Supporting Information for

Tumor-Acidity-Induced Surface Charge Modulation in Covalent Nanonetworks for Activated Cellular Uptake: Targeted Delivery of Anticancer Drugs and Selective Cancer Cell Death

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Materials and Methods:

All the chemicals and solvents were purchased from commercial sources and used without any further purification. NMR spectroscopic measurements were performed using a Bruker DPX 400 MHz NMR instrument in $CDCl_3$ solvent. The molecular weight and PDI was investigated by gel permeation chromatography (GPC) experiment using waters GPC system fitted with a waters 515 HPLC pump and water 2414 RI (refractive index) detector using DMF solvent and calibrated from poly (methyl methacrylate) (PMMA) standards. Mass spectrometry was executed using a Q-tof microYA-263 mass spectrometer by following an ESI technique. Optical fluorescence microscopic images were obtained from Olympus Fluorescence Microscope. The TEM images were captured on JEOL-JEM 2100HR with EELS using 200 kv transmission electron microscope. The DLS and zeta-potential data was collected from a Malvern Nano-zetasizer instrument. Emission and UV-Vis spectroscopic measurement were done in Fluoromax-4 spectrometer from HORIBA Jobin Yvon and Labtronics spectrometer with model

no LT-291. The ITC data were interpreted by use of MicroCal Origin software package (ver. 7.0).

Synthesis of acrylate-ester and tertiary amine based small molecular amphiphile, ATA (3):

The target compound, ATA was synthesized in multistep process and the synthetic scheme is represented below (Scheme S1). Compound **1** and **2** were synthesized in the laboratory following the procedure mentioned in the literature.[1] Synthetic procedure of compound **ATA** is described below.

Compound 1: ^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ (ppm): 6.29 (t, 1H), 6.45 (dd, 1H), 5.70 (dd, 1H), 4.17 (q, 2H), 3.40-3.62 (m, 10H), 3.24 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 166.11, 130.91, 128.22, 71.87, 70.57, 70.55, 70.52, 69.07, 63.64, 58.97.

ESI-MS: m/z calculated for $C_{10}H_{18}O_5Na$ $[M+Na]^+$ = 241.1052, Found: 241.1454.

Compound 2: ^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ (ppm): 4.24 (t, 2H), 3.70-3.51 (m, 14H), 3.35 (s, 3H), 2.85-2.62 (t, 4H), 2.51 (t, 2H).

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm): 173.15, 71.80, 70.48, 70.40, 70.39, 69.00, 63.73, 59.48, 58.91, 56.44, 50.03, 32.90.

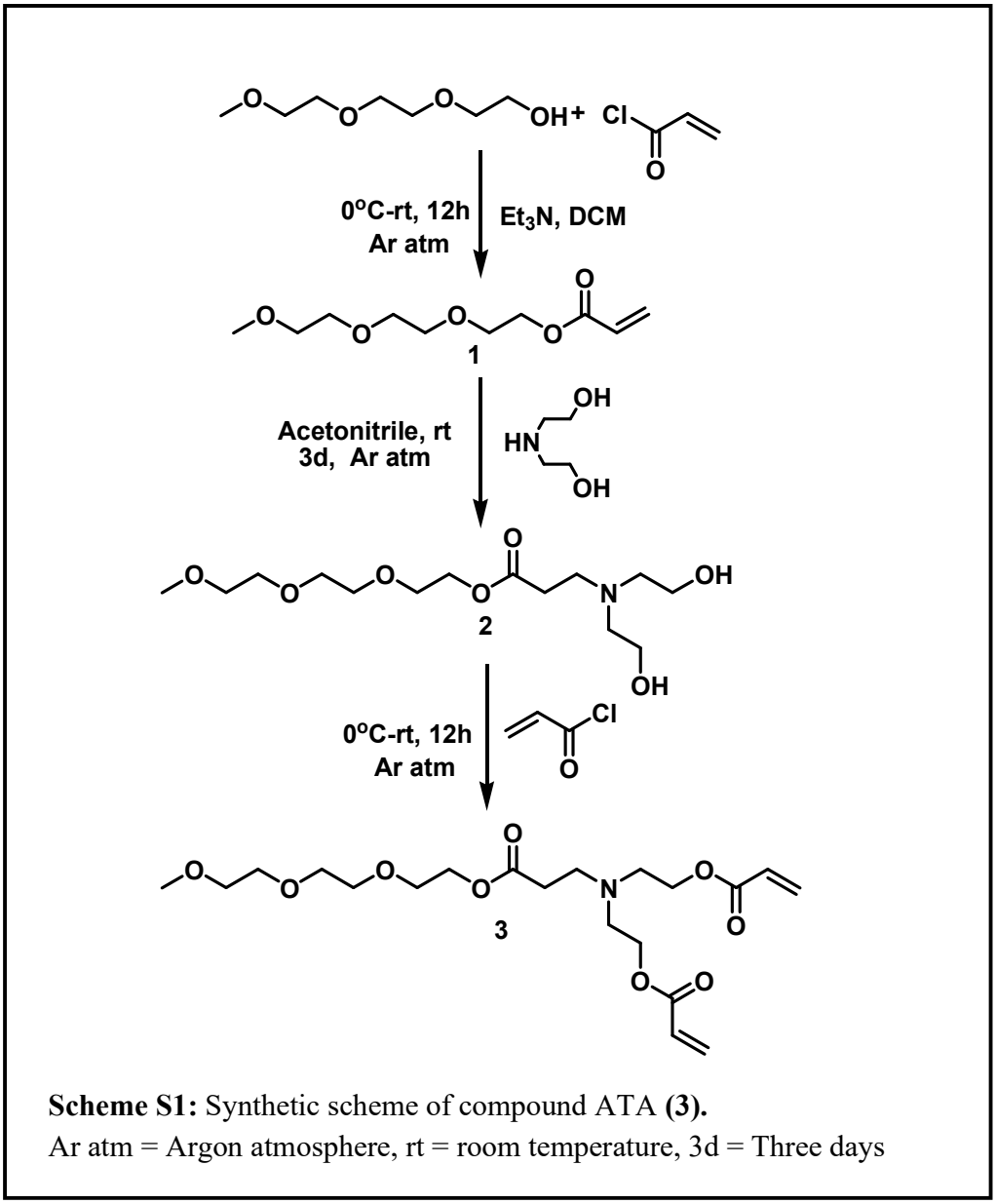
ESI-MS: m/z calculated for $C_{14}H_{30}NO_7$ $[M+H]^+$ = 324.1944, Found: 324.2018.

Compound ATA (3): At first, 0.5 g (1.546 mmol) of compound **2** was dissolved in 10 ml of dry CH_2Cl_2 in a round bottom flask. To this solution, 360 mg (3.556 mmol) of triethylamine was added and the mixture was cooled in an ice bath. Then, acryloyl chloride (308 mg, 3.402 mmol) dissolved in 10 ml of dry CH_2Cl_2 was added drop-wise to the solution with stirring condition under argon atmosphere. After the complete addition, the reaction mixture was settled to room temperature and stirred for 12 hours. Thereafter, the reaction was stopped and the mixture was washed three times (3x25ml) with brine solution. The collected organic part was dried in anhydrous sodium sulfate and evaporated under reduced pressure condition to obtain the crude product as light yellow oil. It was further purified by column chromatography using silica gel as stationary phase and ethyl acetate in hexane mixture as eluent to isolate the desired product as light yellow oil in ~80 % of yield.

^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ (ppm): 6.42 (dd, 2H), 6.11(m, 2H), 5.82(dd, 2H), 4.22(t, 6H), 3.48-3.78 (m, 10H), 3.36 (s, 3H), 2.96 (t, 2H), 2.89 (t, 4H), 2.52 (t, 2H).

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm):174.93, 166.23, 131.12, 128.10, 71.73, 70.45, 70.41, 70.34, 68.98, 63.61, 58.88, 49.74, 38.62, 37.23, 30.50.

