time [s]	10.1600
Scan Type	Continuous
PSD Mode	Scanning
PSD Length [°2Th.]	2.12
Offset [°2Th.]	0.0000
Divergence Slit Type	Fixed
Divergence Slit Size [°]	0.4785
Specimen Length [mm]	10.00
Measurement Temperature [°C]	25.00
Anode Material	Cu
K-Alpha1 [Å]	1.54060
K-Alpha2 [Å]	1.54443
K-Beta [Å]	1.39225

K-A2 / K-A1 Ratio	0.50000
Generator Settings	30 mA, 40 kV
Diffractometer Type	0000000011024644
Diffractometer Number	0
Goniometer Radius [mm]	240.00
Dist. Focus-Diverg. Slit [mm]	91.00
Incident Beam Monochromator	No
Spinning	N

Spectroscopic measurements

Absorption spectra were measured with an Evolution 220 UV-vis spectrophotometer (Thermo Scientific). The spectra were recorded in the continuous mode between 200 and 700 nm, with a wavelength increment of 1 nm and a bandwidth of 1 nm. A 1 mm path length quartz cuvette was used.

Fluorescent spectra were carried out in a JASCO spectrofluorometer FP-8200, by fixing the excitation value at 325nm for dodecanol and DMSO. Selection of excitation value is based on the absorbance maximum of probes in different solvent.

Cell proliferation assay:

The anti-proliferation activity of **5a-c** were tested by MTS assay on fibroblast and PC-3 cell line using the fluorescent dye, yellow tetrazolium dye, which when treated with cells, form purple colored formazan product. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by using a spectrophotometer (1420-040 Victor3 Multilabel Counter, PerkinElmer, USA) at 490nm dissolved in PBS buffer. Absorbance maximum for our proposed probes is around 360nm (DMSO-PBS buffer) and it won't interefere with the MTS assay. Viable cells with active metabolism convert MTT into a purple colored formazan product. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells.

Cells were seeded into 96-well plate 48 h prior treatment. They were then exposed to different concentrations (20, 40, 80 and 250µg/1000µL) of **5a-c** in DMSO-water mixture and to control (DMSO-water mixture). The relative viability was expressed as a percentage of the control well that was treated with the solvent DMSO-water mixture only. Cell viability (%) was estimated as

a ratio of the absorbance of treated cell (N_t) to absorbance of untreated cells (solvent) (N_u) multiplied by 100.

$$\text{Cell viability (\%)} = (N_t / N_u) \times 100$$

Cell imaging/cell uptake studies: The Cell imaging / Cell uptake study of our fluorescent compounds **5a-c** were tested on PC-3 and normal (Fibroblast) cell lines. The cells were seeded into 6-well plate 24 h prior treatment. They were then exposed to **5a-c** in DMSO-PBS buffer mixture with the concentration of 250µg/1000µL. After 4 h of exposure, the media were drained and the cells were washed with PBS for more than 2 times. In order to differentiate the nucleus the Hoechst stain was then added and incubated for 15 minutes. After 15mins incubation, the stain was drained and the cells were again washed with PBS and taken for imaging. The cells were imaged using Confocal microscope.

Synthesis

General procedure for the Ortho formylation of substituted phenols:

Dry paraformaldehyde (35 mmol) was added to a mixture of 3-alkyl phenol (4 mmol), anhydrous magnesium chloride (6 mmol) and triethylamine (15 mmol) in acetonitrile (25 mL) and the mixture was heated under reflux for about 12-15h. After the completion of the reaction identified using TLC, the reaction mixture was cooled to room temperature and 5% aq. HCl was added. The product was extracted with ethylacetate, dried under Na_2SO_4 and purified using column chromatography.

Synthesis of cardanol-aldehyde 2b: Dry paraformaldehyde (1.05g, 35 mmol) was added to a mixture of cardanol (1.21 g, 4 mmol), anhydrous magnesium chloride (570 mg, 6 mmol) and triethylamine (2.1 mL, 15 mmol) in acetonitrile (25 mL) and refluxed for about 12-15h. The product was extracted with ethylacetate, dried under Na_2SO_4 and purified using column chromatography. Yellow liquid (88%)

^{1}H NMR (300MHz, $CDCl_3$) δ = 0.88 (t, J = 6.9 Hz, 3H); 1.25-1.30 (m, 16H); 1.61-1.64 (m, 4H); 1.95-2.05 (m, 2H); 2.61 (t, J = 7.5Hz, 2H); 6.80 (s, 1H); 6.83 (d, J = 8.1 Hz, 1H); 7.44 (d, J = 8.1Hz, 1H); 9.83 (s, 1H); 11.05 (s, 1H).

^{13}C NMR (75MHz, $CDCl_3$) δ = 195.81, 161.81, 153.84, 130.49, 130.01, 129.76, 120.51, 118.86, 117.08, 36.45, 32.62, 32.59, 31.94, 31.86, 31.80, 30.66, 29.72, 29.67, 29.54, 29.45, 29.34, 29.30, 29.25, 29.21, 29.16, 29.01, 28.91, 18.86, 27.24, 27.17, 14.12.

Synthesis of PDP-aldehyde, 2c: Dry paraformaldehyde(1.05g, 35 mmol) was added to a mixture of 3-pentadecylphenol (1.22g, 4 mmol), anhydrous magnesium chloride (570 mg, 6 mmol) and triethylamine (2.1 mL, 15 mmol) in acetonitrile (25 mL) and the mixture was refluxed for about 12-15h. The product was extracted with ethylacetate, dried under Na_2SO_4 and purified using column chromatography.Yield: 92%; White solid; mp = 53-56 ºC.

^{1}H NMR (300MHz, $CDCl_3$) δ 0.88 (t, J = 6.6Hz, 3H); 1.25-1.59 (m, 26H); 2.61 (t, J = 7.5Hz, 2H); 6.8(s, 1H); 6.85 (d, J = 9.3Hz, 1H); 7.45 (d, J = 7.8Hz, 1H); 9.83 (s, 1H); 11.05 (s, 1H).

^{13}C NMR (75MHz, $CDCl_3$) δ 195.80, 161.81, 153.84, 120.50, 118.85, 117.08, 36.45, 31.94, 30.67, 29.71, 29.68, 29.55, 29.45, 29.38, 29.25, 22.71, 14.13.

Synthesis of 3-acetyl-7-alkyl-2H-chromen-2-one, 3a-c: To the compound **2a-c** (1 mmol) in ethanol (10 mL), ethylacetoacetate (1.3 mmol.), 0.3 mL of piperidine and 2-3 drops of glacial acetic acid were added. The mixture was refluxed for 4h. After the completion of the reaction, the reaction mixture was cooled to room temperatutre and 20 ml of cold ice water was added. The product was extracted with chloroform and dried over anhydrous sodium sulfate. The product was further purified using column chromatography.

Compound 3b: Yellow liquid; Yield = 82%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.80 (t, J = 6.3Hz, 3H); 1.14-1.24 (m, 16H); 1.56-1.60 (m, 4H); 1.9-1.96 (m, 2H); 2.65 (t, J = 8.1Hz, 2H); 2.65 (s, 3H); 4.88-4.9 (m, 1H); 5.25-5.34 (m, 1H); 7.09 (d, J = 7.5Hz, 1H); 7.1 (s, 1H); 7.47 (d, J = 8.1Hz, 1H); 8.43 (s, 1H).

^{13}C NMR (75MH_z, $CDCl_3$) δ 194.54, 158.52, 154.58, 150.44, 146.54, 128.97, 128.66, 124.64, 122.21, 115.09, 49.72, 44.25, 35.30, 30.9, 30.7, 29.81, 29.55, 28.68, 28.49, 28.34, 28.28, 28.11, 28.04, 27.96, 27.81, 27.28, 26.19, 26.11, 21.67, 21.63, 14.16.

Compound 3c: White solid; Yield = 89%; ^{1}H NMR (300MHz, $CDCl_3$) δ = 0.85 (t, J = 7.2Hz, 3H); 1.18-1.32 (m, 24H); 1.59-1.67 (m, 2H); 2.71 (s, 3H); 2.72 (t, J = 6.9Hz, 2H); 6.90-7.14 (d, 8.1Hz, 1H) 7.17 (s, 1H); 7.54 (d, J = 7.8Hz, 1H); 8.5 (s, 1H).

^{13}C NMR (75MH_z, $CDCl_3$) δ 194.99, 158.96, 154.99, 150.88, 146.96, 129.35, 125.05, 122.62, 115.52, 115.49, 35.71, 21.29, 30.22, 20.97, 29.06, 29.02, 28.99, 28.89, 28.78, 28.73, 22.06, 13.49.

Synthesis of coumarin coupled pyrene derivatives (5a-c):

Optimization Procedures:

The 3-acetyl coumarin (1mmol.) was dissolved in 10ml of n-butanol under heating. To this 1-pyrenecarboxyaldehyde (1.3mmol.), 3 drops of glacial acetic acid and 0.3ml of piperidine was added and refluxed for 12h. After reflux, the solvent was removed under vacuum. The residue was triturated with 10ml of ethanol until the precipitate was formed. The precipitate was then filtered off and crystallized from appropriate solvent (methanol).

Synthesis of 5a: Melting point – 212-214ºC; Yellow solid; Yield = 77%; ^1H NMR (300MHz, $CDCl_3$) δ = 7.47 (t, J = 7.5Hz, 1H); 7.54 (d, J = 8.1Hz, 1H); 7.79 (t, J = 7,5Hz, 1H); 7.78 (d, J = 9.0Hz, 1H); 8.0 (d, J = 6.6Hz, 1H); 8.15 (t, J = 7.5Hz, 1H); 8.24-8.41 (m, 6H); 8.59 (d, J = 8.4Hz, 1H); 8.67 (d, J = 9.3Hz, 1H); 8.79 (s, 1H); 8.89 (d, J = 15.6Hz, 1H).

HRMS (ES+): m/z calcd for $C_{28}H_{16}O_3$ $(M-H+2Li)^+$ = 413.1320; observed = 413.2647.

