Supplementary Information

Ruthenium(II) polypyridyl complexes as carriers for DNA delivery

Satish S. Bhat,[a] Avinash S. Kumbhar,*[a] Anupa A. Kumbhar,[a] Ayesha Khan,[b] Peter Lönnecke,[c] and Evamarie Hey-Hawkins[c]

Experimental Section

Chemicals. All chemicals and solvents were purchased commercially and were distilled before use. 2,3-Bis(bromomethyl)quinoxaline was purchased from Sigma Aldrich. $RuCl_3 \cdot 3H_2O$ was purchased from S. D. Fine chemicals, Mumbai (India) and calf thymus DNA was purchased from SRL, Kolkata (India) and used as received. *pBR322* DNA was purchased from Chromos Biotech Pvt. Ltd. India.

Syntheses. The ligands 1,10-phenenthroline-5,6-dione (phendione),[1] bpg [4b,5,7,7a–tetrahydro-4b,7a-epiminomethanoimino-6H-imidazo[4,5-f] [1,10] phenanthroline-6,13-dione][2-4] were synthesized according to the literature. The precursor complexes of the type *cis*-$[Ru(N\text{-}N)_2Cl_2]$[5, 6] are prepared according to the literature method.

Synthesis of bis(2,3- dimethylquinoxaline)bipyridine glycoluril (bqbg) (1). DMSO (8 mL) was purged with nitrogen for 30 minutes. Powdered KOH (343 mg, 6.1 mmol) is added and stirred for another 30 minutes then bipyridine glycoluril (300 mg, 1.02 mmol) and 2, 3-bis(bromomethyl)quinoxaline (645 mg, 2.04 mmol) were added in one portion and the reaction mixture was cooled in a water bath (15 °C). The reaction mixture was stirred for 8 h. Then reaction mixture was poured into 300ml of water and product was extracted with chloroform (3×100 mL) and the combined organic layers were washed with water and then dried ($MgSO_4$). After filtration and rotary evaporation, the residue was purified by column chromatography (SiO_2, $CHCl_3$/MeOH 25:1). Yield: 217 mg (40%). ^{1}H NMR (DMSO-d_6, 300 MHz, 25 °C): ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): δ_{ppm} = 9.01 (d, 2H), 8.04 (m, 6H), 7.75 (m, 4H), 7.42 (dd, 2H), 5.45 (4H, NCH_2Ar), 5.03 (4H, NCH_2Ar); ^{13}C{1H) NMR ($CDCl_3$, 75.47MHz): δ_{ppm} = 157.57, 152.03, 150.49, 147.53, 140.58, 135.56, 130.69, 128.81, 126.51, 124.04, 76.51, 47.77; IR (KBr pellet, cm^{-1}): ν= 3068, (ArH), 2889, 2820 (CH_2), 1711 (C=O), 1579, 1568, 1446, (C=C, C=N); ESI-MS (m/z, (%) positive mode) : 603.6 $(M+H)^+$ (~100%). Anal. Calcd for $C_{34}H_{22}N_{10}O_2 \cdot CH_3OH$; C, 66.24; H, 4.13; N, 22.07; found: C, 66.01; H, 4.49; N, 21.89.

$[Ru(bpy)_2(bqbg)]Cl_2$ (2). The precursor complex cis-$[Ru(bpy)_2Cl_2] \cdot 2H_2O$ (100 mg, 0.0192 mmol) and bqbg (115 mg, 0.0192 mmol) were dissolved in methanol-water (1:1, 50 mL) and

the mixture was heated to reflux for 8 h, whereupon the color of the solution changed from dark purple to red. The red solution was filtered hot and was cooled to room temperature. The solvent was removed under vacuum to obtain a red solid. The product was purified by column chromatography on active alumina using acetone and methanol as eluent. The red fraction was collected and concentrated in vacuum to get the pure product. Yield: 135 mg (65%). ^{1}H NMR (DMSO-d_6, 300 MHz, 25 °C); δ = 9.25 (d, 2H), 8.88 (d, 4H), 8.23 (m, 4H), 7.99 (m, 8H), 7.84 (m, 4H), 7.82-7.55 (m, 8H), 5.44 (d, 2H), 5.23 (dd, 4H), 5.02 (d, 2H); ^{13}C{1H) NMR (DMSO-d_6, 75.47MHz): δ_{ppm} = 156.56, 156.18, 155.01, 152.7, 152.16, 151.98, 151.22, 149.85, 148.21, 145.85, 143.95, 143.89, 139.60, 138.45, 136.45, 131.02, 130.41, 128.50, 124.65, 77.05, 46.95; IR (KBr pellet, cm^{-1}) ν = 3412 (H_2O), 3052, 3020 (ArH), 2949, 2862 (CH2), 1712 (C=O), 1579, 1566, 1458, 1425 (C=C, C=N), ESI-MS (m/z, (%) positive mode) : 1052 ($[M - Cl]^+$) (~5%), 508 ($[M - 2Cl]^{2+}$) (~100%). Anal. Calcd for $C_{54}H_{38}N_{14}O_2Cl_2Ru{\cdot}2H_2O$, C, 57.75; H, 3.77; N, 17.46; found: C, 57.89; H, 3.91; N, 17.12.