ESI-MS: m/z calculated for $C_{20}H_{34}NO_9$ $[M+H]^+$: 432.2228, Found: 432.2210.



Scheme S1: Synthetic scheme of compound ATA **(3).**
Ar atm = Argon atmosphere, rt = room temperature, 3d = Three days

NMR Characterization:

^{1}H and ^{13}C NMR spectroscopic measurements were performed using a Bruker 400 MHz NMR spectrometer at temperature 25°C in $CDCl_3$ solvent, purchased from Sigma-Aldrich. All chemical shifts were represented in ppm (δ) units relative to tetramethylsilane (singlet δH= 0.00).

Calibration was processed by the use of the residual solvent signal of chloroform at δH = 7.26. Analysis method followed first-order and the following abbreviations were used throughout the text: s ~ singlet, br. s ~ broad singlet, d ~ doublet, t ~ triplet, q ~ quartet, m ~ multiplet.

Electrospray Ionization Mass Spectrometry (ESI-MS):

The samples were dissolved in accurate solvents and then injected in the column for recording mass spectra at room temperature.

Determination of Critical Aggregation Concentration (CAC):

At first, a measured amount (6.5mg) of compound ATA was dissolved in 400 µl of acetone and 10 mL of HPLC water was drop-wise added to it and finally kept open for 6 hours to evaporate the acetone. After that, it was used as stock solution (1.5mM) of ATA. Various concentrations of ATA solution (1.2mM, 1.0mM, 0.8mM, 0.6mM, 0.4mM, 0.2mM, 0.1mM, 0.05mM, 0.001mM, per 1ml of HPLC water) were taken in several screw capped vials and a certain amount of DiI dye in acetone (20 µL, 1 mM) was added to the each vial followed by stirring of the solution at uncapped condition for 2 hours to evaporate the acetone, where the concentration of DiI was maintained at 2×10^{-6} M. Then, the DiI dye-encapsulated ATA solution was used for the spectroscopic analysis. Next, the concentration of the dye encapsulated ATA solution was plotted against the absorption intensity of dye at 558 nm and the inflection point was considered as the CAC of ATA.

Isothermal Titration Calorimetric (ITC) Experiments:

In a typical ITC measurement, concentrated solution of ATA (0.53mg/0.1ml HPLC water, 12mM, 1µL is injection volume, and number of injections = 40) was filled in the sample cell and

the deionized water was filled in the reference cell. Then, the sample was injected maintaining the time gap of 120 s between each addition with stirring speed at 400 rpm and temperature at 25°C. The free energy (ΔG) of micellization for ATA can be derived from equation-1 given below, where CAC, T and R are the critical aggregation concentration, temperature and universal gas constant respectively. The entropy (ΔS) of micellization can also be derived from Gibb's-Helmholtz equation (eq.2).

$\Delta G = RT \ln CAC$ ……. eq.1

$\Delta G = \Delta H - T\Delta S$ ...……...eq.2

Fabrication of Nanonetwork (NN):

A calculated amount (1.74mg, 2mM) of compound ATA was dissolved in 200 µl of acetone and then 2.0ml of HPLC water was drop wise added to it and finally kept open for 6 hours get the nanoassemblies solution by evaporation of acetone. Next, hexanedithiol (0.62µl, 0.004 mmol in 0.2ml acetone) and hexylamine (0.13µl, 0.001 mmol in 0.2ml acetone) were simultaneously added to the nanoassembled solution to fabricate the nanonetwork. Then the solution was stirred for 48 hours and after that it was filtered with a 0.45 µm syringe filter.

Gel Permeable Chromatography (GPC):

At first, a measured quantity (1mg) of the monomer (ATA) and cross-linked nanonetwork (NN) were dissolved in 1ml of DMF. Then the solutions were sonicated and vortexed for 3 minutes. The homogeneous solutions were produced by allowing them to settle at room temperature for few hours and then the solutions were filtered using a membrane filter with 0.45 µm pore size.

Finally, the solutions were injected into the GPC column one by one to estimate the molecular weight of the monomer and nanonetwork (NN), where the flow rate and the column temperature were kept at 0.8ml /min and 25°C, respectively.

Transmission Electron Microscope (TEM):

To investigate the TEM images, 1.0mM solutions of nanoassembly (NA) and nanonetwork (NN) were drop-casted on a carbon coated copper grid (300-mesh) and air dried for 24 hours, before measurements were carried out.

Dynamic Light Scattering (DLS):

To analyze the DLS data, the solutions (1.0mM) of nanoassembly (NA) and nanonetwork (NN) were filtered using a membrane filter with a 0.22 μm pore size and measurement were carried out at room temperature.

Dilution and stability test:

The variations of hydrodynamic diameter were recorded in a DLS instrument by continuous dilution even below the CAC. The time dependent DLS for both of the nanoassembly (NA) and nanonetwork (NN) was carried out in FBS at 37^{o}C to check the stability in serum.

Fluorescence Microscopy Study:

The encapsulation of DiI inside the micelle core of ATA was checked under the fluorescence microscope (OPM). In brief, DiI encapsulated micelle solution (50 μl) was drop-casted on a clean glass slide and a cover glass was placed on it. After that images were collected at a magnification of 40X using an OLIMPUS BX-51 fluorescence microscope.

DiI Encapsulation and release Studies:

A measured amount of DiI in acetone (10 μL, 1 mM) was added gradually to the solutions of ATA and allowed to stir for 6 hours for evaporation of the acetone from the mixture at room temperature. Next, this dye encapsulated solution of ATA was used for the absorption measurement to examine the extent of dye encapsulation. After confirmation of DiI encapsulation, the DiI encapsulated nanonetwork was fabricated by cross linking of the DiI loaded nanoassembled solution using dithiol following Michael Addition reaction. Then, the unencapsulated DiI were removed from the DiI loaded nanoassembly and nanonetwork solutions by filtering through a membrane filter (0.45 μm pore size). The pH responsive guest release study was performed with the DiI loaded solutions (both nanoassembly and nanonetwork) and the pH of the solutions (both nanoassembly and nanonetwork) was adjusted to pH ~7.4 and pH ~5.0 using tris buffer, respectively. Finally, time dependent UV−Vis spectra were monitored to examine the DiI release. The % release of guest molecule can be calculated using absorbance intensity of DiI at 558 nm following the equation: $[(A_o-A_t)/A_o] \times 100$, where A_0 is the initial absorbance intensity and A_t is the intensity of absorbance at any time 't'.

Doxorubicin encapsulation and release Studies:

To the self-assembled solution of ATA (2ml, 0.8mM), a certain amount (1.12 mg) of hydrophobic DOX was added and vortexed for 5minutes followed by sonication for few minutes. After that, the solution was filtered with a 0.45 μm syringe filter and cross-linked by above described procedure to fabricate DOX loaded nanonetwork (NN-DOX). Then, dialysis was performed to remove all the small molecules from DOX-loaded nanoassembly (NA-DOX) and nano-network (NN-DOX) with a 3500 MWCO dialysis bag against 60 mL of triple-distilled water. The DOX release was monitored overtime and % release was calculated by fluorescence spectroscopy.

Forster Resonance Energy Transfer (FRET):

For the FRET experiment, stock solutions (1mg/ml) of two FRET pairs (DiO as FRET donor and DiI as FRET acceptor) were prepared at first in acetone solvent. From the stock solutions of DiI and DiO, 15 µl of each solution were taken and mixed together. Then, this mixture of DiO and DiI was added to the 1.0mM solution of nanoassembly and the mixed solution was kept open for 6hours to evaporate acetone. After that, the solution was cross-linked using dithiol to fabricate the nanonetwork followed by filtration with a 0.45 µm pore sized membrane filter.

This mixed dye loaded solution were used for the FRET experiment, where the solution was excited at wavelength of 470 nm and slit width was kept at 3/3 with scan speed of 5nm/s. Then time dependent emission of the solution was monitored to calculate the FRET ratio $I_a/(I_a+I_d)$ at different time period, where I_a and I_d are the respective emission intensities of the acceptor (DiI, 585 nm) and the donor (DiO, 512 nm) dye.

