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Supporting information

Stimuli-responsive fluorescent nanogel: A nonconventional donor for the ratiometric temperature and pH sensing

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EXPERIMENTAL SECTION:

Materials: 1,4-Diaminobutane (99%), Deuterium oxide (D_2O, 99.9 atom % D), Doxorubicin-hydrocholoride, Quinine sulphate dihydrate were purchased from sigma-Aldrich and N, N-methylene(bis)acrylamide (MBA) 3x cryst. extrapure AR, Lithium bromide, Dimethylformamide (DMF), Tetrahydrofuran (THF), N – isopropyl acryl amide (NIPAM) were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL). Sodium hydroxide pellets and acetic acid glacial were purchased from CDH. All chemicals were of extra pure or HPLC grade used without further purification. HPLC grade water was used throughout the experiments.

Synthesis of temperature and pH responsive (NANO-PAMAM) nanogel:

The temperature and pH responsive hyperbranched polyaminoamide (NANO-PAMAM) was synthesised via an aza-Michael addition reaction between N, N'-methylene-bisacrylamide (A_2 monomer) and 1, 4 -diaminobutane (B_4 monomer). The distinctive procedure for the synthesis of (NANO-PAMAM) nanogel was described as follows: N, N'-Methylene(bis)acrylamide (MBA, 0.250 g, 1.62 mmol) was suspended in 2.5 ml of water and stirred for 10 min at room temperature. 1,4-butane diamine (mole ratio of MBA to di-amine is 2:1) was then added to the aqueous suspension of MBA to start the polymerisation. Within 20 min the suspension became clear. After 90 min from the addition of diamine, the aqueous solution of N – isopropylacrylamide (NIPAM 0.906 g, 8.1 mmol, excess) was added and the solution was stirred for more 48 hours at the room temperature. The resulting solution was purified by precipitation in tetrahydrofuran (THF). The precipitated product was washed with THF 3 times to remove unreacted excess NIPAM. The product was then dried and dispersed

in water immediately to prepare the nanogel solution with a concentration of 10 mg/ml. This solution was then used for further experiments.

Preparation of (NANO-PAMAM-DOX).

With the variation of doxorubicin content, such as 10 wt% [NANO-PAMAM-DOX (0.1)] and 20 wt% [NANO-PAMAM-DOX (0.2)] with respect to NANO-PAMAM. To prepare two different samples the aqueous solution of doxorubicin was added to the aqueous solution of nanogel and stirred for 48 hours at the room temperature. The reaction is monitored by NMR spectra and finally, (NANO-PAMAM-DOX) pair dispersed in water was then used for the ratiometric temperature and pH sensing.

Table-S1

Samples	Reagents		Solvent	Time of the reaction
	NANO-PAMAM	DOX		
NANO-PAMAM-DOX (0.1)	0.030 g	0.0030 g	Water (3 ml)	48 hours
NANO-PAMAM-DOX (0.2)	0.030 g	0.0060 g	Water (3 ml)	48 hours

Nuclear Magnetic Resonance (NMR) Spectroscopy– A Bruker 400 MHz spectrometer was used for ^{1}H-NMR spectra. D_2O was used as a NMR solvent.

Size Exclusion Chromatography (SEC) – Size Exclusion Chromatography (SEC) coupled with triple detector was used for the relevant analysis. The system contains Shimadzu i-series plus integrated HPLC attached with Refractive index detector (Wyatt Optilab T-rEX), Viscometer detector (Wyatt Visco Star III) and Multi angle light scattering detector (Wyatt DAWN HELEOS LS II). DMF containing 0.01% LiBr was used as the eluent, temperature of the column was 40 ˚C and the flow rate was 0.75 mL/min. Data collection and processing was done by ASTRA 7.3.0 software (Wyatt Technological Corporation).

Determination of the Volume Phase Transition Temperature (VPTT):

Generally, the aqueous dispersion of the temperature responsive hyperbranched polyaminoamide, (NANO-PAMAM) (10 mg/ml) was prepared and the obtained solutions were then transferred to a 1cm quartz cell (2 windowed) with 1 cm path length. The transmittance (%) of the aqueous solutions at 356 nm was recorded by an UV−vis spectrophotometer (Jasco V- 760 nm) attached with a temperature regulator.

Hydrodynamic Radius (R_h) determination:

The aqueous solution of the temperature responsive hyperbranched polyaminoamide (NANO-PAMAM) (10 mg/ml) was prepared and the obtained solutions were then transferred

to a 10 mm glass cell (4 windowed). Dynamic light scattering (DLS) was used to observe the hydrodynamic diameter of the nanogel particle using Litesizer 500 particle analyzer from Anton Paar. From CONTIN plot, size and the particle size distributions were calculated.

Transmission Electron Microscopy (TEM):

Transmittance electron microscope (TEM) images were analyzed by using JEOL TEM with accelerating potential of 200 kV.

Fluorescence spectrophotometer:

Fluoromax-4P spectrofluorometer (Horiba jobin Yvon) was used to acquire fluorescence excitation and emission spectrum. Four winded quartz cuvette was used.

Lifetime measurement:

Temperature dependent lifetime measurement of the donor was carried out by using time-correlated single photon counting (TCSPC) technique with excitation at 375 nm by using a diode laser as an excitation source. For this measurement, fluorescence spectrophotometer from Edinburgh instrument (model: lifeSpec II, U.K) was used. A detector from Hamamtsu MCP PMT (3809U) was used to collect the signal. By using Peltier controlled cuvette holder (model: TLC 50) from Quantum Northwest, temperature variation experiment was conducted. Also, by using four winded quartz cuvettes with path length of 1cm, lifetime measurement was carried out. Finally, F 900 decay analysis software was used for the data collection and fluorescence decay emission were measured at the magic angle 54.7˚C.

By using the following equation, we have calculated the weight-average lifetime –

$$\langle\tau\rangle = \sum_{i=1}^{N} (C_i \tau_i)$$

$$C_i = B_i / \sum_{i=1}^{N} B_i$$

Where, τ_i = The fluorescence decay time

B_i = The pre-exponential factor

Determination of Quantum Yield:

Quinine sulphate dihydrate in 0.1(M) H_2SO_4 was used as the standard solution to obtain the fluorescence quantum yield of NANO-PAMAM nanogel.