$[Ru(phen)_2(bqbg)]Cl_2$ (3). The synthesis and purification of compound **3** was similar to that of **2** using $[Ru(phen)_2Cl_2]{\cdot}2H_2O$ (100 mg, 0.0176 mmol) and bqbg (106 mg, 0.0176 mmol). Yield: 147 mg (73%). Crystals were grown by solve evaporation of perchlorate salt of complex, which was synthesized by redissolving purified product in water to that aqueous solution of sodium perchlorate was added the bright red precipitate formed was collected by filtration and washed with diethylether. ^{1}H NMR (DMSO-d_6, 300 MHz, 25 °C); δ = 9.26 (d, 2H), 8.92 (d, 2H), 8.77 (d, 2H), 8.44 (m, 6H), 8.08-7.94 (m, 8H). 7.84 (m, 4H), 7.74 (m, 4H), 7.55 (m, 2H), 5.47 (d, 2H), 5.24 (dd, 4H), 4.99 (d, 2H); ^{13}C{1H) NMR (DMSO-d_6, 75.47MHz): δ_{ppm} = 155.99, 153.24, 153.02, 152.32, 152.15, 151.86, 149.97, 147.01, 146.72, 139.41, 137.26, 136.95, 136.46, 130.74, 130.54, 130.27, 130.14, 128.32, 128.12, 128.02, 126.39, 76.85, 46.74; IR (KBr pellet, cm^{-1}); ν = 3416 (H_2O), 3070 (ArH), 2937, 2843 (CH2), 1716 (C=O), 1639, 1440, 1358(C=C, C=N). ESI-MS: (m/z, (%) positive mode): 1099 ($[M - Cl]^+$) (~6%), 532 ($[M - 2Cl]^{2+}$) (~100%). Anal. Calcd. for $C_{58}H_{38}N_{14}O_2Cl_2Ru{\cdot}H_2O$, C, 60.41; H, 3.49; N, 17.01; found: C, 60.17; H, 3.31; N, 16.83.

Methods and Instrumentation

Spectroscopy and Electrochemistry

^{1}H NMR spectra were measured on a Varian-Mercury 300 MHz spectrometer with chloroform (d_1) and DMSO (d_6) as a solvent at room temperature and all chemical shifts are given relative to TMS. The infrared spectra of solid samples dispersed in KBr were recorded

on a Shimadzu FTIR-8400 spectrophotometer. Microanalysis (C, H, and N) were carried out with a Thermo Quest microanalysis instrument capable of carrying out C, H, N, S (carbon, hydrogen, nitrogen and sulfur) analysis. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer using water/methanol as solvent. The ESI capillary voltage was set at 3.5 kV and the cone voltage was 40V. UV–Vis spectra were recorded on a Jasco UV–Vis spectrophotometer. Steady-state emission experiments were carried out on a Shimadzu RF-5301 spectrofluorometer at room temperature.

Emission quantum yields (ϕ) were calculated by integrating the area under the fluorescence curves and by using equation 1[7]

$$\phi_{Sample} = \{OD_{Standard} \times A_{Sample}\}/\{OD_{Sample} \times A_{Standard}\} \times \phi_{Standard} \quad \text{---------- (1)}$$

Where, OD is optical density of the compound at the excitation wavelength (450 nm) and A is the area under the emission spectral curve. The standard used for the fluorescence quantum yield measurements was $[Ru(bpy)_3]Cl_2$.[8]

X-ray Crystallography. A crystal of complex **3** with perchlorate anion suitable for single-crystal X-ray diffraction with a size of 0. 4 x 0.3 x 0.2 mm^3 was selected. The data was collected on an Xcalibur-S diffractometer (Agilent Technologies) using Mo-K_α radiation and ω-scan rotation. Data reduction was performed with CrysAlisPro,[9] including the program SCALE3 ABSPACK[10] for empirical absorption correction. The structure was solved by direct methods and the refinement of all non-hydrogen atoms was performed with SHELXL-97.[11] H atoms are calculated on idealized positions. The Structure figure was generated with ORTEP[12]. CCDC 826878 contains the supplementary crystallographic data for **3**. The data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44)1223-336-033; or deposit@ccdc.cam.uk). Crystal parameters and details of the data collection and refinement are given in Table 1. Selected bond lengths and bond angles are given in Table 2.