Synthesis of 5b: Melting point – 126-128ºC; Yellow solid; Yield = 74% ^1H NMR (300MHz, $CDCl_3$) δ = 0.81 (t, J = 4.5Hz, 3H); 1.20-1.26 (m, 14H); 1.51-1.60 (m, 8H); 1.9-1.96 (m, 2H); 2.67 (t, J = 7.2 Hz, 2H); 5.26-5.32 (m, 2H); 7.11 (d, J = 7.8Hz, 1H); 7.15 (s, 1H); 7.52 (d, J = 7.8Hz, 1H); 7.94-8.17 (m, 7H); 8.2 (d, J = 15.6Hz, 1H); 8.45 (d, J = 8.1Hz, 1H); 8.54 (d, J = 9.3Hz, 1H); 8.59 (s, 1H), 9.0 (d, J = 15.6Hz, 1H).

^{13}C NMR ($CDCl_3$, 75MHz) δ 184.82, 158.41, 154.15, 149.88, 139.66, 131.77, 129.92, 129.34, 129.21, 128.41, 127.41, 127.32, 127.28, 126.06, 124.90, 124.73, 124.57, 124.39, 124.23, 123.82, 123.43, 123.22, 122.64, 121.25, 115.10, 114.72, 34.99, 29.50, 28.41, 28.32, 29.02, 27.91, 27.70, 21.37, 12.83.

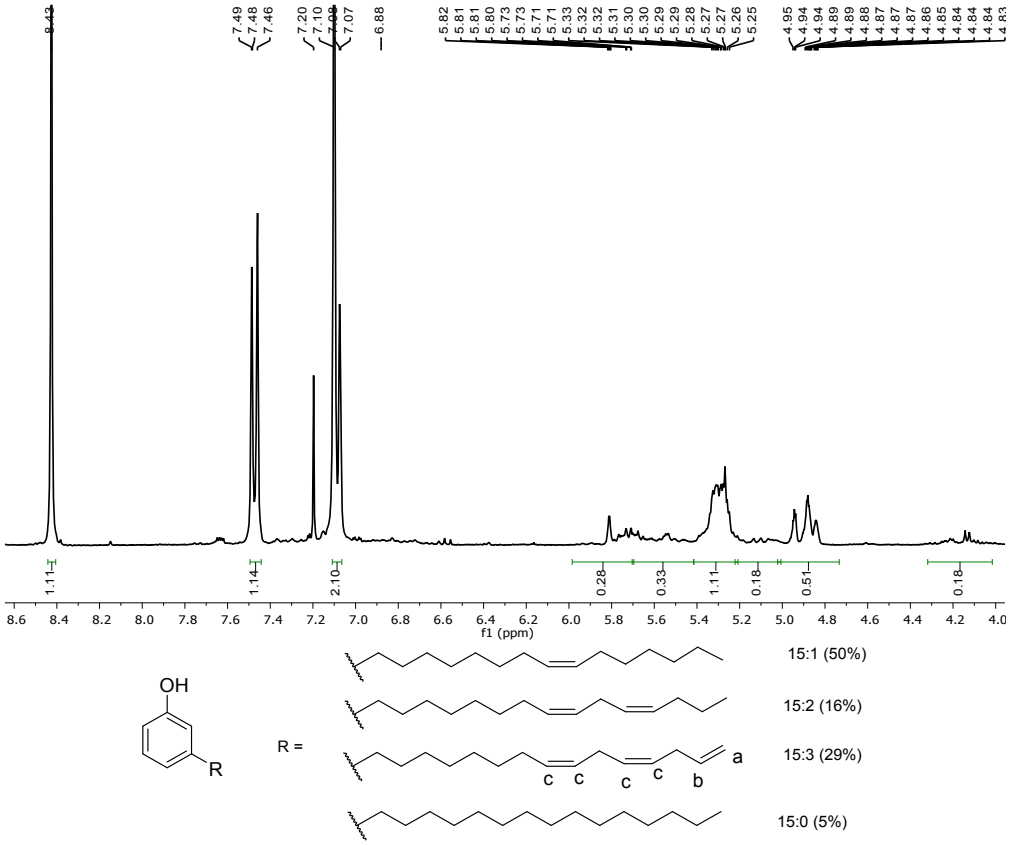
HRMS (ES+): m/z calcd for $C_{43}H_{45}O_3$ $(M+Na)^+$ = 632.3266; observed = 632.2545

Synthesis of 5c: Melting point – 158-160ºC; Yellow solidYield = 82%; ^{1}H NMR (300MHz, $CDCl_3$) δ = 0.88 (t, J = 6.3Hz, 3H); 1.2-1.5 (m, 24H); 1.65-1.69 (m, 2H); 2.74 (t, J = 7.5Hz, 2H); 7.19 (d, J = 7.8Hz, 1H); 7.23 (s, 1H); 7.60 (d, J = 7.8Hz, 1H); 8.02-8.16 (m, 3H); 8.19-8.25 (m, 4H); 8.20 (d, J = 15.6Hz, 1H); 8.54 (d, J = 8.1Hz, 1H); 8.62 (d, J = 9.3Hz, 1H); 8.68 (s, 1H); 9.07 (d, J = 15.6Hz, 1H).

^{13}C NMR ($CDCl_3$, 75MHz) δ 184.73, 18.25, 154.00, 149.77, 146.78, 139.54, 131.60, 129.75, 129.16, 129.05, 128.25, 127.24, 127.14, 127.11, 125.88, 124.71, 124.54, 124.38, 124.24, 124.10, 123.63, 123.39, 123.26, 123.06, 122.53, 121.08, 114.94, 112.59, 30.40, 28.17, 28.13, 28.01, 27.90, 27.84, 27.69, 21.17, 12.60.

HRMS (ES+): m/z calcd for $C_{43}H_{47}O_3$ $(M+Na)^+$ = 634.3439; observed = 634.5325

Calculation of composition of cardanol by NMR



The ^{1}H NMR signals at 4.9 ppm (m, 0.51H) are assigned to terminal vinyl group of the triene moiety. The fraction of the triene moiety is evaluated to be 0.26 from the equation, 0.51H/2H. (26%)

The ^{1}H NMR signal due to H-b of triene moiety was observed at 5.8 ppm (m, 0.28H). The fraction of the triene moiety is evaluated to be 0.28 from the equation, 0.28H/1H. (28%)

The ^{1}H NMR signals due to H-c at 5.3 ppm (m, 1.62H) are related to the cis-olefin protons of the triene, diene and monoene moieties, which is approximately equal to the calculated value.

Reference:

Mitsuhiro Shibata, Yusuke Itakura and Hironori Watanabe, *Polymer Journal*, 2013, 45, 758–765

Tables and figures

Table S1- Solvents/vegetable oil used for gelation studies

S.No	Solvent	5a	MGC	5b	MGC	5c	MGC
1	Ethanol	P	**-**	P	-	P	**-**
2	n-Butanol	PG	**-**	PG	-	P	**-**
3	Octanol	G	2mg/150µL (1.3% wt/v**)**	G	2mg/150µL (1.3%wt/v)	P	**-**
4	Decanol	G	**2mg/700µL (0.28%wt/v)**	G	2mg/150µL (1.3% wt/v)	P	**-**
6	Dodecanol	G	2mg/500µL (0.4% wt/v)	G	**2mg/200 µL (1% wt/v)**	G	**4mg/100µL (4%wt/v)**
7	Toluene	S	**-**	S	-	S	**-**
8	Chloroform	I	-	I	-	I	**-**
9	Hazelnut oil	S	**-**	G	2mg/150µL (1.3%wt/v)	P	**-**
10	Olive oil	S	**-**	G	2mg/150µL (1.3%wt/v)	P	**-**
11	Heavy paraffin oil	PG	**-**	S	-	PG	**-**
12	Light paraffin oil	S	**-**	S	-	PG	**-**
13	Sesame oil	S	**-**	S	-	PG	**-**

G, S, P, PG and I denote Gelation, Solution, Precipitation, Partial Gelator and Insoluble respectively.

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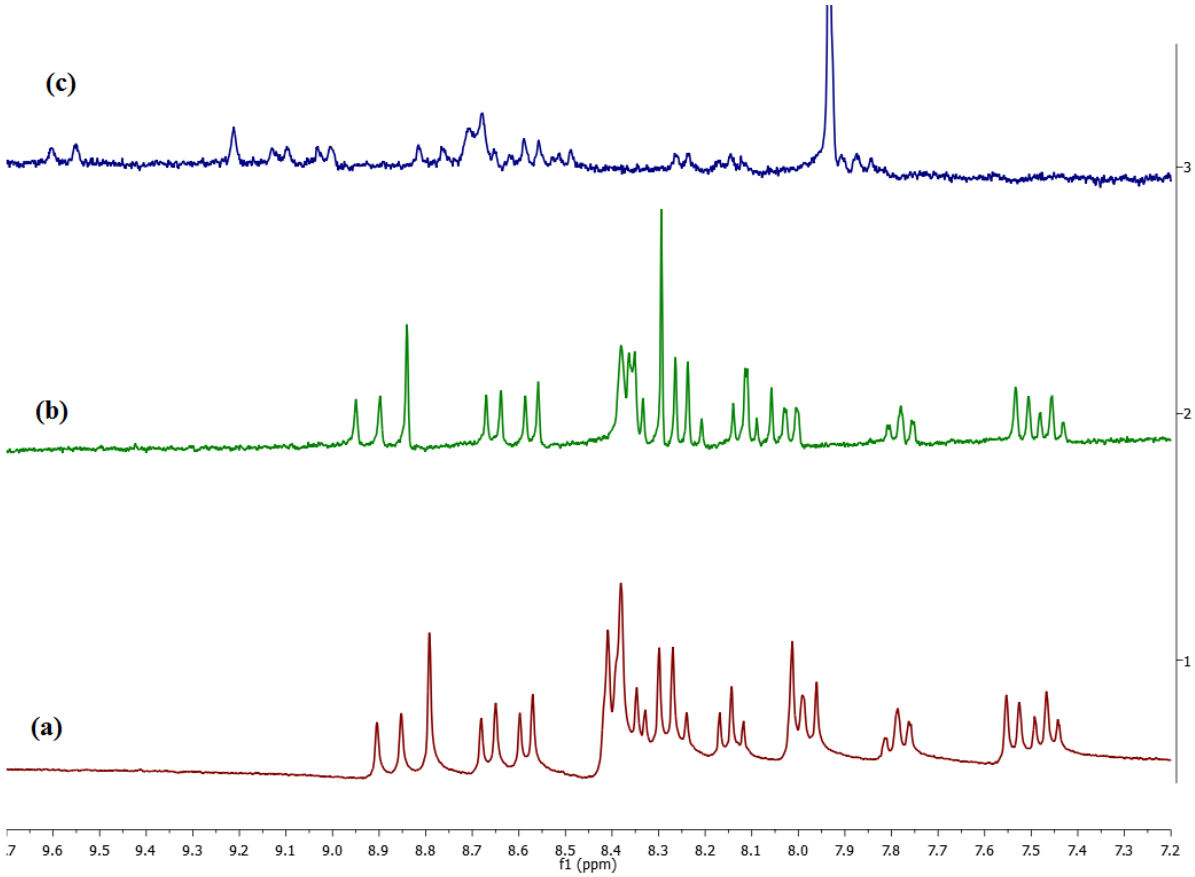

Figure S1 - ^{1}H NMR spectra (aromatic region alone) of the compound, **5a**; (a) in DMSO-d_6; (b) in DMSO-d_6 + dodecanol (1:1 ratio); (c) DMSO-d_6 + dodecanol (1:2 ratio).