Fluorescence quantum yield (ϕ_f) of NANO-PAMAM nanogel was calculated according to the following equation:

$$\Phi_f = \phi_{std} [I_s / I_{std}] \times [A_{std} / A_s] \times [\eta_s / \eta_{std}]^2$$

Where, ϕ_{std} = fluorescence quantum yield of the standard solution (ϕ_{std} is 0.54 for quinine sulphate in 0.1 M H_2SO_4)

I_s = Integrated emission intensity of the sample

I_{std} = Integrated emission intensity of the standard solution

A_s = Absorbance of the sample

A_{std} = Absorbance of the standard solution

η_s = Refractive index of the sample (and it is 1.33 as the sample was dissolved in water)

η_{std} = Refractive index of the standard solution (and it is 1.33 in 0.1 M H_2SO_4)

All the values of the corresponding parameters were putted in the above equation and the quantum yield was obtained. The quantum yield of the NANO-PAMAM nanogel was 0.66 %.

Determination of pka value:

The pka value of (NANO-PAMAM-DOX), nano-sensor using pH meter (OAKTON PC 2700 model) was determined via the half equivalence point determination by the titration between the acetic acid (weak acid) containing (NANO-PAMAM-DOX) nanogel and sodium hydroxide (strong base). The current experiment is defined to closely resemblance the pH dependent fluorescence study. Here, doxorubicin acted as the indicator and changed its colour from red to purple at the equivalence point. We also determined the pka value of NANO-PAMAM.

At the half of the equivalence point, [acid] = [conjugate base] and applying the Hendersen Hasselbalch equation, we have

$$pH = pka + \log [acid]/[conjugate\ base]$$

$$\log [acid]/[conjugate\ base] = \log (1) = 0$$

$$pH = pka$$

At the half of the equivalence point, the pH of the solution was 5.42 and hence the pka value was determined as 5.42 for NANO-PAMAM-DOX. While compared with earlier literature, the value closely matches with the earlier reported pKa values of PAMAM dendrimers (Anal. Methods, 2016,8, 263-269, Polymer Journal, 1985, 17, 117–132)

Energy transfer efficiency (ETE):

Energy transfer efficiency of donor, NANO-PAMAM was calculated by using the following equation: -

$$E_T = 1 - I_{DA} / I_D$$

Where,

I_{DA} = Intensity of donor, NANO-PAMAM in presence of acceptor

I_D = Intensity of donor, NANO-PAMAM in absence of acceptor

MTT assay:

For the MTT assay, L929 MTT assay (fibroblast) cells were used for invitro cytotoxicity assessment. L929 (Fibroblast) cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma aldrich, USA). L929 cell lines was first cultured in 25 cm^2 tissue culture flask with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L glutamine, sodium bicarbonate, antibiotic solution comprising penicillin (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (2.5 µg/ml) at 37 °C in a humidified CO_2/air (5:95).After two days of incubation, L929 cells were seeded in a 96-well tissue culture plate with 100 µl cell suspension (50000 cells/well)incubated at 37˚C in a humidified with 5% CO_2 incubator. After 24 hours the growth medium was removed, freshly prepared each compound in 5% DMEM were five times serially diluted by two-fold dilution (20µg, 10µg,5µg, 0.5µg, 0.1µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also monitored for control.

Theoretical Parameters:

The spectral overlap integral J(λ) between donor emission spectrum and acceptor absorption spectrum can be determined according to eqn (1).

$$J_{(\lambda)} = \int F_D (\lambda).\ \varepsilon_A (\lambda).\ \lambda^4\ d\lambda \qquad (1)$$

Where, $F_D(\lambda)$ is the normalized emission spectrum of donor, and $\varepsilon_A(\lambda)$ is the molar extinction coefficient with the unit M^{-1} cm^{-1} of the acceptor and λ in nm and the spectral overlap integral J(λ) for (NANO-PAMAM-DOX was calculated by using the a/e (Fluor Tools) software to be 1.420 e^{+13} nm^4 M^{-1} cm^{-1} (shown in Figure S5).

Afterward, the Förster radii (R_0) can be calculated using eqn (2) as follows:

$$R_0 = 0.211\ [(k^2\ \phi_D\ J(\lambda)/n^4]^{1/6} \qquad (2)$$

where, k^2 is the dipole orientation factor and a value of $k^2 = 2/3$ was used considering randomly oriented transition dipoles, ϕ_D was the quantum yield of donor and n was the refractive index of the solvent which was used to disperse (NANO-PAMAM-DOX).