DNA Binding Studies

The concentration of CT-DNA was calculated from its known extinction coefficient at 260 nm (6600 M^{-1} cm^{-1}). Solutions of calf thymus DNA in phosphate buffer gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} of 1.8–1.9 indicating that the DNA was sufficiently free of protein.

Absorption titration experiments were performed by maintaining a constant metal complex concentration (10 μM) and varying nucleotide concentration (0–140 μM) in buffer. After addition of DNA to the metal complex, the resulting solution was allowed to equilibrate at 25 °C for 20 minutes, after which absorption readings were noted. The data were then fit to equation 2 [13] to obtain intrinsic binding constant K_b.

$$[DNA]/[\varepsilon_a-\varepsilon_f] = [DNA]/[\varepsilon_b-\varepsilon_f] + 1/K_b\,[\varepsilon_b-\varepsilon_f] \text{ ----------(2)}$$

Where, [DNA] is the concentration of DNA in base pairs, ε_a is the extinction coefficient observed for the MLCT absorption band at the given DNA concentration, ε_f is the extinction coefficient of the complex free in solution and ε_b is the extinction coefficient of the complex when fully bound to DNA. A plot of $[DNA]/[\varepsilon_a-\varepsilon_f]$ versus [DNA] gave a slope $1/[\varepsilon_a-\varepsilon_f]$ and Y intercept equal to $1/K_b\,[\varepsilon_b-\varepsilon_f]$, respectively. The intrinsic binding constant K_b is the ratio of the slope to the intercept.[13]

DNA melting experiments were carried out by monitoring the absorption at 260 nm of CT–DNA (100 μM) with a JASCO V–630 spectrophotometer equipped with a Peltier temperature-controlling programmer ETC–717 (± 0.1 °C) in phosphate buffer at various temperature in the presence and absence of the complexes. UV melting profiles were obtained by scanning A_{260} absorbance monitored at a heating rate of 1 °C/min for solutions of CT–DNA (100 μM) in the absence and presence of ruthenium(II) complexes (20 μM) from 30 to 90 °C with the use of the thermal melting program. The melting temperature Tm which is defined as the temperature where half of the total base pairs is unbound was determined from the midpoint of the melting curves.

Emission titration experiments were performed by using a fixed metal complex concentration to which increments of the stock DNA solutions were added. Typical concentration of metal complex used was 20 μM and [DNA]/[Ru] ratios ranged between 0–30. After the addition of DNA to the metal complex, the resulting solution was allowed to equilibrate for 20–30 min at room temperature before being excited in their intense metal to ligand charge-transfer band between 400 and 500 nm and emission is measured at 550–750 nm. The excitation and emission slit widths employed were 5 nm each.

Electrophoresis Mobility Shift Assay and DNA Cleavage Study.

For the gel electrophoresis experiments the super coiled *pBR 322* DNA (200ng in nucleotide) in TBE buffer (89 mM Tris–borate acid, 2 mM EDTA, pH 8.2) was treated with the metal

complexes (1 to 6μM) and the mixture is incubated at 37°C in dark for 30minutes after incubation were quenched by the addition of 2 μL gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% glycerol and 2 mM EDTA). The samples were subjected to electrophoresis at 60V on 1% agarose gel in TBE (Tris–Boric acid–EDTA) buffer (pH 8.2). The gel was stained with a 0.5 μg/mL ethidium bromide and visualized by UV light and photographed for analysis.

Cytotoxicity: Cell viability assay

The cell lines HeLa and HL-60 were obtained from National Centre for Cell Sciences Repository, University of Pune, Pune-411007. The cells were maintained in DMEM media with 10% FBS and 1% antibiotic solution at 37°C at 5% CO_2 in the steri-cycle CO_2 incubator with HEPA Class 100 filters, Thermo Electron Corporation.