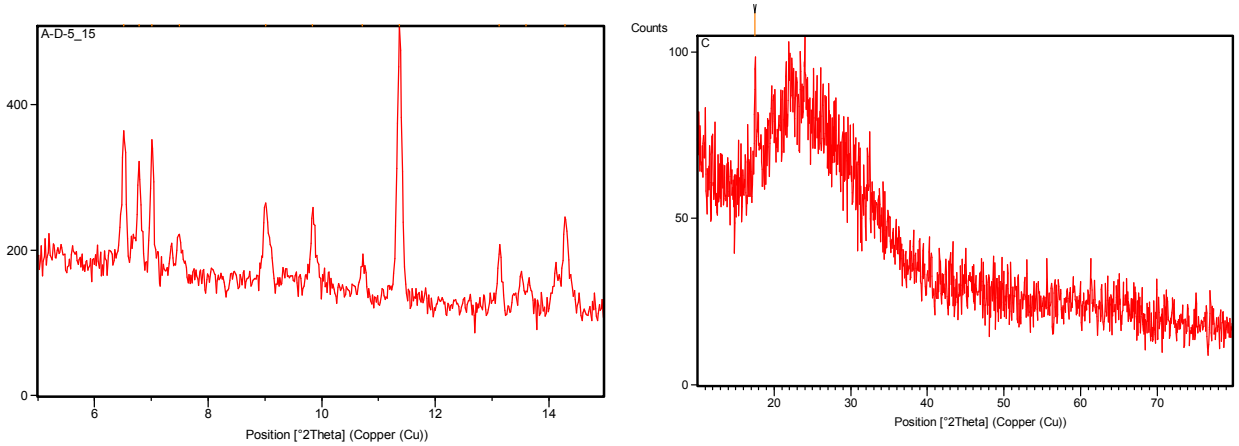

Figure S2 – SAXD and WAXD data for decanol gel of **5a**

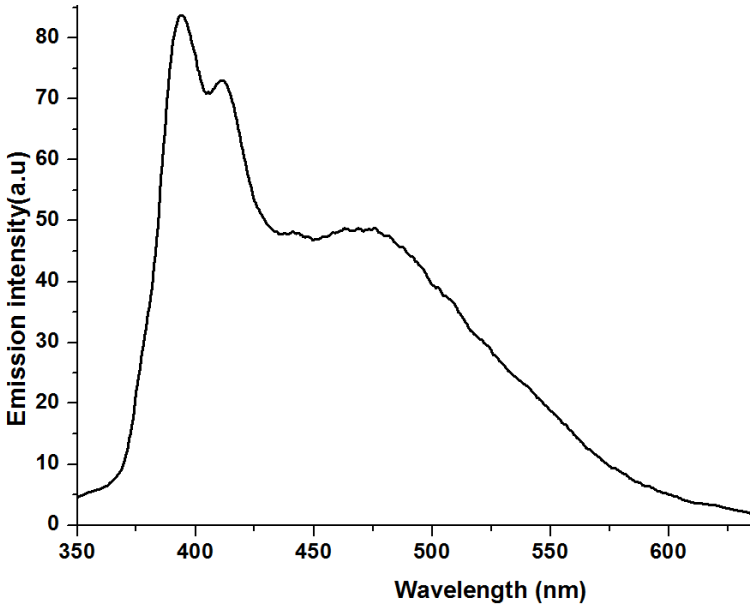

Figure S3 - Emission spectra of **5a** in DMSO ($1X10^{-5}$ M)

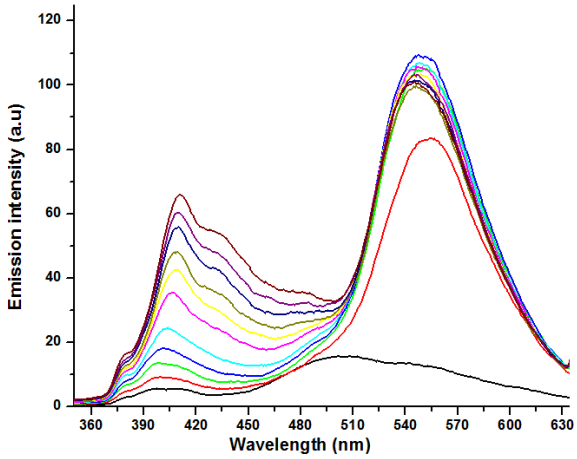

Figure S4 - Fluorescence titration of **5b** in DMSO ($1X10^{-5}$) with PBS buffer. Piecemeal addition of 100mL of corresponding solvent was performed for titration experiments [λ_{ex} = 325nm].

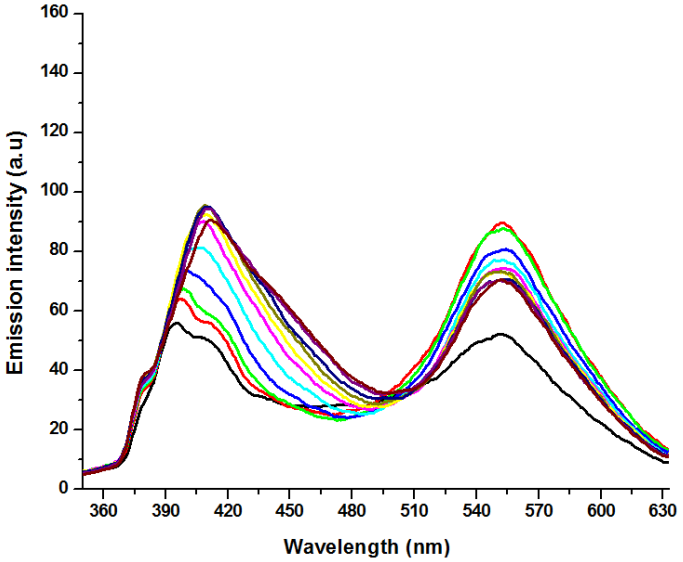

Figure S5 - Fluorescence titration of **5c** in DMSO ($1X10^{-5}$) with PBS buffer. Piecemeal addition of 100mL of corresponding solvent was performed for titration experiments [λ_{ex} = 325nm].

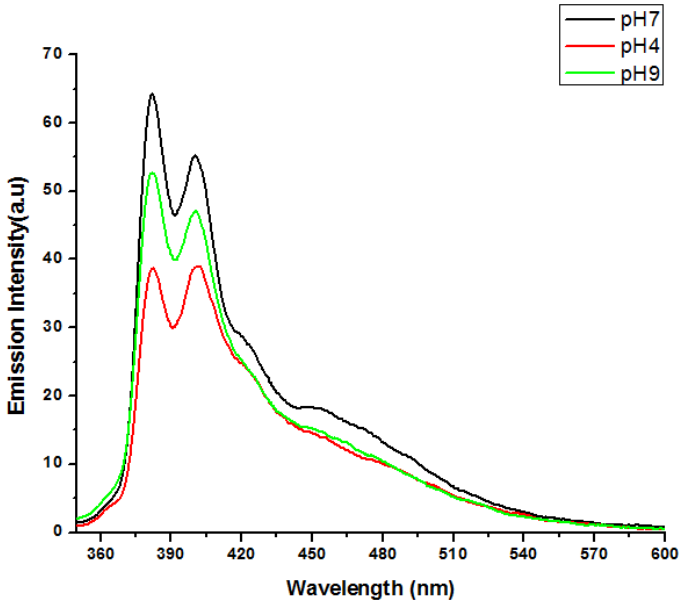

Figure S6 - Emission spectra of **5a** in DMSO-under the influence of different pH ($1X10^{-5}$ M) [λ_{ex} = 325nm].

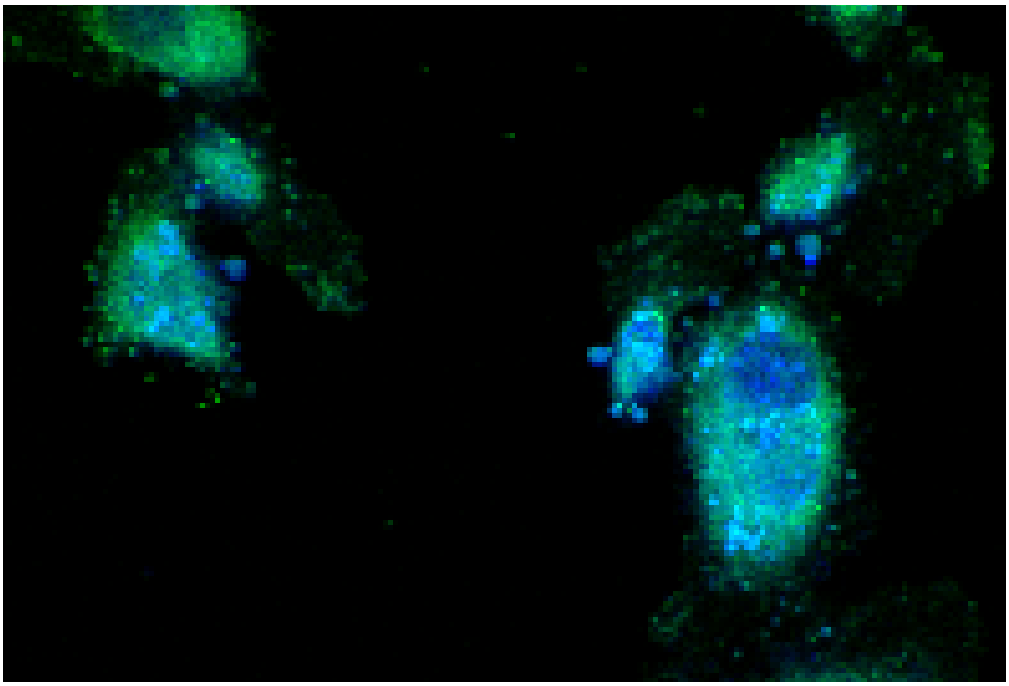

Figure S7 - LCSM images PC3 prostate cancer cells incubated with **5a** (magnified image shows cell membrane rupture and the overflow of cytoplasm)