Figures and tables

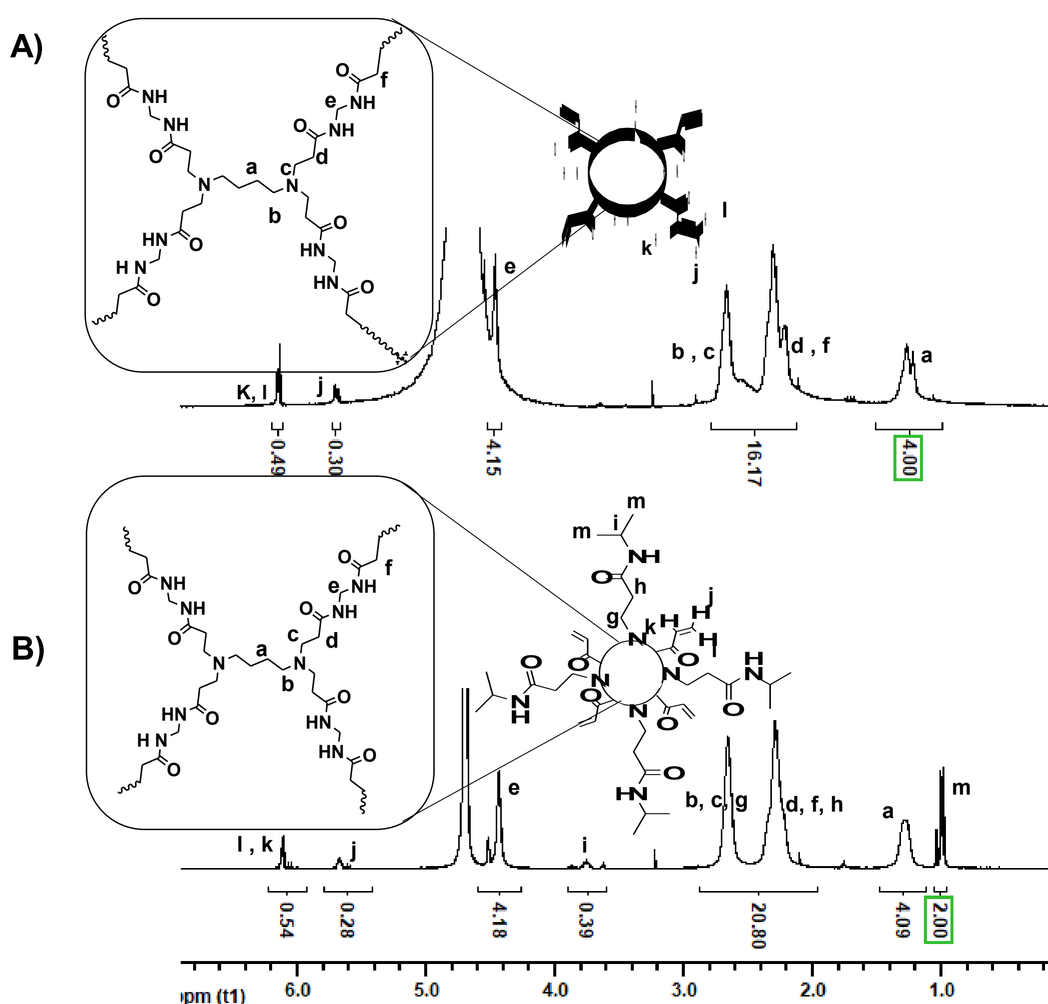

Figure S1: ^{1}HNMR spectra of (NANO-PAMAM) A) before NIPAM addition and B) after NIPAM addition in D_2O. The content of the vinylic proton of surface acrylamide groups remains unchanged with the appearance of isopropyl moieties of NIPAM, it confirms the desired reaction.

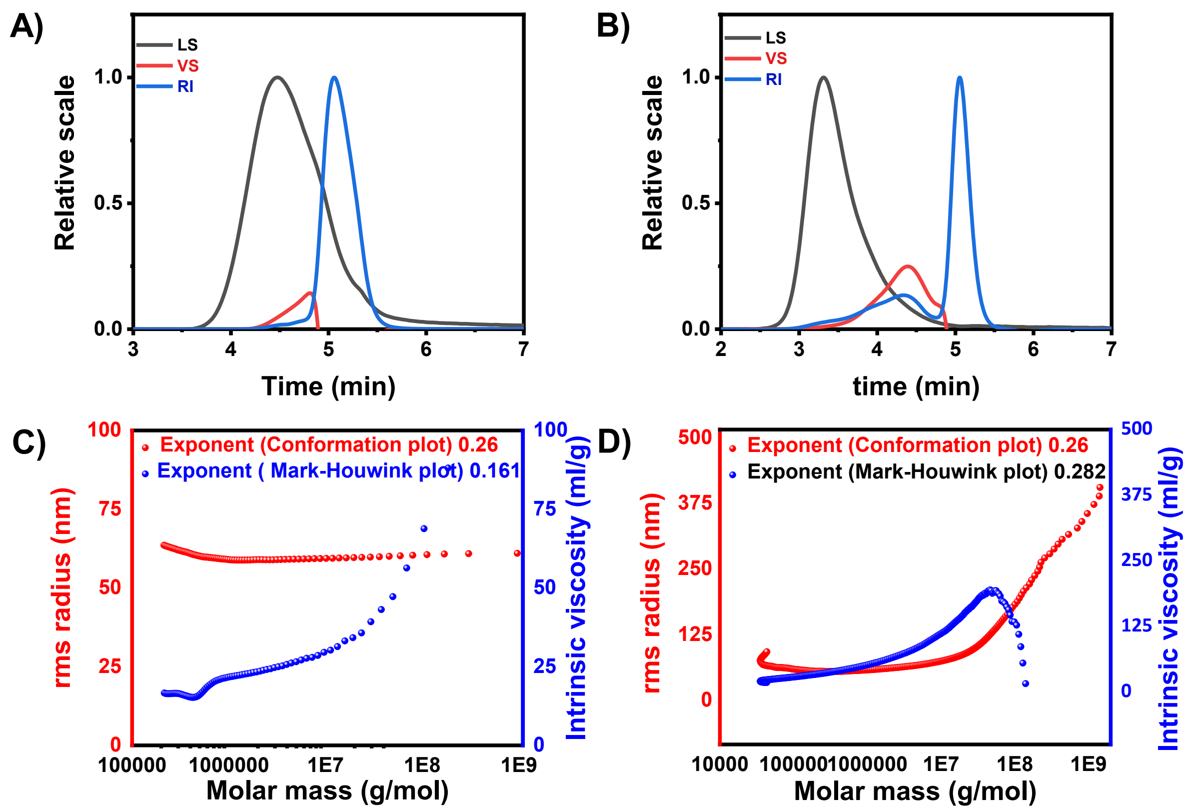

Figure S2: SEC chromatogram (using LS, Viscometer, RI detector) of NANO-PAMAM A) before addition of NIPAM B) after addition of NIPAM. Conformation plot and Mark-Houwink plot of NANO-PAMAM C) before addition of NIPAM and D) after addition of NIPAM.

Table-S2: Results from the size exclusion chromatography (SEC) with triple detector (analysis were done by using ASTRA 7.3.0 software – Wyatt technology corporation).

Sample	Mn (g/mol)	Mw (g/mol)	PDI	Mark-Houwink - Sakurada exponent (a)	Exponent of the Conformation plot	Mark-Houwink -Sakurada k
NANO-PAMAM before addition of NIPAM	3.789×10^{5} (±1.546%)	6.472×10^{5} (±1.344%)	1.708 (±2.049%)	0.161 (±0.152%)	0.26	2.148 (±0.314%) mL/g
NANO-PAMAM after addition of NIPAM	6.080×10^{4} (±3.727%)	5.397×10^{5} (±1.677%)	8.877 (±4.087%)	0.284 (±0.046%)	0.26	1.072 (±0.152%) mL/g