Cell Culture:

For cell culture of the H9c2 and Hela cells, DMEM media along with 10% FBS, 100 µg/ml streptomycin sulphates and 100 units/ml penicillin G sodium was used in presence of 5% CO_2 at 37°C. After trypsinisation, the cells were seeded at required density according to respective experiments to allow them to re-equilibrate a day before the start of the experiment.

Cytotoxicity Assay:

Cell toxicity was determined by the MTT assay. Hela and H9c2 cells were seeded at a density of 1×10^6 cells per well in 96-well plate. Then, different concentrations of Nanoassemblies (NA), nanonetworks (NN), Nanoassembly-DOX (NA-DOX) and nanonetwork-DOX (NN-DOX) were

added to the cell medium and incubated for 24 hours. 5mg/ml of MTT stock solution was diluted in a ratio of 1:10 in 1X PBS. After incubation, the culture medium was changed with 40µL of the diluted MTT solution. The cells were then incubated for 3 hours in 5% CO_2 at 37°C. After 3hrs, the MTT solution was discarded from each well and 50µl of extraction buffer (80% isopropanol, 20% Triton X-100 and 12(N) HCl) was added to each well. The absorbance was measured at 570nm.

Cell Imaging by Fluorescence Microscopy:

H9c2 and Hela cells (1×10^9 cells per) were platted in 35 mm dish with a fresh cover slip and incubated for 24 hours for the cell imaging studies by Fluorescence Microscopy. After obtaining the appropriate confluency, the cells were treated with free DOX, nanoassembly-DOX and nanonetwork-DOX. After 3, 6, 12 and 24 hours of incubation, the cells were washed for two times with PBS (Phosphate Buffer Saline) and they were fixed then by using 500 µL of 4% Paraformaldehyde for 10 minutes. The cells were washed with PBS again after the fixation and the nuclei were stained using DAPI at room temperature for 15 minutes. After that, cells were further washed with PBS for three times and with the mounting media (0.5% N-propyl gallate, 90% glycerol, 20 mM Tris, pH 8.0) cover slips were mounted on the slides. Finally, the images were captured by Leica DM2000 across several areas.

Additional Figures:

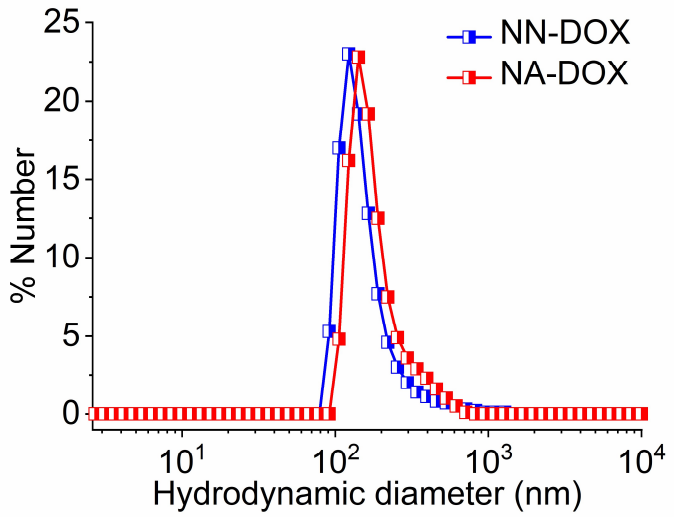


Fig. S1: DLS size distribution of DOX encapsulated nanoassembly (NA-DOX) and nanonetwork (NN-DOX). Temperature=25°C.

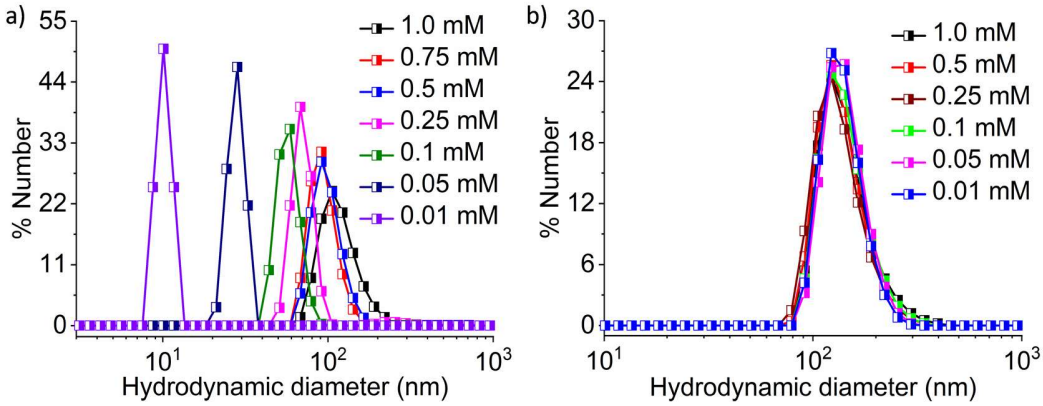


Fig. S2: DLS size distribution plot of (a) nanoassembly (NA) (b) nanonetwork (NN) upon dilution with water. Temperature = 25°C.

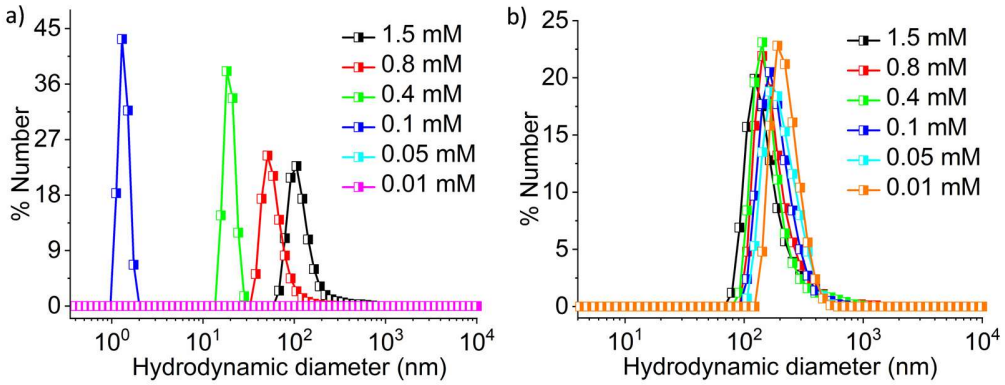


Fig. S3: DLS size distribution plot of (a) nanoassembly (NA) (b) nanonetwork (NN) upon dilution with DMF. Temperature = 25ºC.

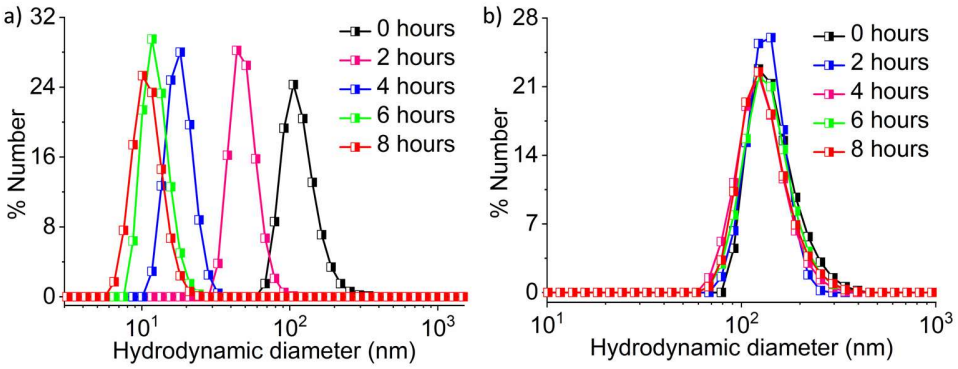


Fig. S4: DLS size distribution plot of (a) nanoassembly (NA) (b) nanonetwork (NN) in FBS (Fetal Bovine Serum). Concentration of ATA= 1.0mM, Temperature=37ºC.