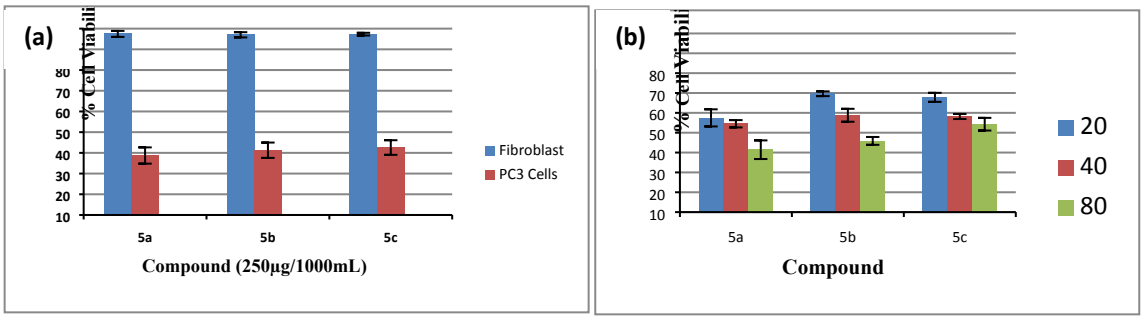

Figure S8 - Graphical representation of (a) Fibroblast and PC3 cell viability when exposed to 250µg/1000µL [**5a**: 0.6X10^{-3}M, **5b**: 0.4 X10^{-3}M and **5c**: 0.4 X10^{-3}M (250µg/1000µL)] of compound **5a-c**. and (b) PC3 cell viability when exposed to increasing concentration of **5a-c** (20-80µg/1000µL). In fibroblast % of cell viability was around 95-98%

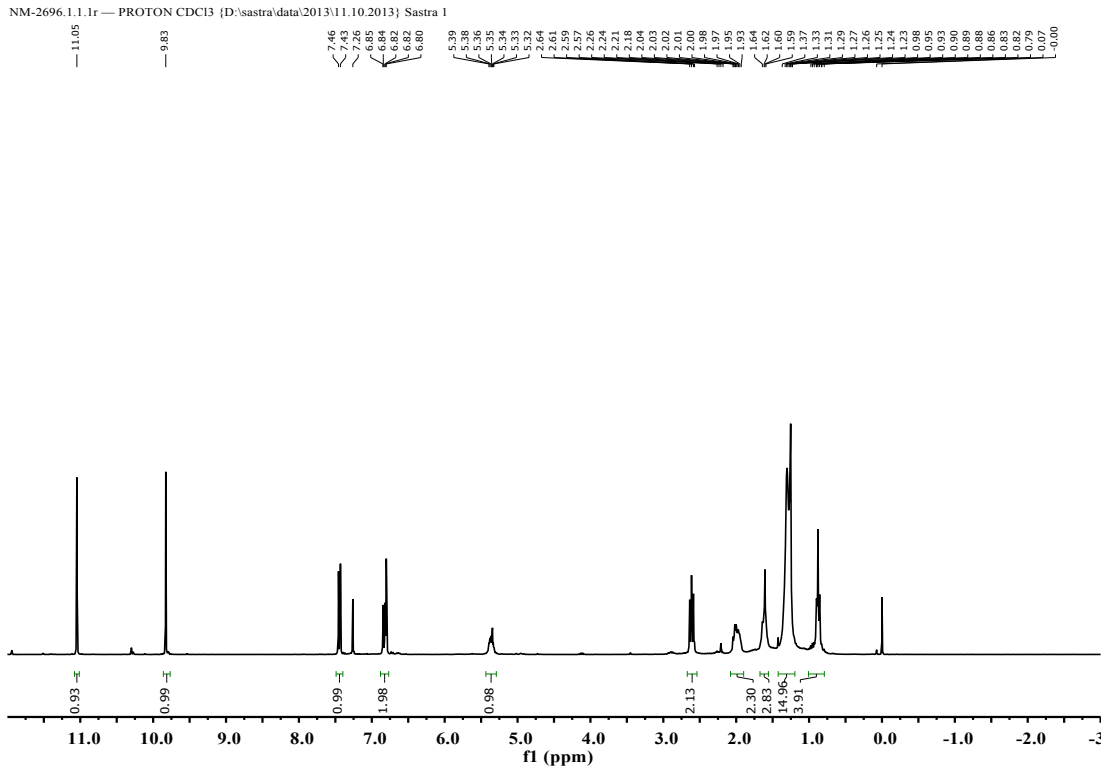

Figure S9- ^{1}H NMR spectrum of **2b** in $CDCl_3$ at 300 K (300 MHz)

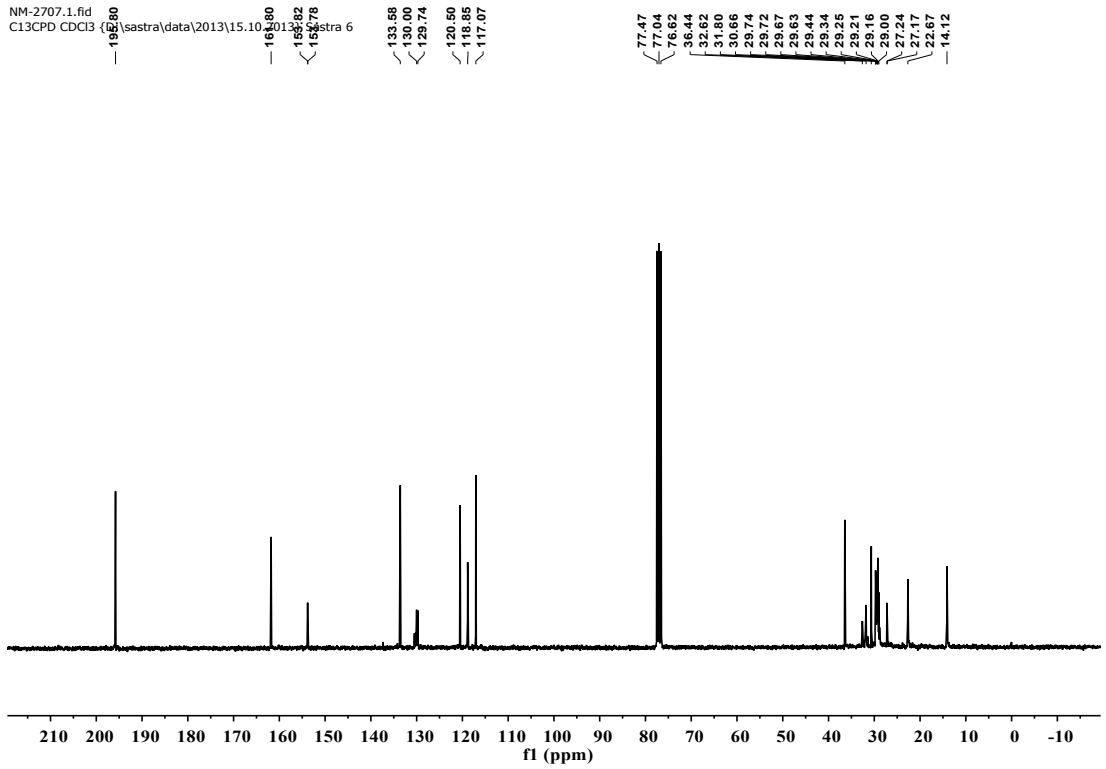

Figure S10- ^{13}C NMR spectrum of **2b** in $CDCl_3$ at 300 K (75 MHz)

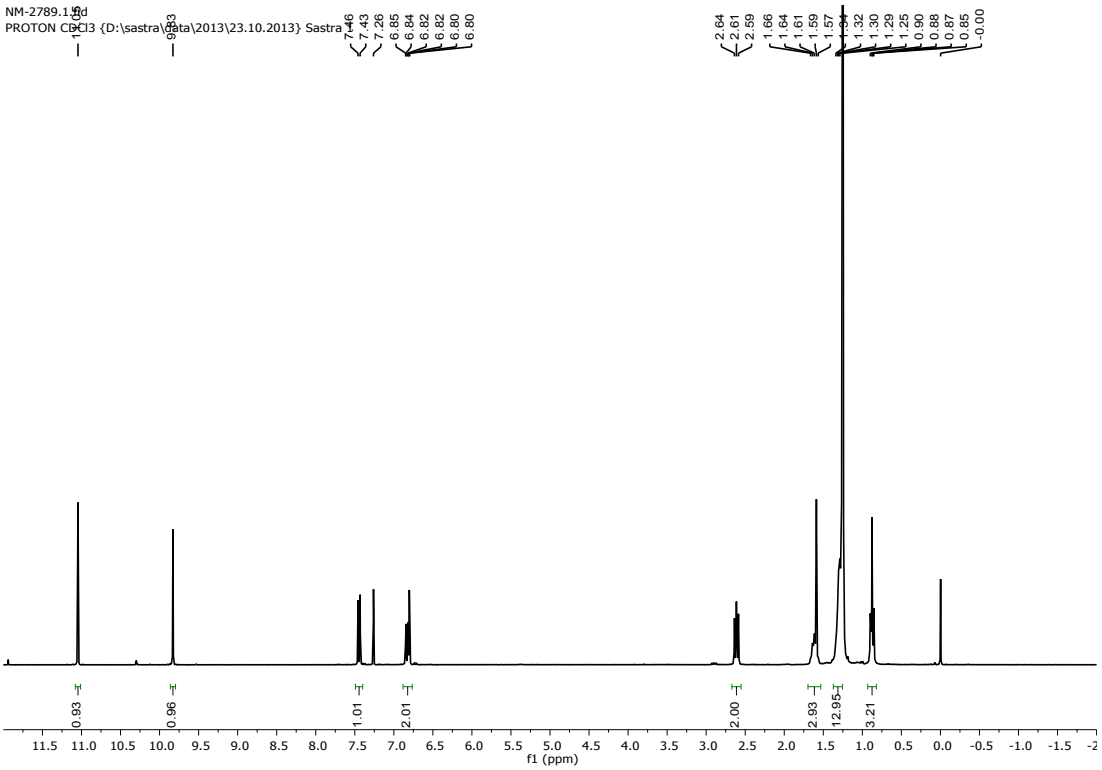

Figure S11- ^{1}H NMR spectrum of **2c** in $CDCl_3$ at 300 K (300 MHz).