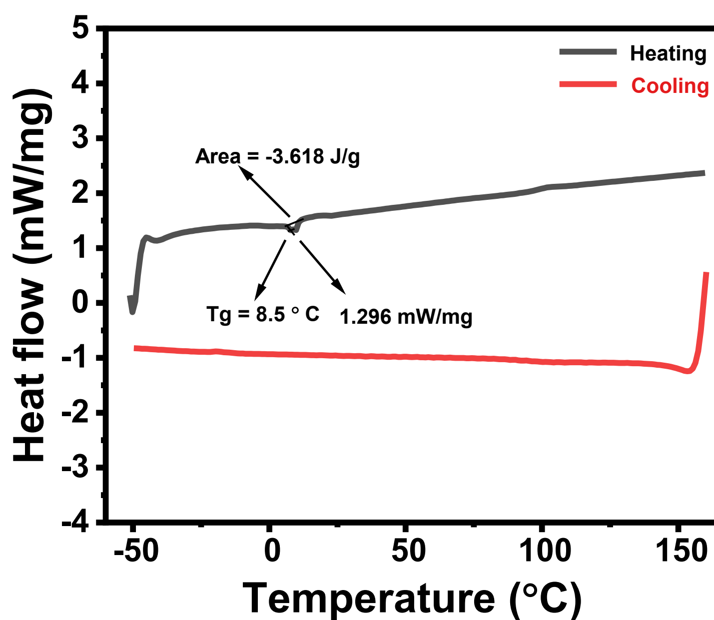

Figure S3: DSC thermogram of NANO-PAMAM on heating and cooling.

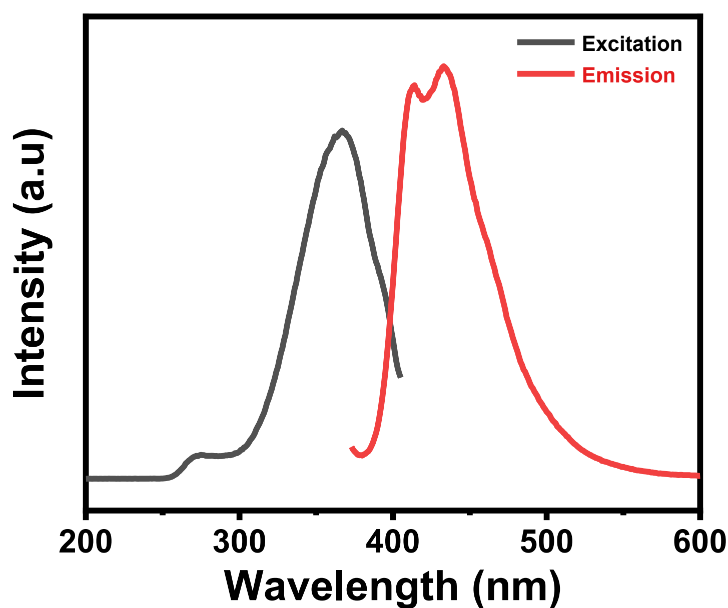

Figure S4: Excitation and Emission spectra of (NANO-PAMAM) nanogel in water.

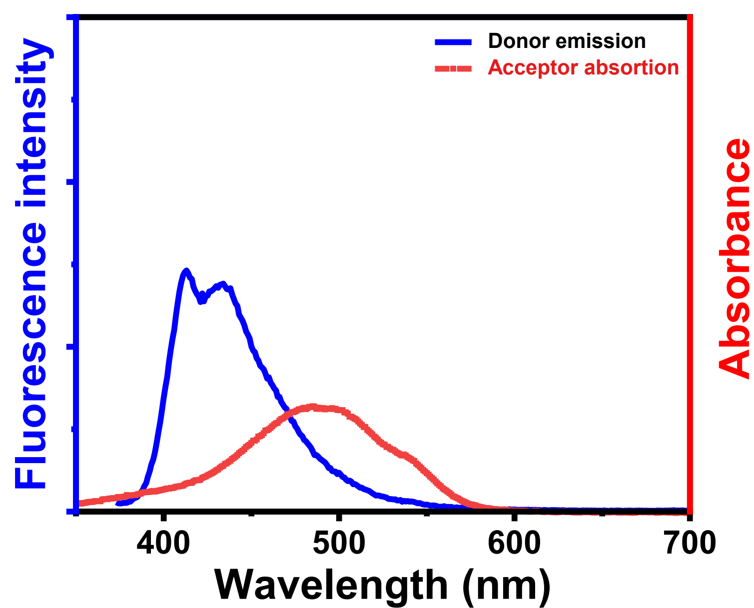

Figure S5: Spectral overlap between donor (NANO-PAMAM) emission and acceptor (DOX) absorption. (Overlap integral value, J(λ) is 1.420 e^{+13} nm^4, M^{-1} cm^{-1})

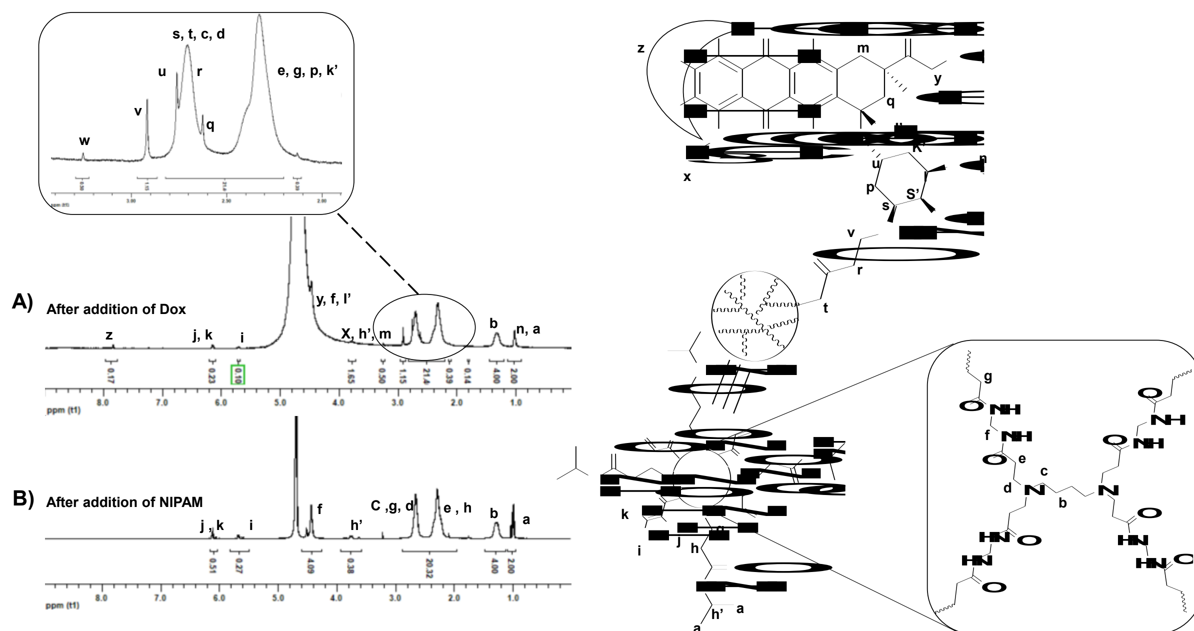

Figure S6: A comparative ^{1}HNMR spectra analysis of A) NANO-PAMAM and B) NANO-PAMAM-DOX (0.2).