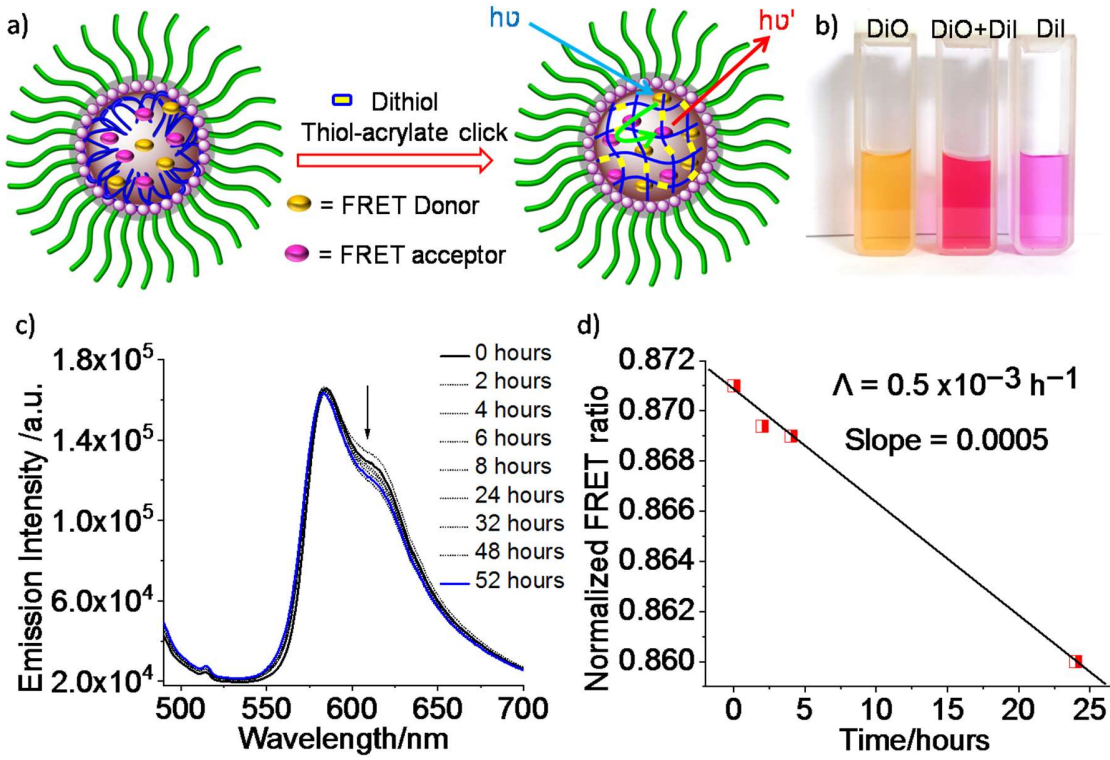


Fig. S5: (a) Schematic representation of FRET by co-encapsulating DiI and DiO; (b) Images of the individually and mixed DiI and DiO loaded nanonetwork solution; (c) monitoring of FRET over 52 hours by emission spectroscopy; (d) Determination of the leakage coefficient from the normalized FRET ratio vs. Time plot.

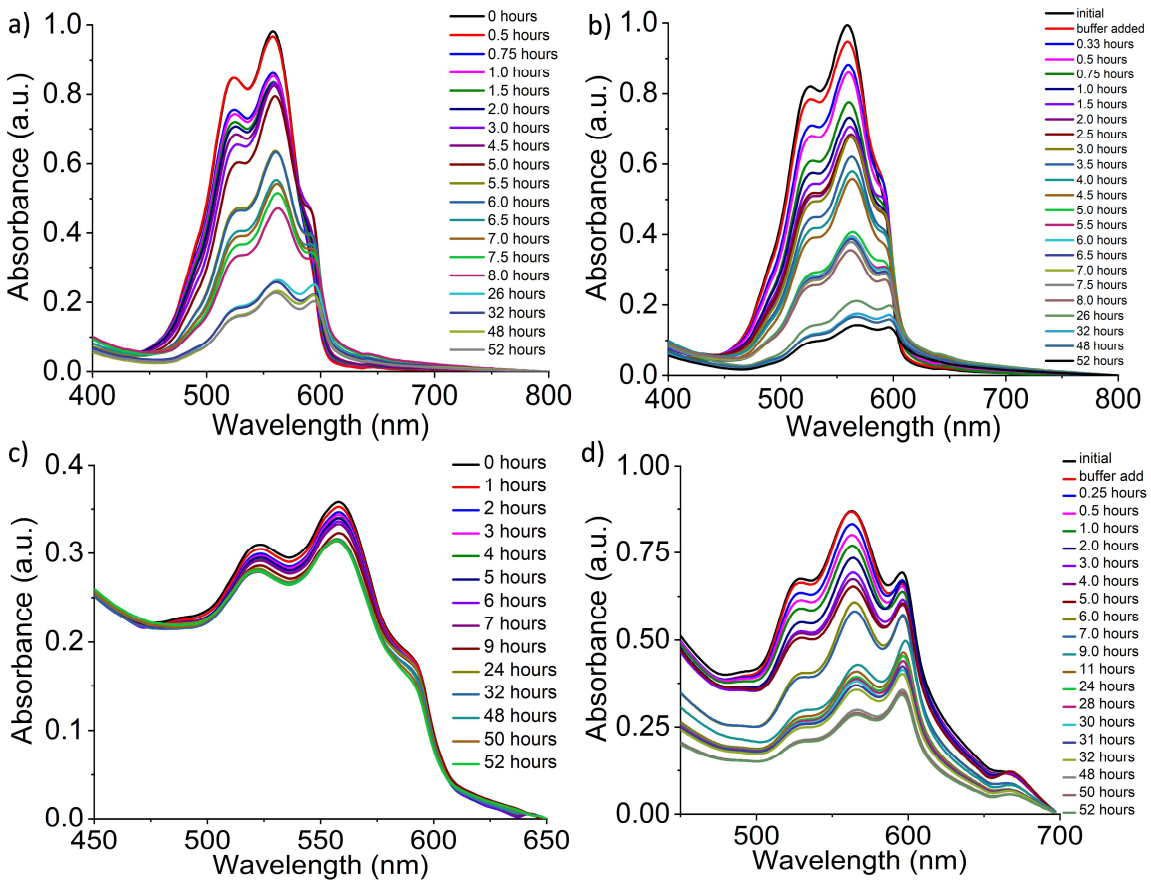


Fig. S6: DiI encapsulated release profile of (a) nanoassembly (NA) at pH~7.4 (b) nanoassembly (NA) at pH~5.0 (c) nanonetwork (NN) at pH~ 7.4 (d) nanonetworks (NN) at pH~5.0. Concentration of ATA= 1.0mM and Temperature=25°C.