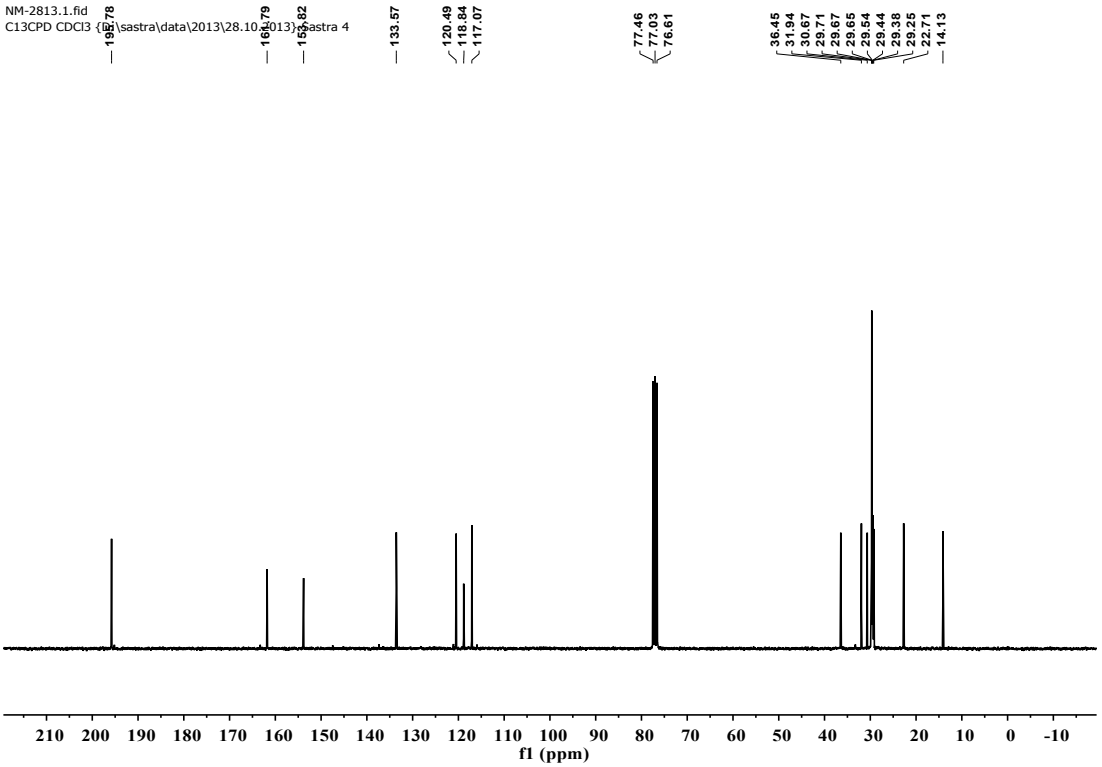

Figure S12- ^{13}C NMR spectrum of **2c** in $CDCl_3$ at 300 K (75 MHz).

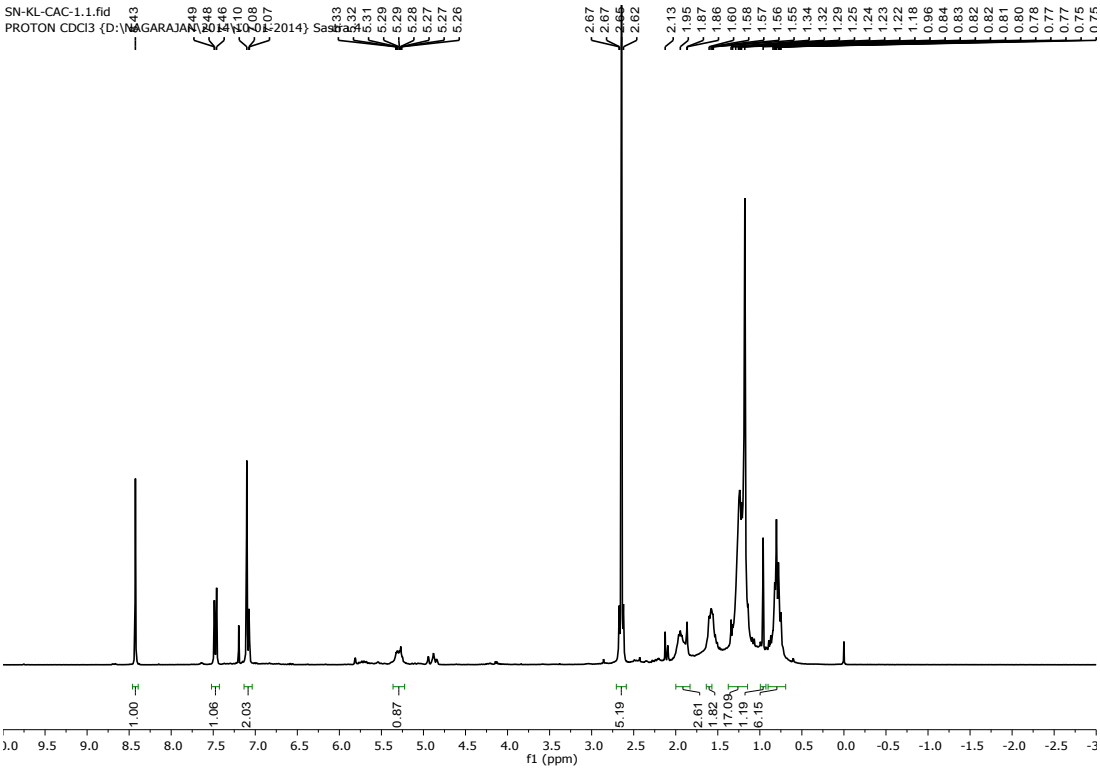

Figure S13- ^{1}H NMR spectrum of **3b** in $CDCl_3$ at 300 K (300 MHz).

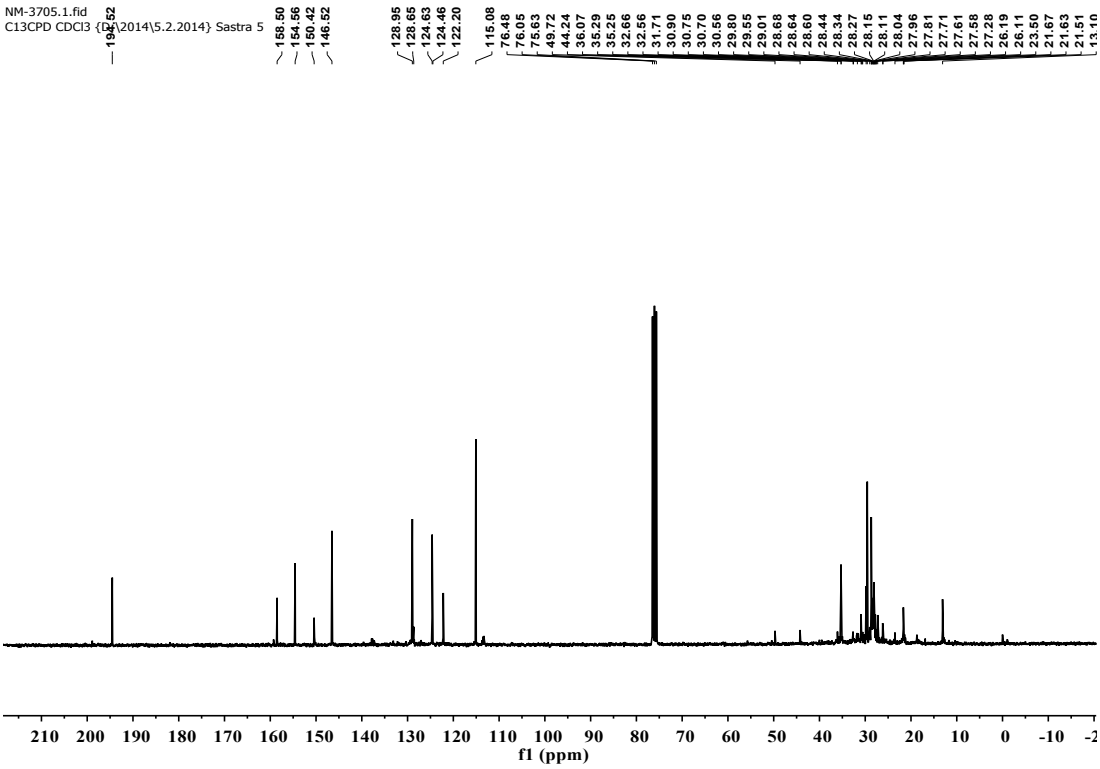

Figure S14- ^{13}C NMR spectrum of **3b** in $CDCl_3$ at 300 K (75 MHz).