The reaction was confirmed via noting the disappearance of acrylamide peaks and equivalent appearance of DOX aromatic peaks around 7.0 ppm.

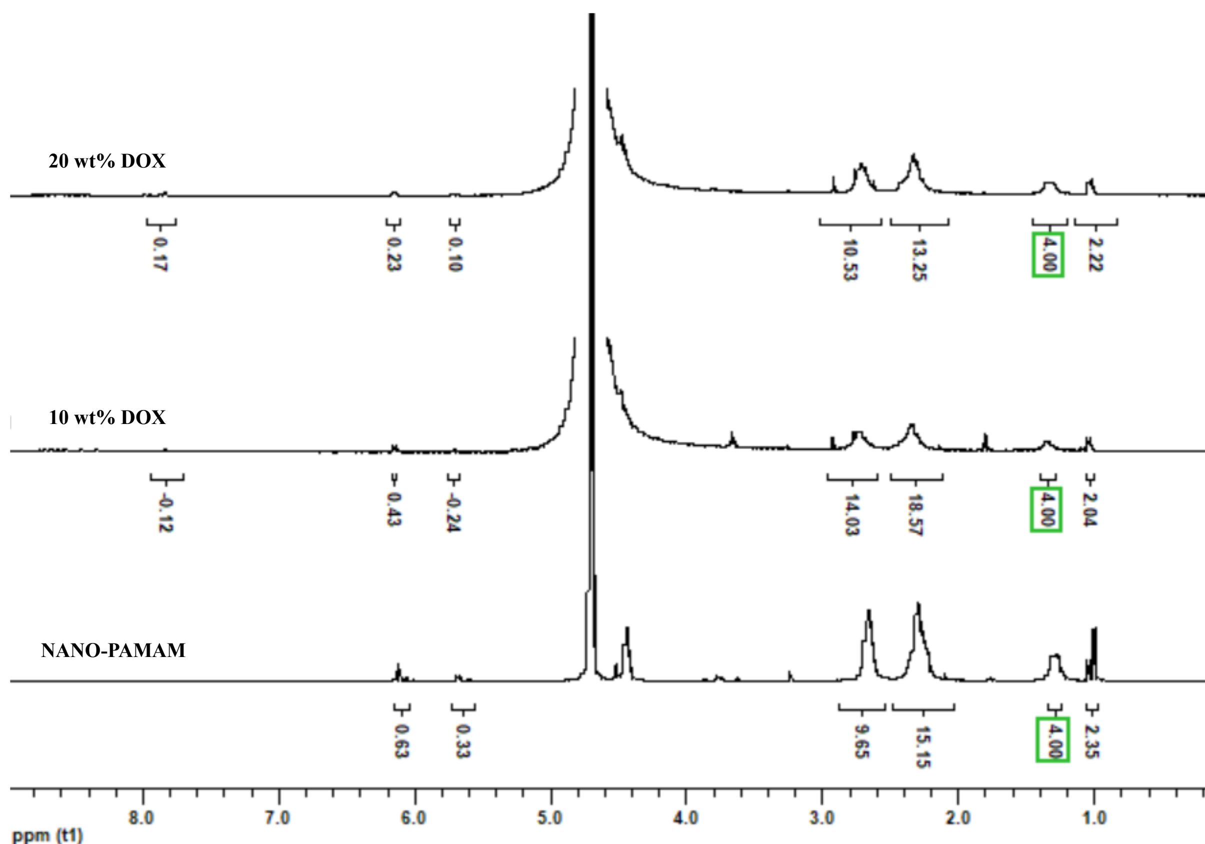

Figure S7: ^{1}HNMR spectra of (NANO-PAMAM) and NANO-PAMAM-DOX with different content of DOX different content of Doxorubicin, 10 wt% and 20 wt% with respect to (NANO-PAMAM).

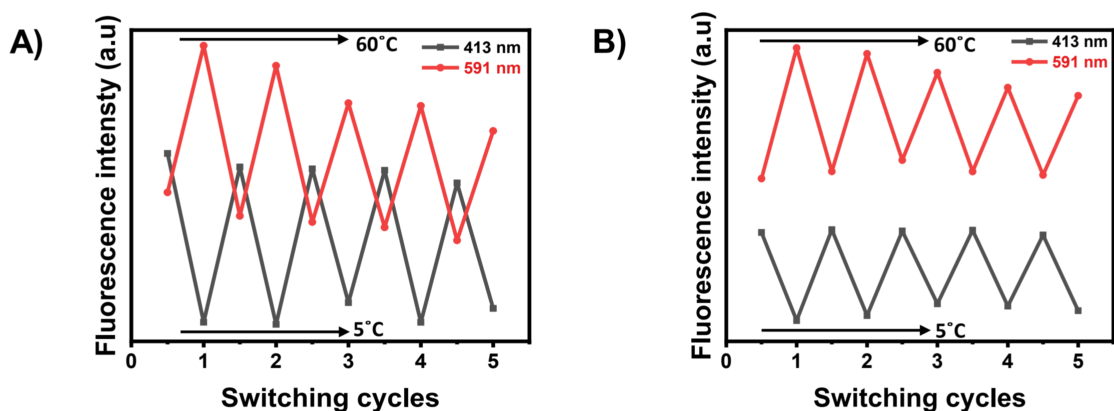

Figure S8: Reversible switching cycle was observed from (5˚C to 60˚C) for (NANO-PAMAM-DOX) solution when Dox content was A) 10 wt% and B) 20 wt% with respect to NANO-PAMAM