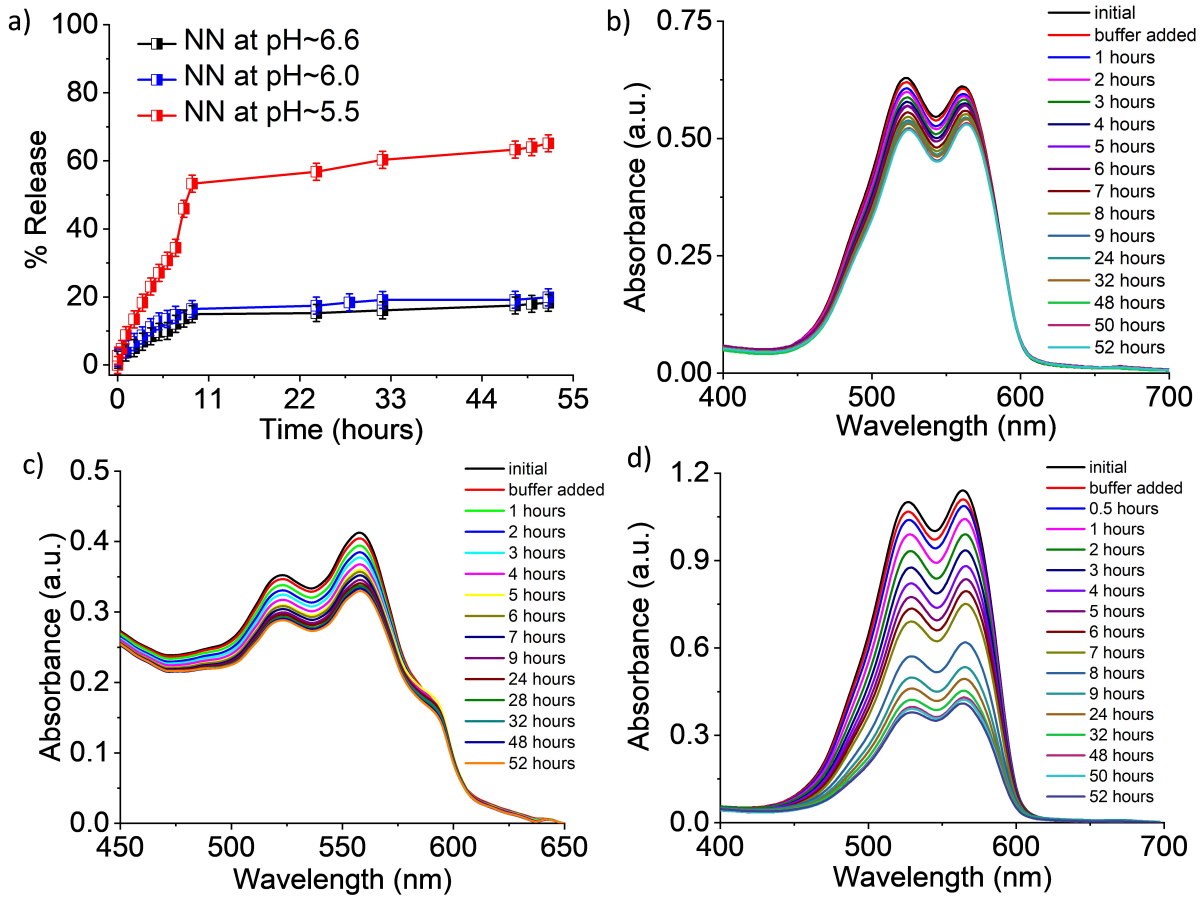


Fig. S7: (a) DiI release profile of nanonetwork at pH~6.5, 6.0 and 5.5. DiI encapsulated release profile of (b) nanonetwork (NN) at pH~6.5 (c) nanonetwork (NN) at pH~6.0 (d) nanonetwork (NN) at pH~ 5.5. Concentration of ATA= 1.0mM and Temperature=25°C.

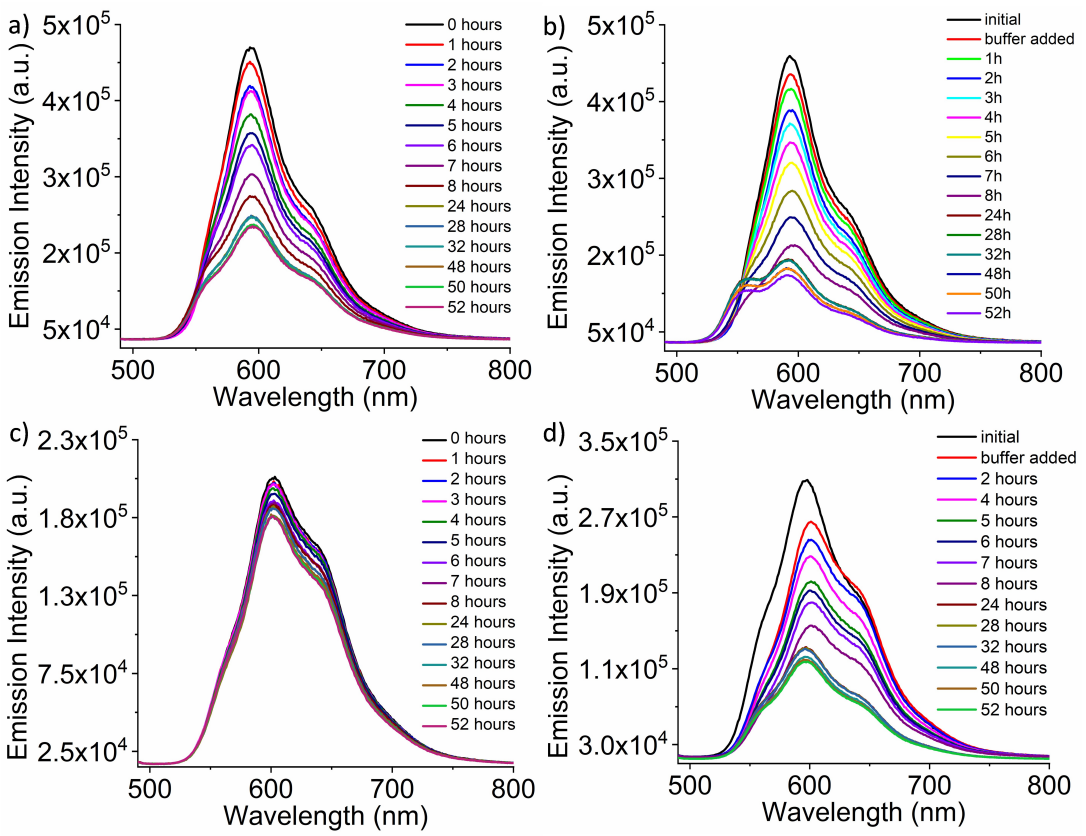


Fig. S8: DOX encapsulated release profile of (a) nanoassembly (NA) at pH~7.4 (b) nanoassembly (NA) at pH~5.0 (c) nanonetwork (NN) at pH~ 7.4 (d) nanonetworks (NN) at pH~5.0. Concentration of ATA= 1.0mM and Temperature=25°C.

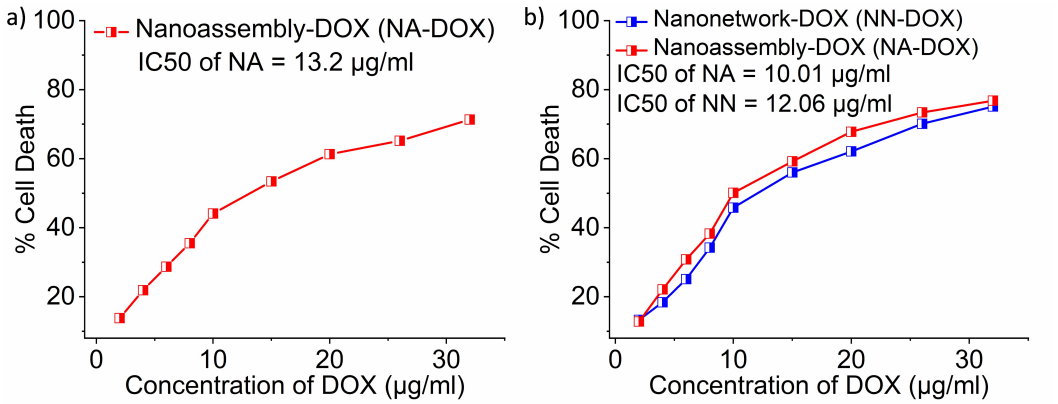


Fig. S9: IC50 calculation plot of DOX loaded nanoassembly (NA-DOX) and nanonetwork (NN-DOX) against (a) H9c2 and (b) HeLa cell lines.

Table S1: IC50 values of DOX loaded nanoassembly (NA-DOX) and nanonetwork (NN-DOX) against different cell lines are listed here.