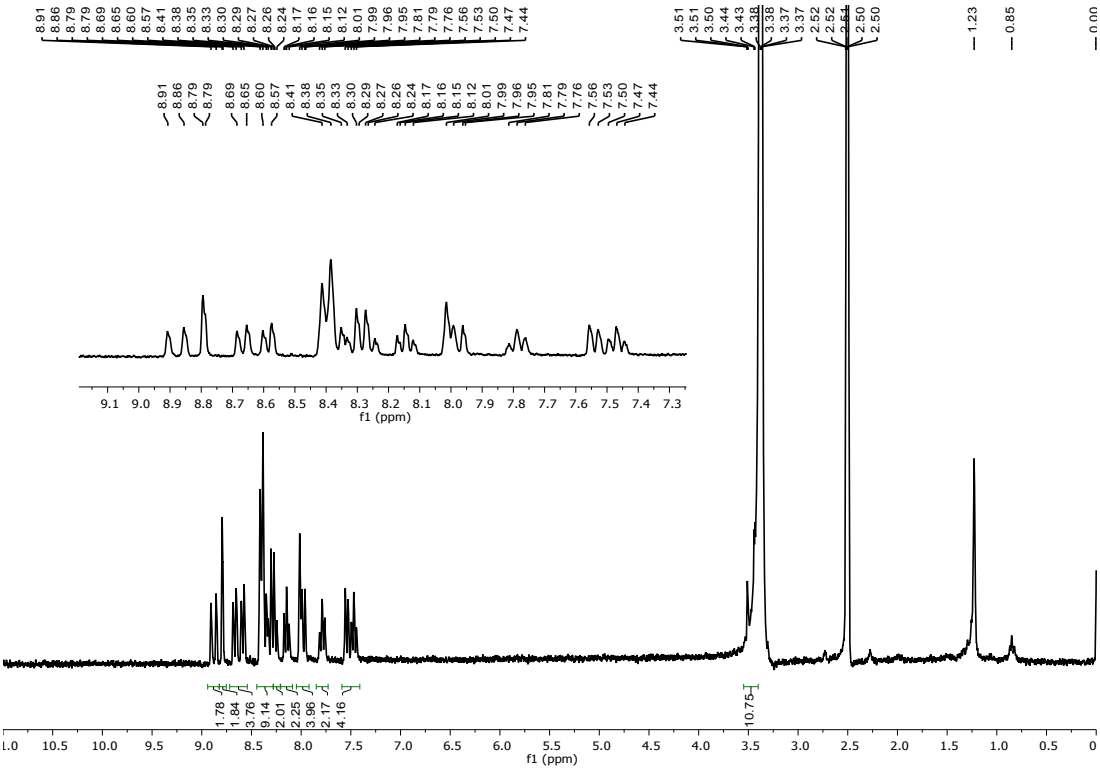

Figure S15- ^{1}H NMR spectrum of **5a** in DMSO-d_6 at 300 K (300 MHz).

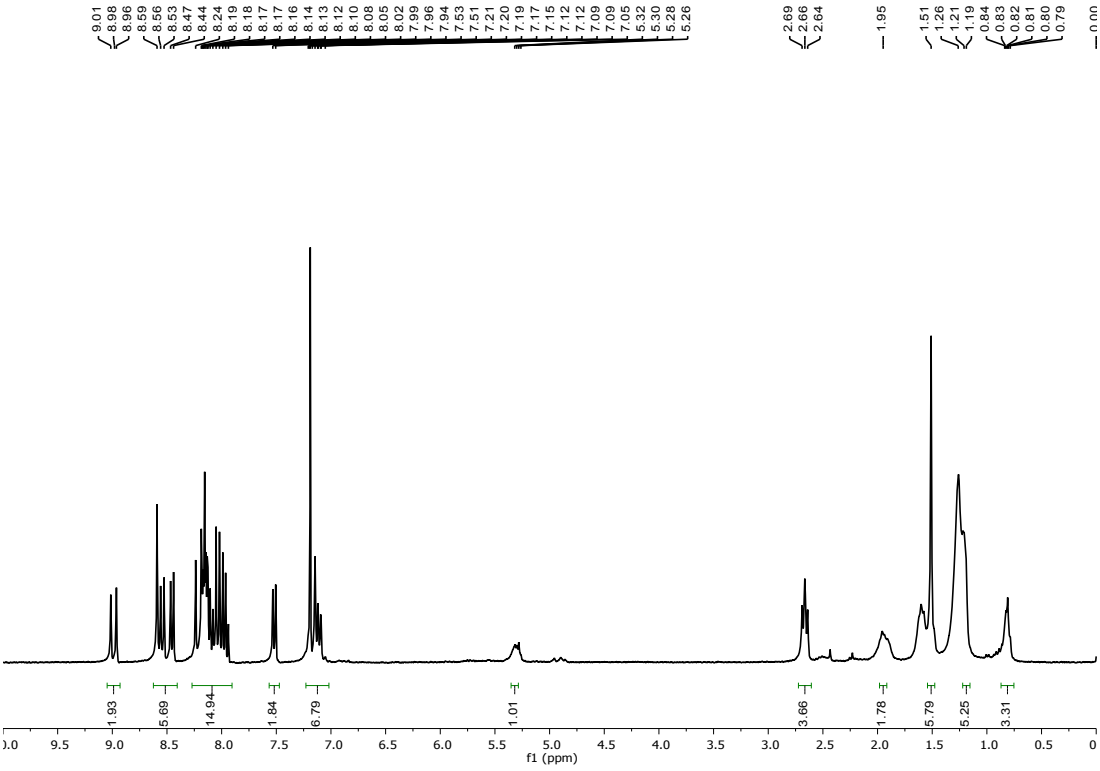

Figure S16- ^{1}H NMR spectrum of **5b** in $CDCl_3$ at 300 K (300 MHz).

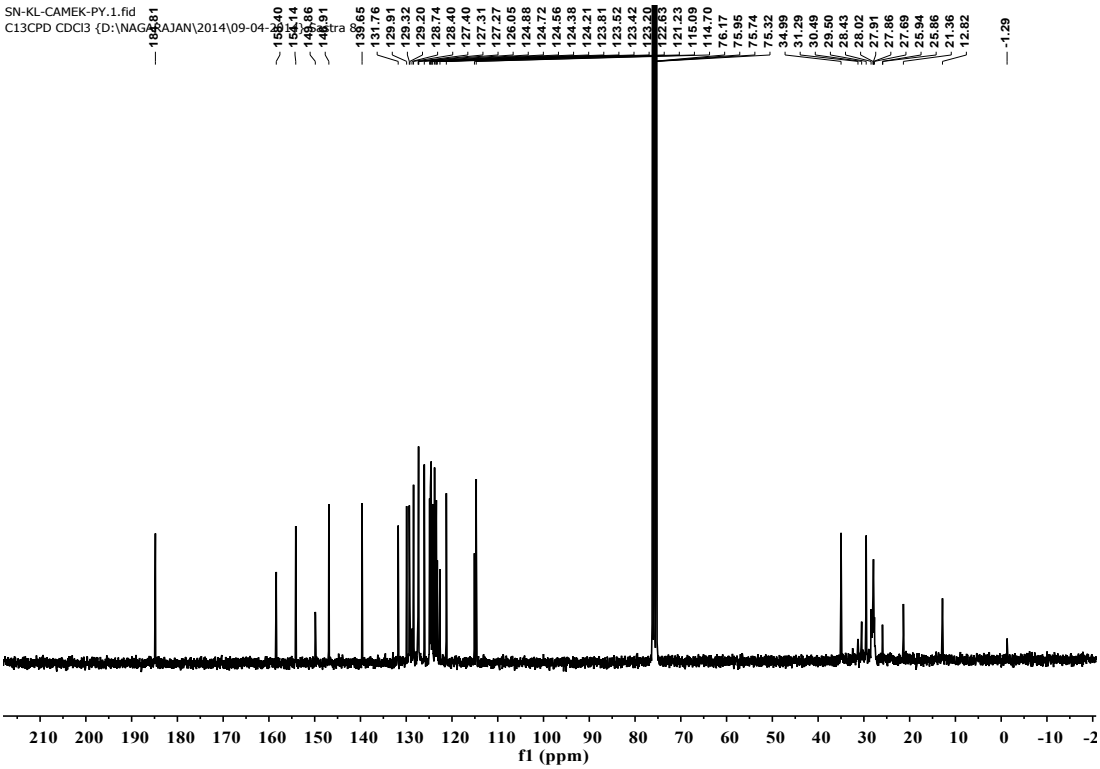

Figure S17- ^{13}C NMR spectrum of **5b** in $CDCl_3$ at 300 K (75 MHz).

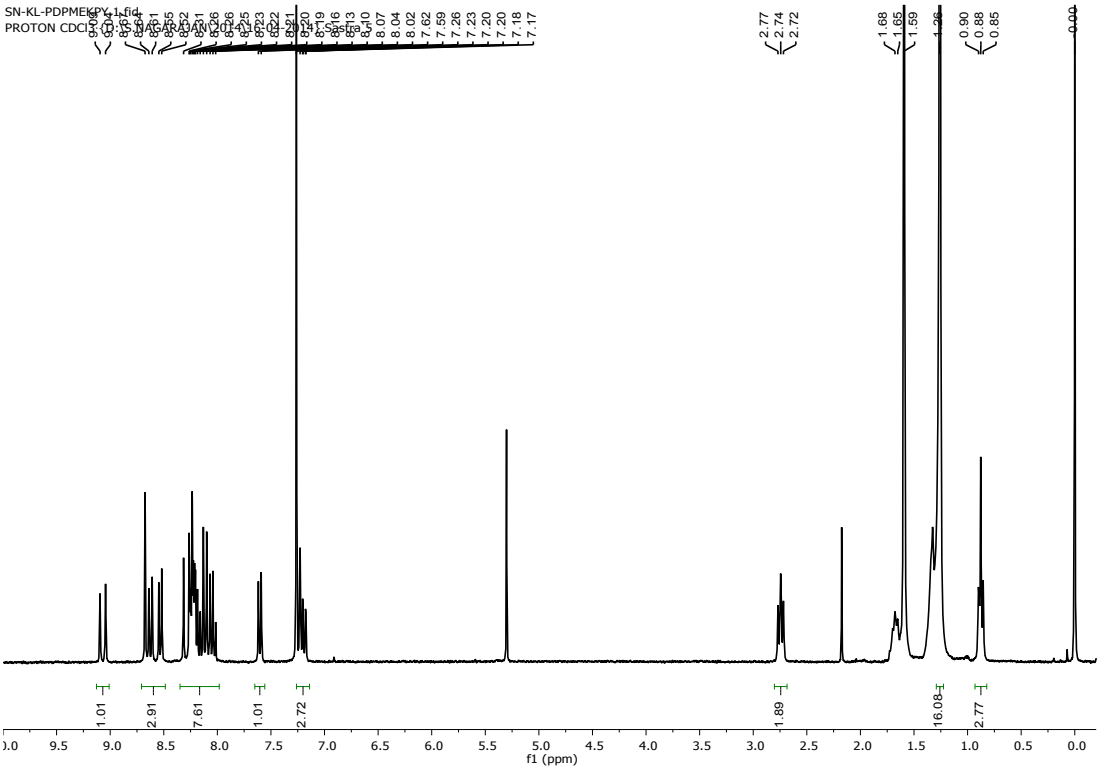

Figure S18- ^{1}H NMR spectrum of **5c** in $CDCl_3$ at 300 K (300 MHz).