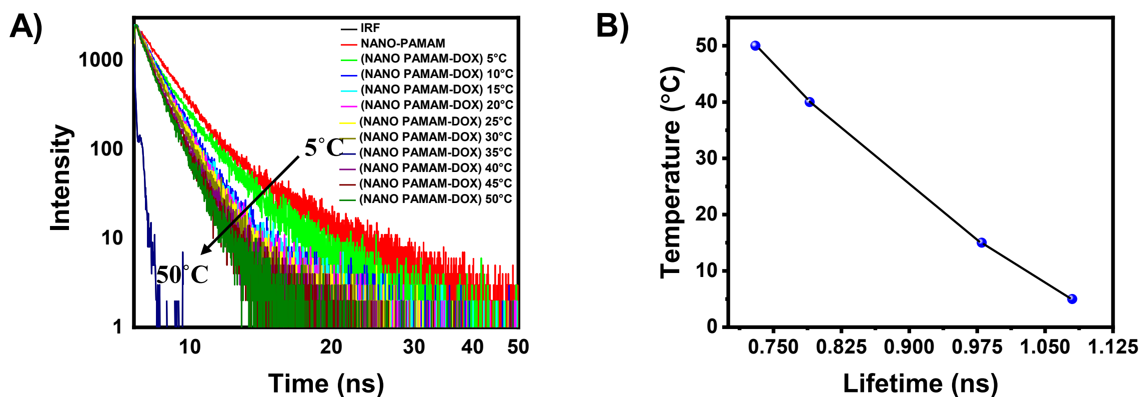

Figure S9: A) Lifetime measurement by using time-correlated single photon counting (TCSPC) technique with excitation at 375 nm for NANO-PAMAM (donor) in absence and presence of Doxorubicin (acceptor) and also with the variation of temperature B) The variation of average lifetime of donor with change in temperature.

Table S3: Average lifetime of NANO-PAMAM (donor) in absence and presence of Doxorubicin (acceptor) and also the variation of donor lifetime with change in temperature.

Sample	Average lifetime (ns)
NANO-PAMAM (Donor)	1.92
(NANO-PAMAM-DOX) 5˚C	1.08
(NANO-PAMAM-DOX) 15˚C	0.98
(NANO-PAMAM-DOX) 40˚C	0.79
(NANO-PAMAM-DOX) 50˚C	0.73

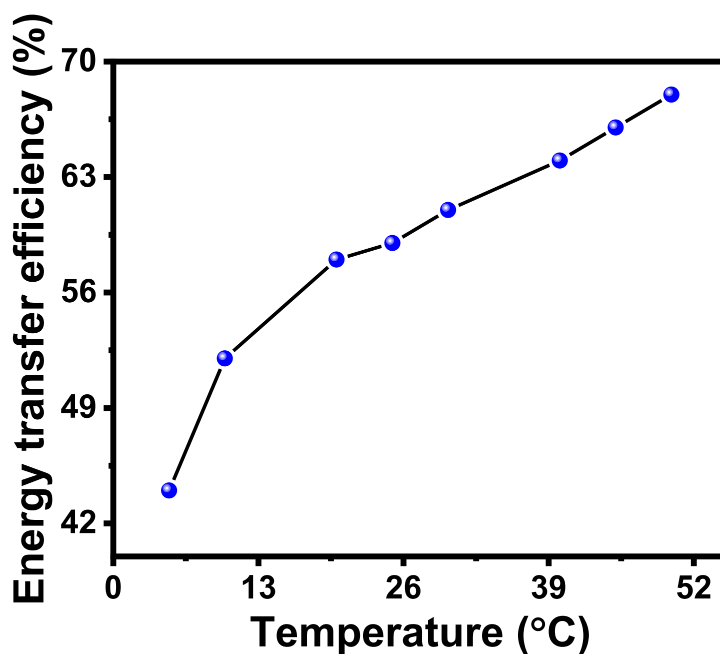

Figure S10: Energy transfer efficiency of NANO-PAMAM with the variation of temperature.

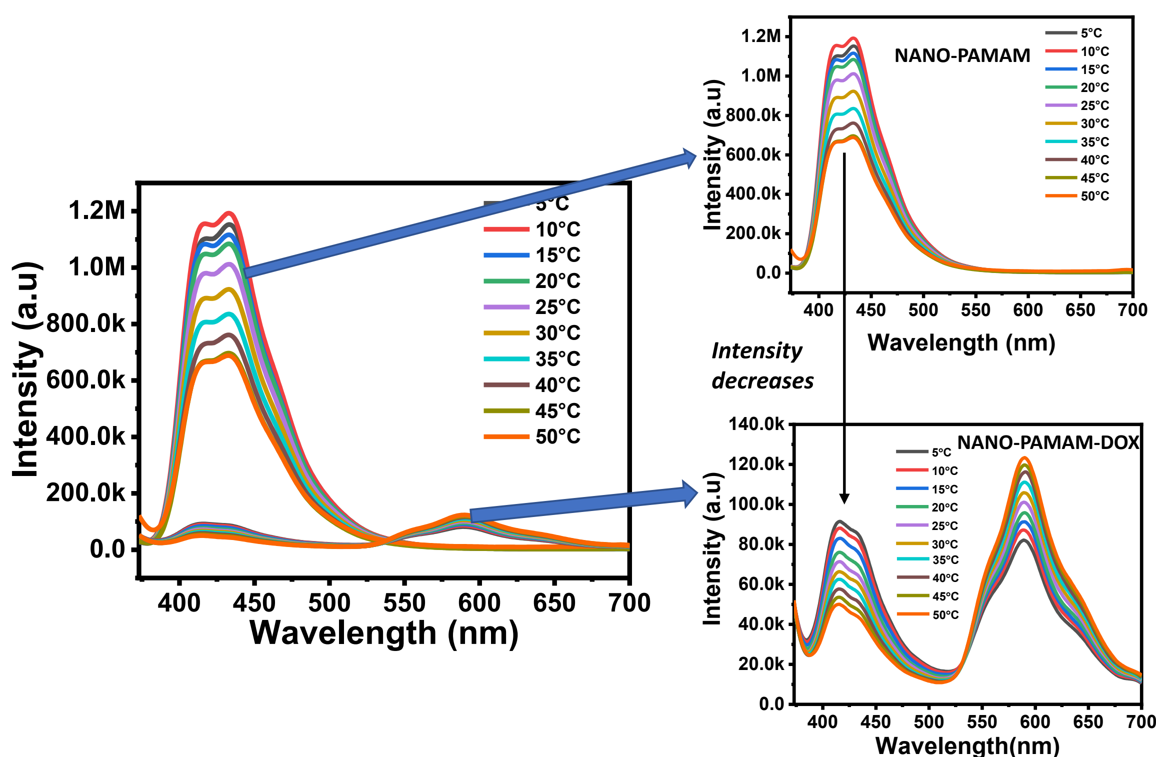

Figure S11: Comparison of the fluorescence emission of NANO-PAMAM and NANO-PAMAM-DOX with the variation of temperature - during FRET the intensity of the donor fluorophore significantly decreases.