Cells	IC50 of NA-DOX (µg/ml)	IC50 of NN-DOX (µg/ml)
H9c2	13.2	-
HeLa	10.01	12.06

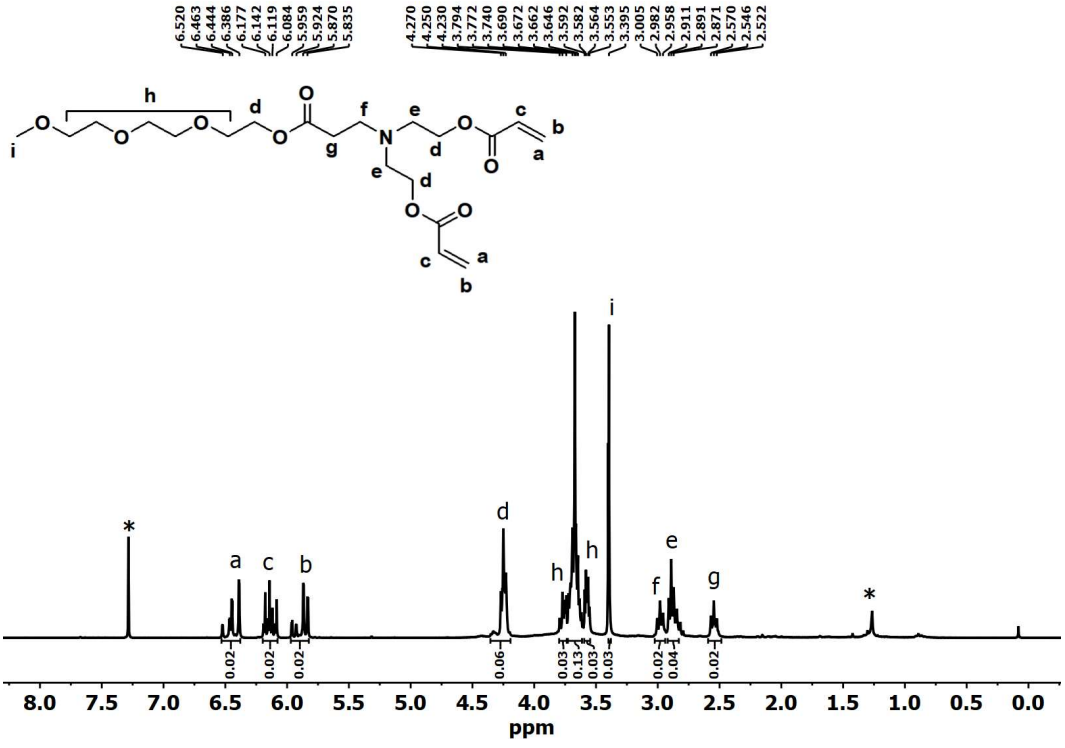


Fig. S10: ^{1}H NMR spectrum of ATA in $CDCl_3$ Solvent. * indicates solvent peaks.

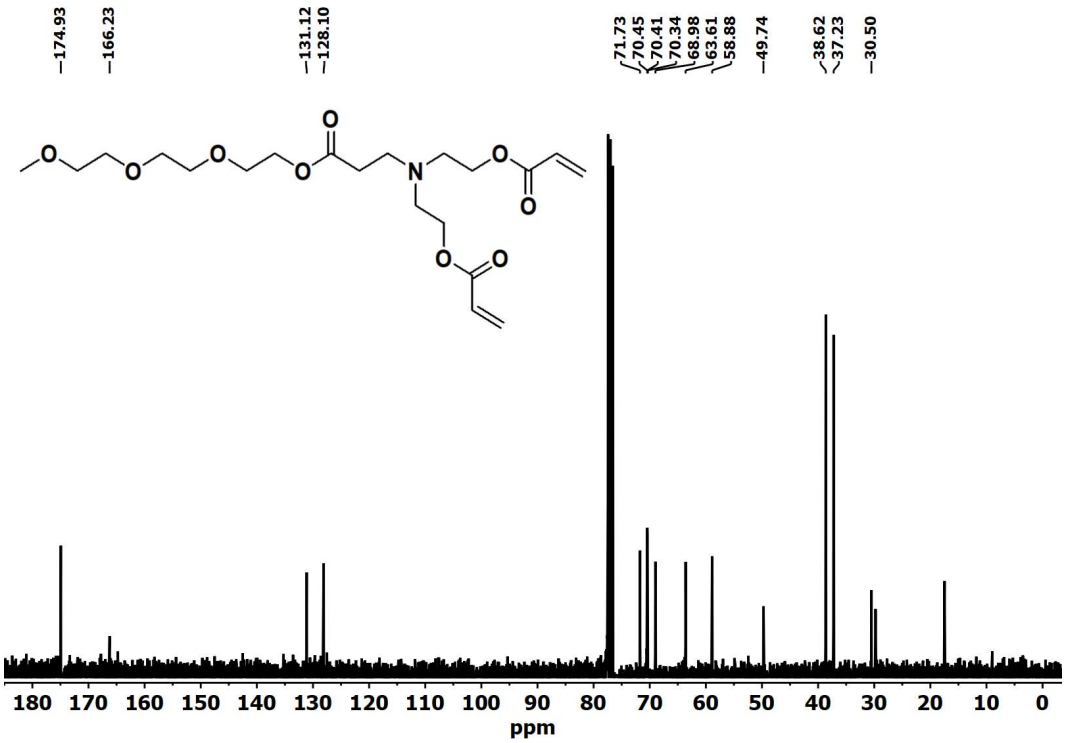


Fig. S11: ^{13}C NMR spectrum of compound ATA in $CDCl_3$ Solvent.

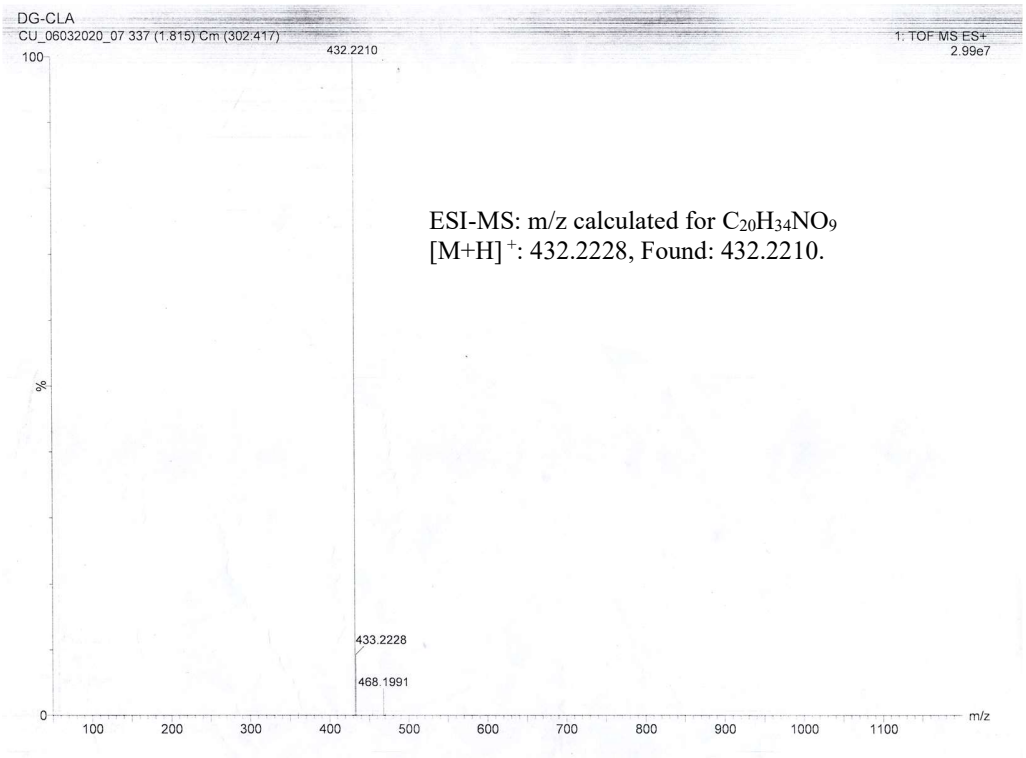


Fig. S12: ESI-MS spectrum of compound ATA.