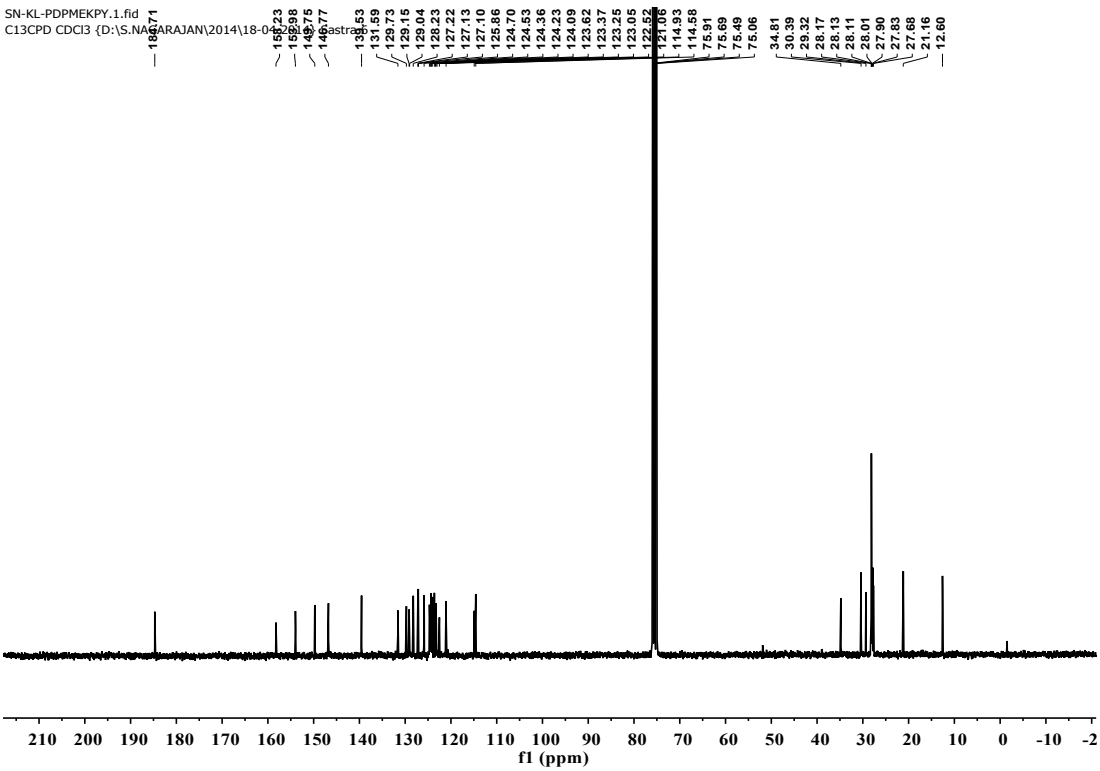

Figure S19- ^{13}C NMR spectrum of **5c** in $CDCl_3$ at 300 K (75 MHz).

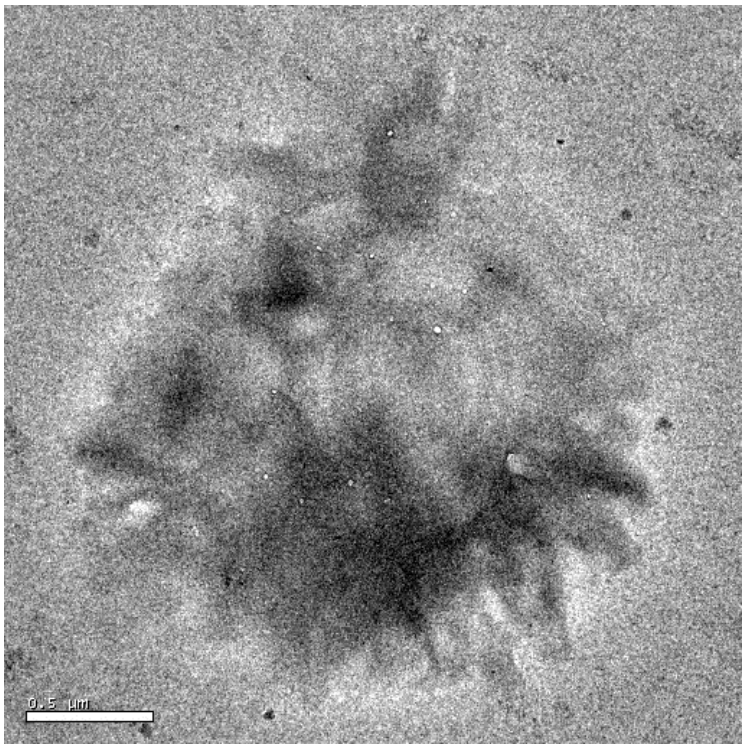

Figure S20- HRTEM image of nanosheet/nanoflake formed by **5c** at lower concentration in DMSO-water mixture (1:1 ratio)

Analysis of particle size using zetasizer (multimodal analysis parameters)

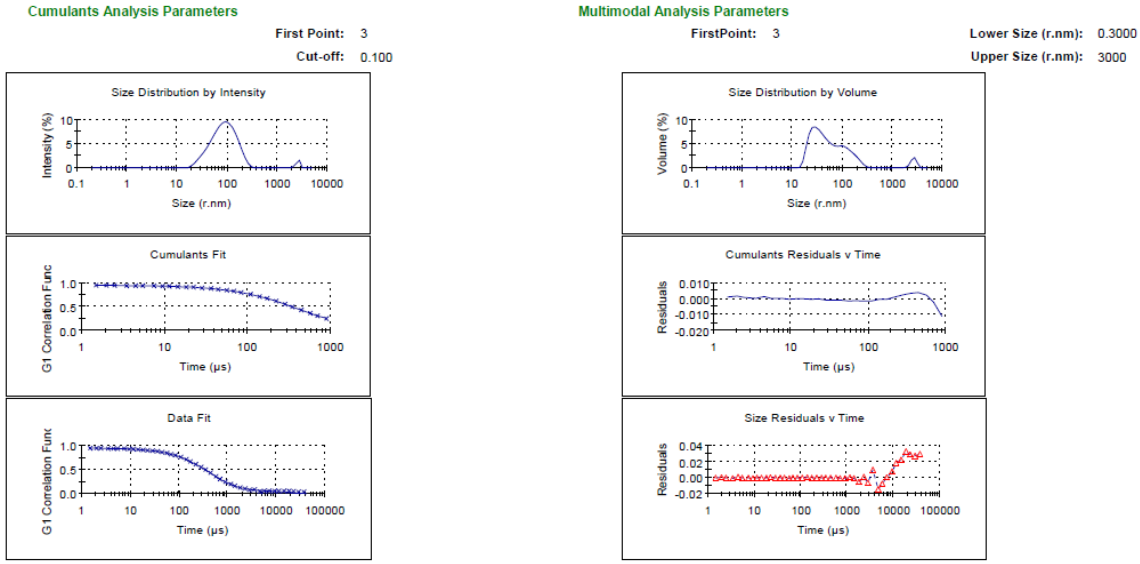

Figure S21 - Average sizes of self-assembled aggregates of 5a in in DMSO-water mixture ($1X10^{-3}$M solution)

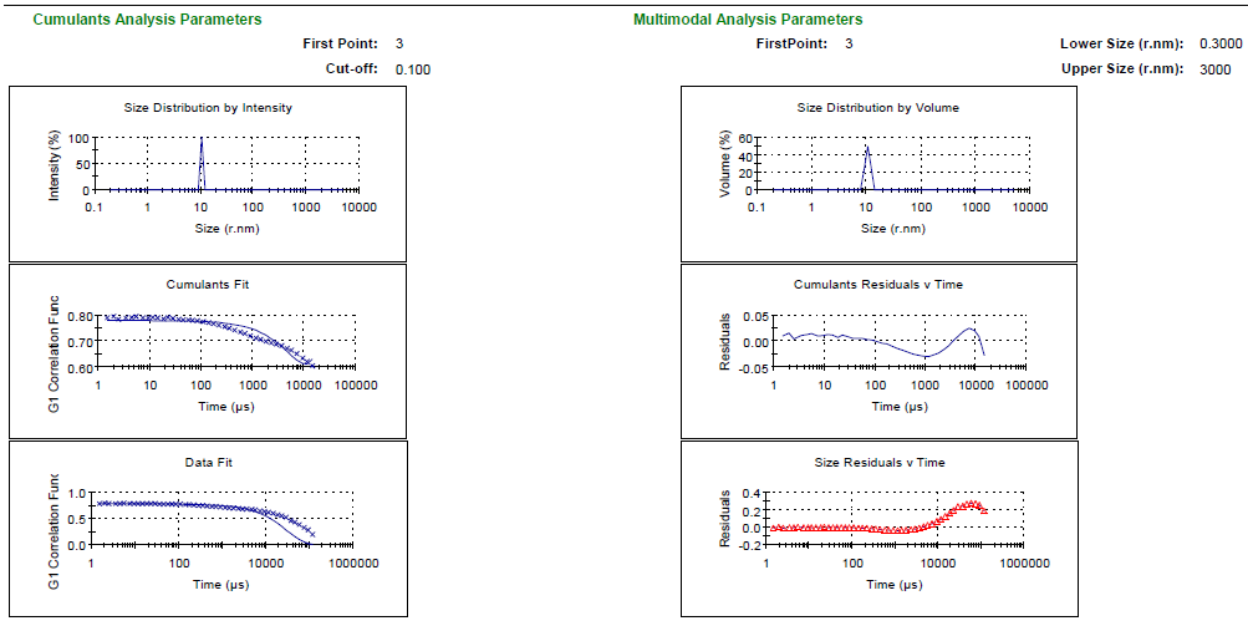

Figure S22 - Average sizes of self-assembled aggregates of 5b in in DMSO-water mixture ($1X10^{-3}$M solution)