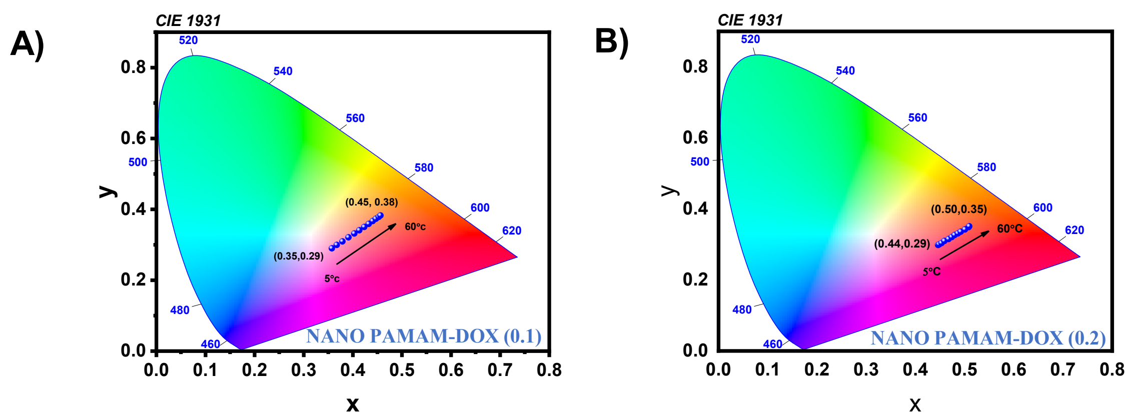

Figure S12: (A) and (B) represent CIE 1931 chromaticity diagram showing the luminescence color at different temperatures for NANO-PAMAM-DOX (0.1) and NANO-PAMAM-DOX (0.2) respectively. The emission colour of the NANO-PAMAM-DOX moves from the purple to the red region and the corresponding color coordinates change from (0.35, 0.29) to (0.45, 0.38) for NANO-PAMAM-DOX (0.1) and from (0.44, 0.29) to (0.50, 0.35) for NANO-PAMAM-DOX (0.2) as the temperature increases from 5 °C to 60 °C.

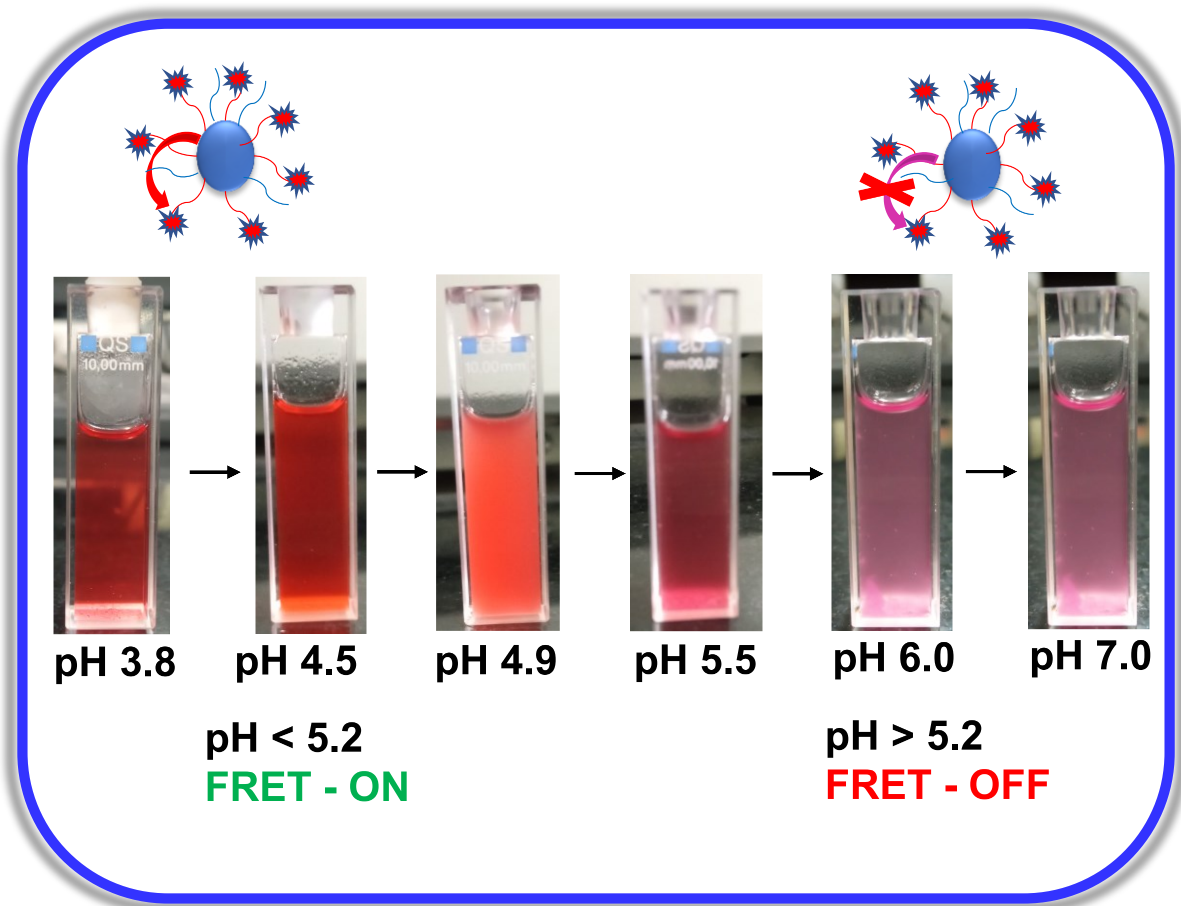

Figure S13: Photographic images of (NANO-PAMAM-DOX) in buffer solution under visible light.