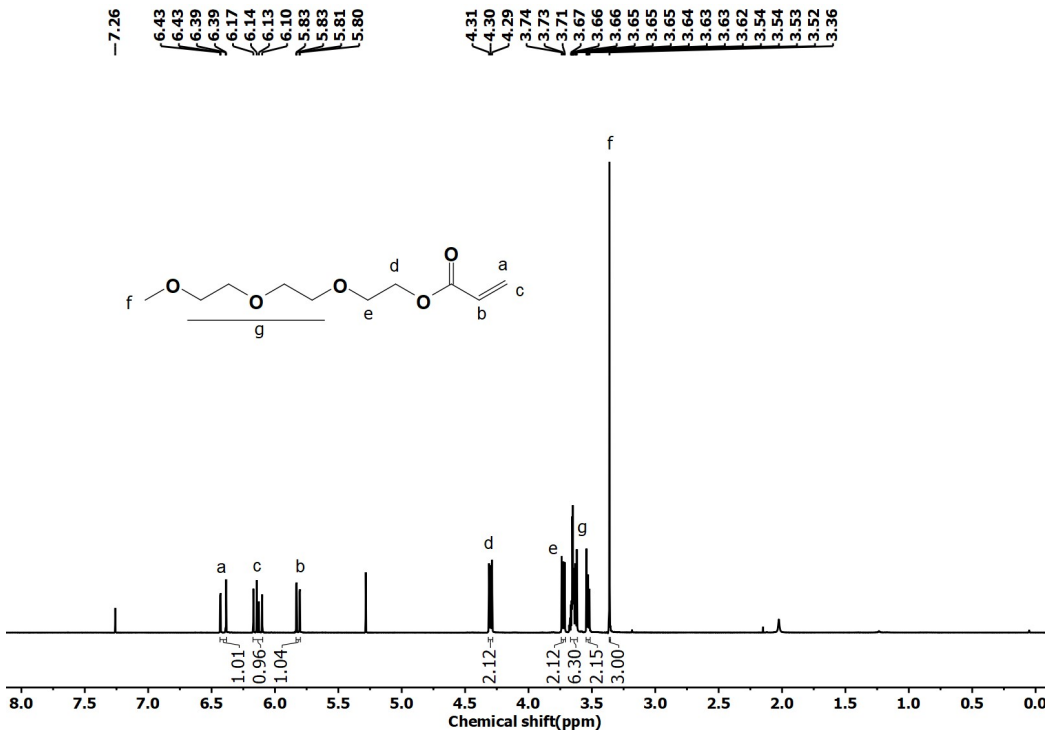


Fig. S13: ^{1}H NMR spectrum of compound-**1** in $CDCl_3$ Solvent.

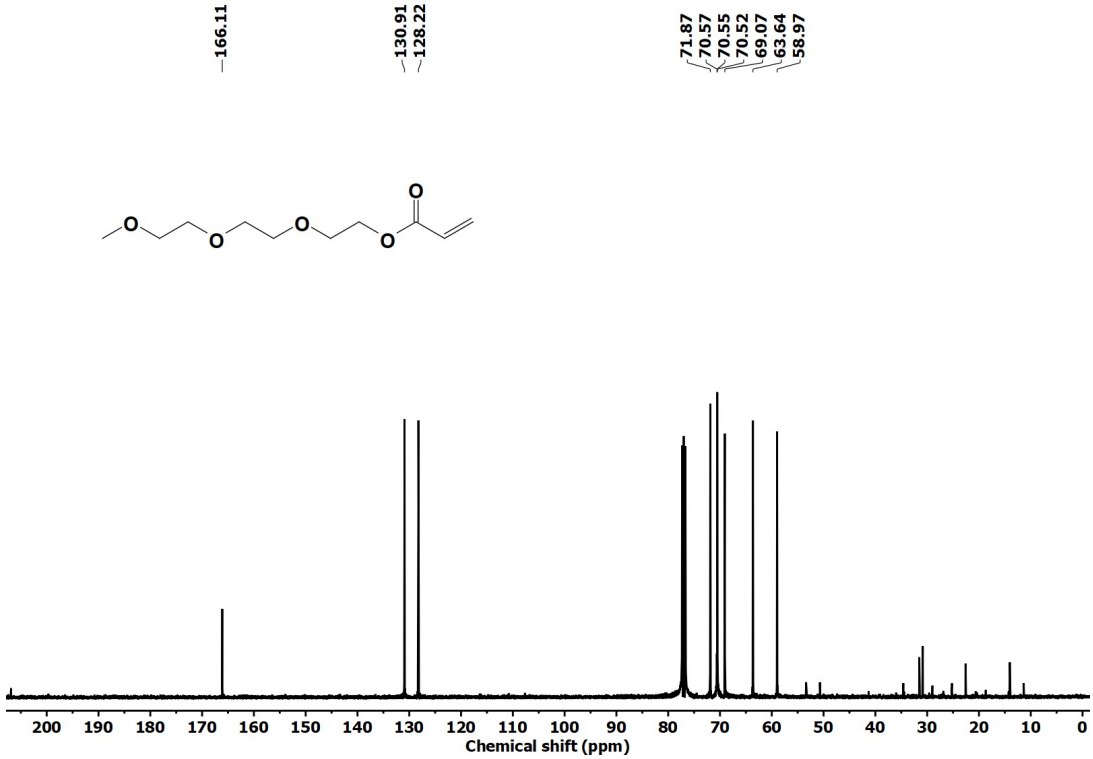


Fig. S14: ^{13}C NMR spectrum of compound-**1** in $CDCl_3$ Solvent.

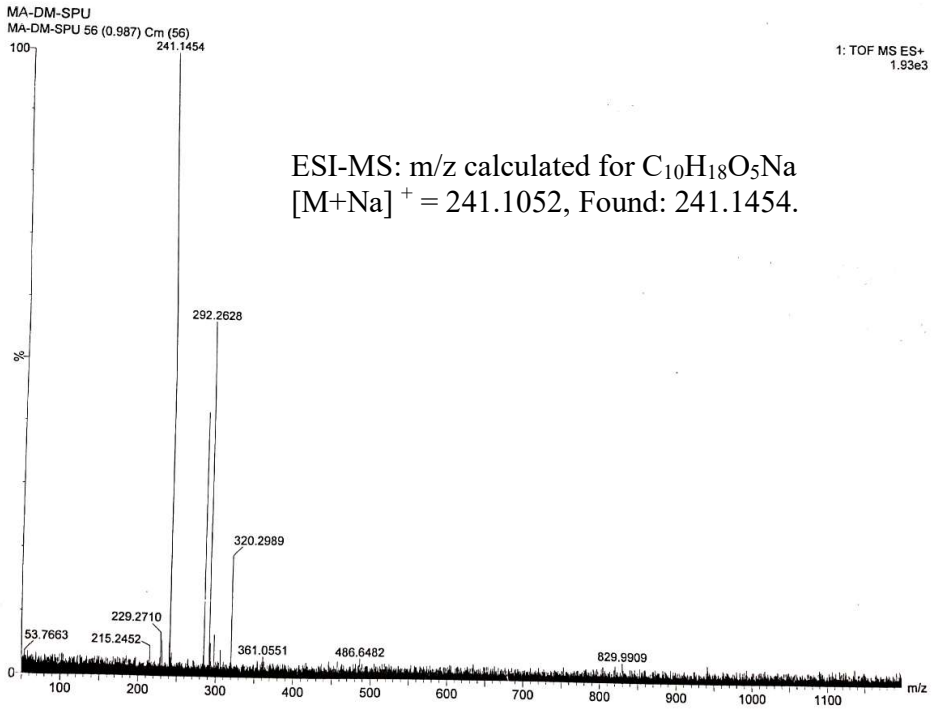


Fig. S15: ESI-MS spectrum of compound-**1**.