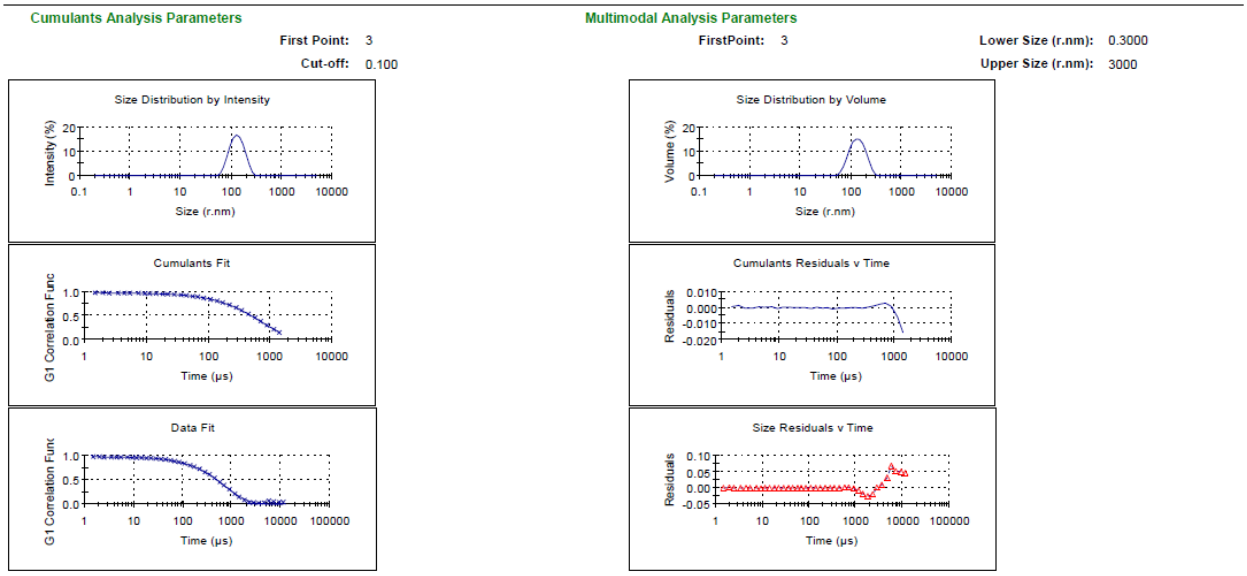

Figure S22 - Average sizes of self-assembled aggregates of **5c** in in DMSO-water mixture ($1X10^{-3}$M solution)

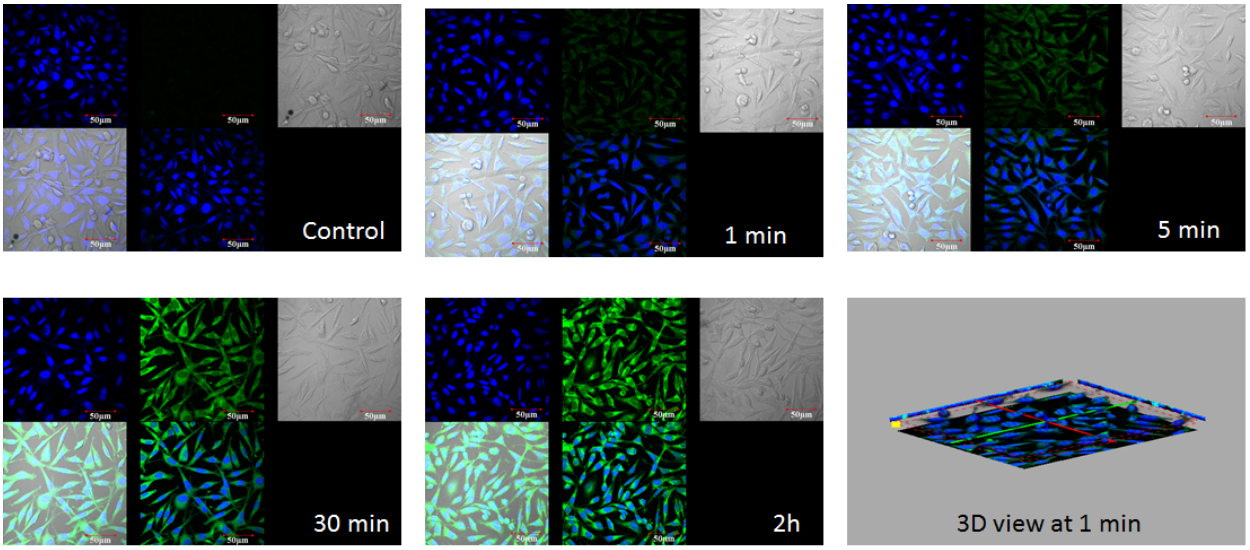

Figure S23: . LCSM images of fibroblast incubated with **5a** at different time interval (0 min- 2 h).

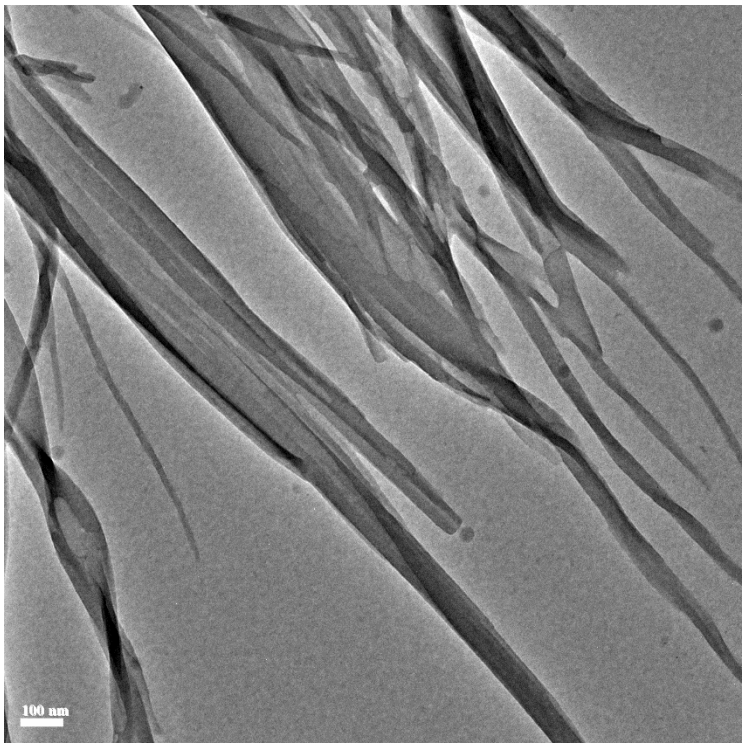

Figure S24: HRTEM image of gel, **5a** in dodecanol.

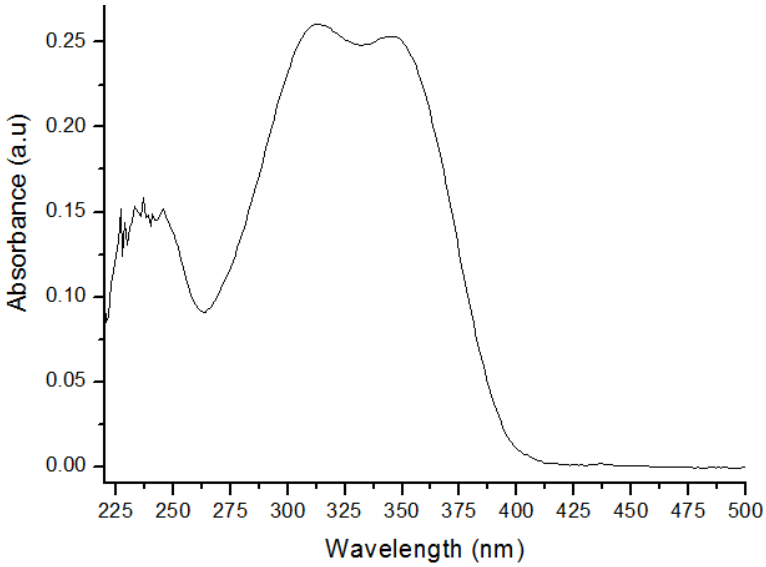

Figure S25: UV-Vis spectra of **5b** in DMSO-PBS buffer mixture ($1X10^{-5}$).

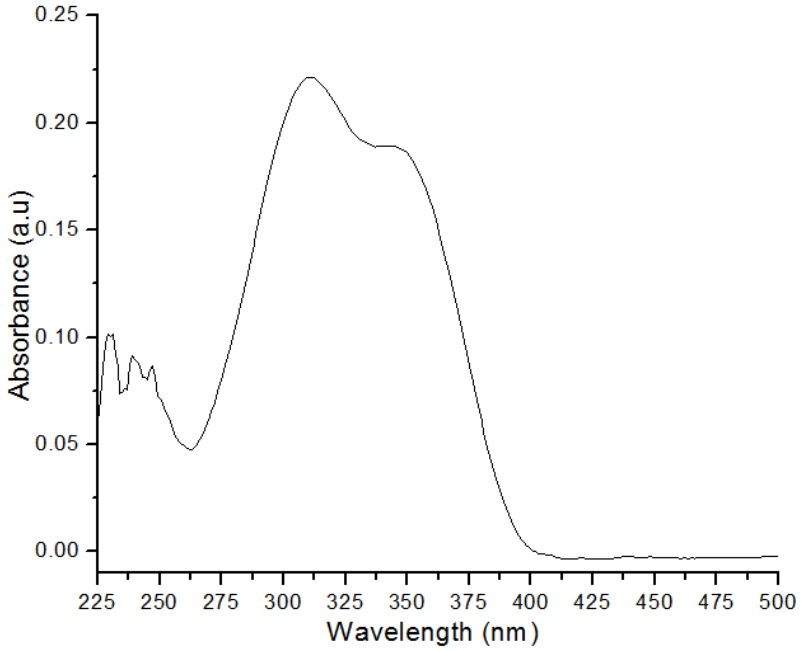

Figure S26: UV-Vis spectra of **5c** in DMSO-PBS buffer mixture ($1X10^{-5}$).