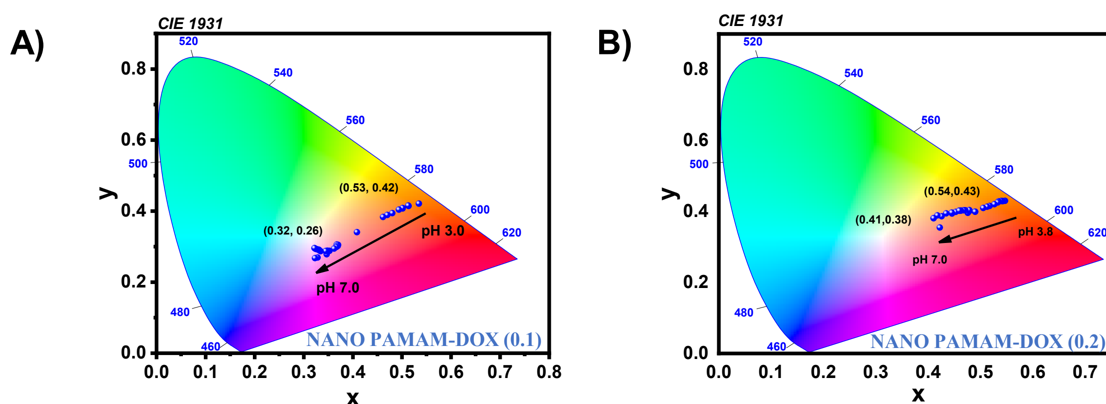

Figure S14: (A) and (B) represent CIE 1931 chromaticity diagram showing the luminescence color at different pH for NANO-PAMAM-DOX (0.1) and NANO-PAMAM-DOX (0.2) respectively.

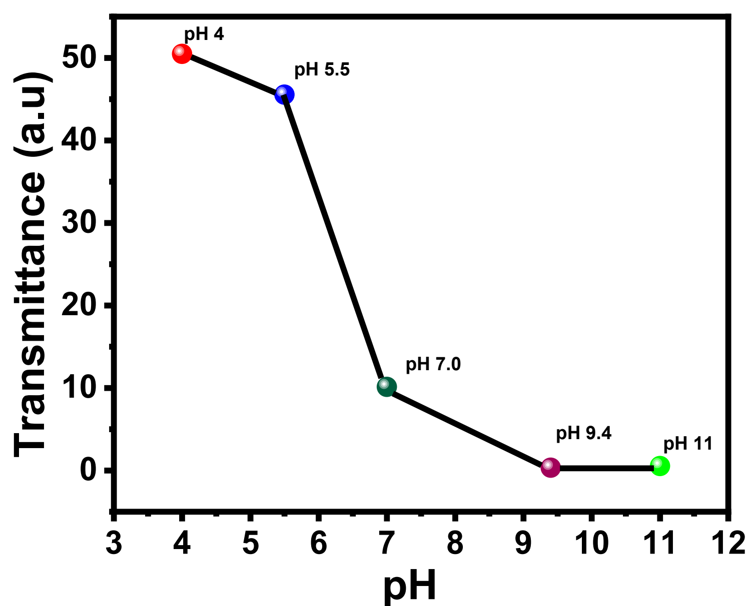

Figure S15: pH induced phase transition behavior of NANO-PAMAM.

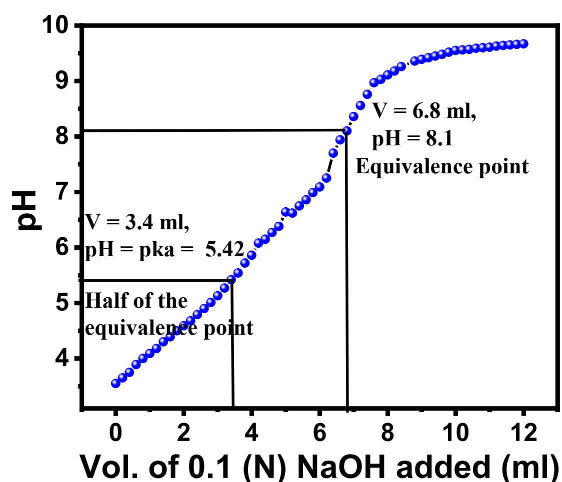

Figure S16: pKa value determination for NANO-PAMAM-DOX. The value also closely matches with the earlier reported pKa values of PAMAM dendrimers (Anal. Methods, 2016,8, 263-269, Polymer Journal, 1985, 17, 117–132) (the polymer backbones are comparable in both cases)

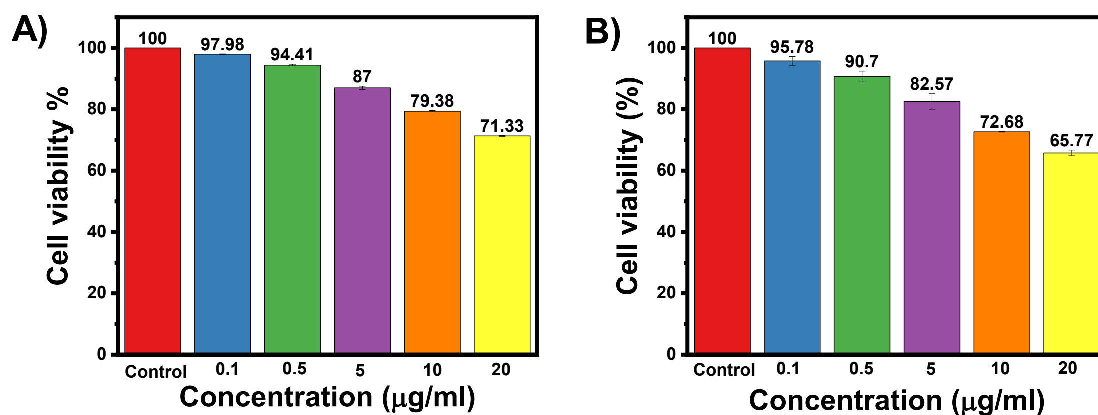

Figure S17: Cell viability of L929 cells with different concentration with (A) NANO-PAMAM and (B) (NANO-PAMAM-DOX), (nanogel with DOX content 20 wt% was used for the study)

Note- Authors also like to acknowledge SAIF, IIT Madras for the DSC analysis and Centre for Research on Molecular Biology and Applied Sciences for the MTT assay.