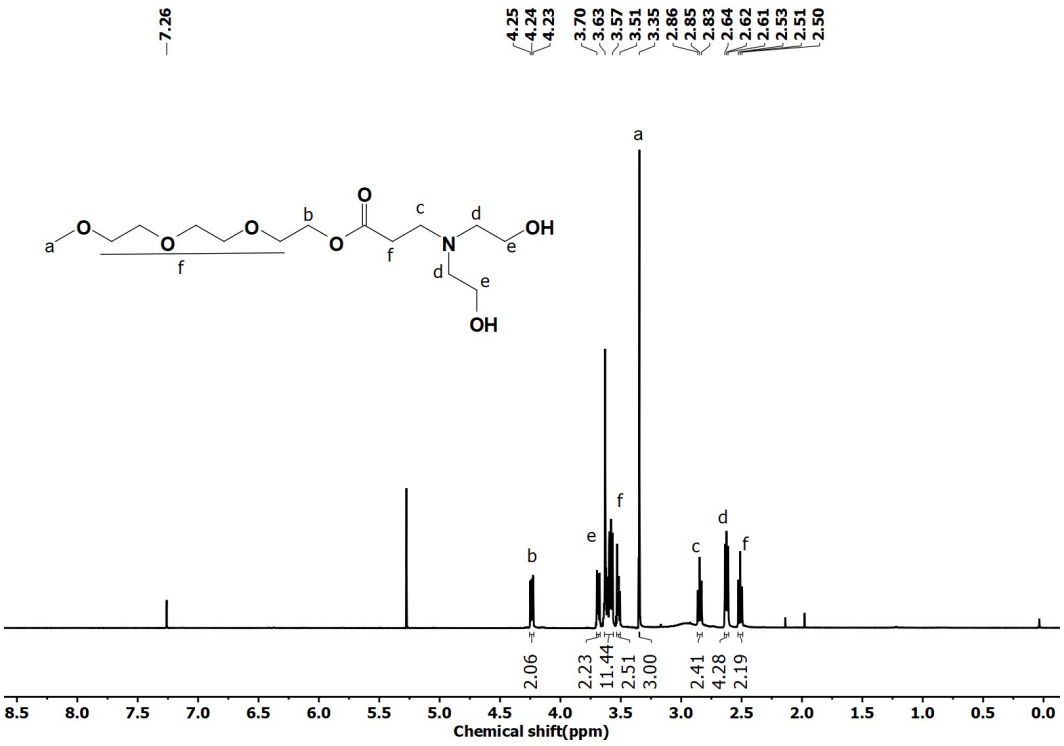


Fig. S16: ^{1}H NMR spectrum of compound-**2** in $CDCl_3$ Solvent.

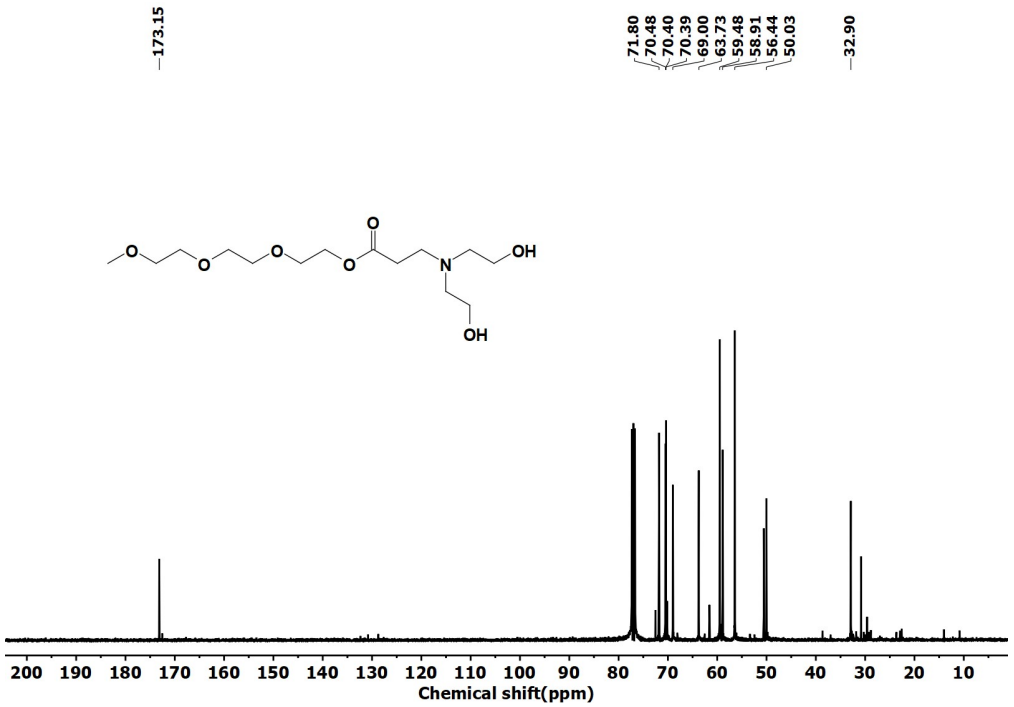
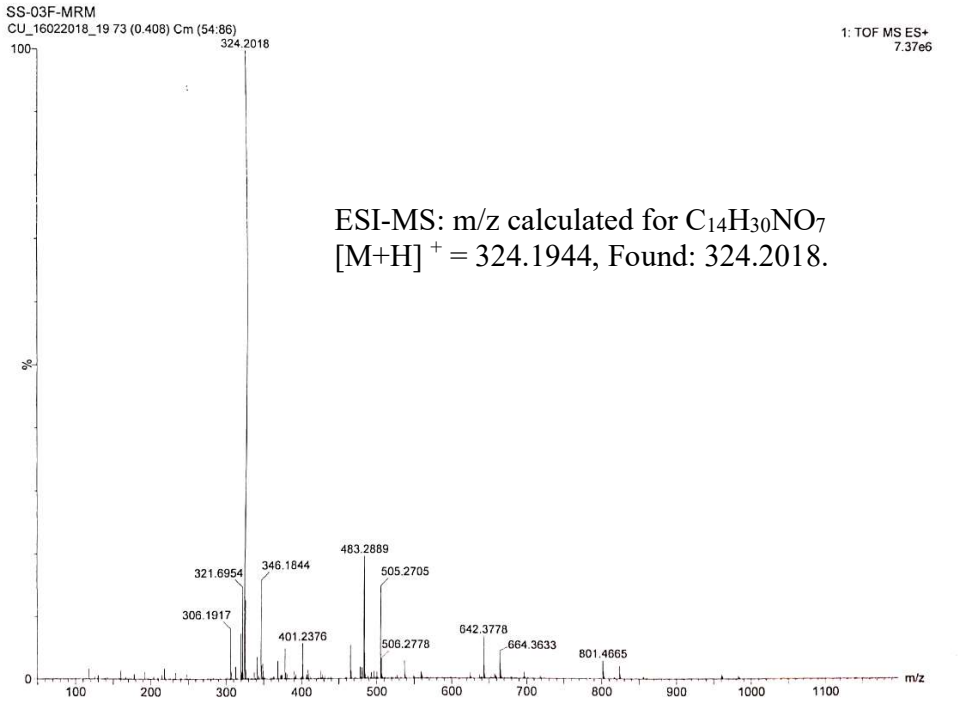


Fig. S17: ^{13}C NMR spectrum of compound-**2** in $CDCl_3$ Solvent.



ESI-MS: m/z calculated for $C_{14}H_{30}NO_7$ $[M+H]^+$ = 324.1944, Found: 324.2018.

Fig. S18: ESI-MS spectrum of compound-**2**.

Reference:

1. S. Santra, S. M. Ali, A. Mondal and M. R. Molla, *Langmuir,* 2020, **36**, 8282–8289.