The number of viable cells remaining after appropriate treatment was determined by 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) assay.[14] Briefly, cells were plated (4000 cells/well per 0.2 mL DMEM medium) in 96-well microtiter plates and incubated overnight. The complexes **2** and **3** were then added at indicated concentrations to quadruplicate wells. After 24, 48, 72 h, and 96 hrs MTT was added to each well at a final volume of 0.5 mg/mL and the microplates were incubated at 37°C for 3h. After the supernatant was removed, the formazan salt resulting from the reduction of MTT was solubilized in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) and the absorbance was read at 570 nm using an automatic plate reader (Thermo Corporation). The cell viability was extrapolated from optical density (OD) 570 nm values and expressed as percent survival.

Fluorescence microscopic images

From the cell lines pre-inoculated with the complexes, in 24 well plate, the supernatant from the wells were transferred to a centrifuge tube and spin at 5000 rpm for 5 minutes. The pellet was resuspend in PBS. 10-15μL of cell content was pipette and fixed before images were taken. For HeLa cell lines which are of adherent type, the cells were trypsinized and washed with PBS buffer before capturing the images. Images were taken in a Carl Zeiss Axio Scope A1 fluorescence microscope with filter set no. 9 and excitation at 450-490 nm.

Restriction Digestion Analysis

Plasmid DNA pBR 322 with known restriction sites Eco R1 and Pst 1 was selected for the studies. Complexes where incubated with *pBR 322* for 2 hrs for complete condensation. Briefly, cells were plated (4000 cells/well per 0.2 mL DMEM medium) in 96-well microtiter

plates and incubated overnight. The condensed *pBR 322* complexes were then added at indicated concentrations to quadruplicate wells. After 36 hrs, the cells were trypsinized and the plasmid DNA was extracted. The plasmid DNA was subjected to Eco R1 and Pst I restriction analysis for 2 hrs at 37°C. The product was run on a 0.8% agarose gel to observe the restriction pattern.

Crystal Structure. Single crystals suitable for X-ray diffraction were grown by slow evaporation of perchlorate salt of complex **3** in acetonitrile and water mixture at room temperature. The compound crystallizes in the triclinic space group $P\bar{1}$ with 5 additional acetonitrile solvent molecules. One of them was found to be slightly disordered on two positions. A summary of the crystallographic data, bond lengths, and bond angles for **3** are given in Table 1 and 2. The ruthenium(II) ion is chelated by the bqbg ligand and two phenanthroline ligands oriented in a *cis*- geometry. The coordination geometry around Ru1 is described as distorted octahedral, with an average bite angle of 79.31° for the three bidentate ligands. The mean Ru–N(phen) bond length is 2.065 Å, and the Ru–N (bqbg) bond length of 2.0505(3) Å.

Table 1. Crystal data and structure refinement for **3**.

Empirical formula	$C_{58}H_{38}Cl_2N_{14}O_{10}Ru \cdot 5\ CH_3CN$
Formula weight	1468.26 $g \cdot mol^{-1}$
Temperature	130(2) K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	$P\bar{1}$
Unit cell dimensions	a = 13.9989(5) Å α = 117.950 (4)°
	b = 16.5857(6) Å β = 100.287(3)°
	c = 17.2342(7) Å γ = 103.188(3)°
Volume	3246.3(2) $Å^3$
Z	2
Density (calcd)	1.502 Mg/m^3
Absorption coefficient	0.401 mm^{-1}
F(000)	1504

Crystal size	0. 4 x 0.3 x 0.2 mm^3
θ range for data collection	2.98 to 26.37°
Index ranges	-17<=h<=17, -20<=k<=20, -21<=l<=21
Reflections collected	43455
Independent reflections	13252 [R(int) = 0.0477]
Completeness of θ = 26.37°	99.8%
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	1 and 0.94697
Refinement method	Full-matrix least-squares on F^2
Restraints / parameters	12 / 896
Goodness-of-fit on F^2	0.967
Final R indices [I>2σ(I)]	R1 = 0.0522, wR2 = 0.1423
R indices (all data)	R1 = 0.0784, wR2 = 0.1519
Largest diff. peak and hole	1.492 and -0.775 e. $Å^{-3}$

Table 2. Selected bond lengths [Å] and angles [°] for **3**.

Bond lengths			
Ru(1)-N(1)	2.073(3)	Ru(1)-N(4)	2.058(3)
Ru(1)-N(2)	2.061(3)	Ru(1)-N(5)	2.047(3)
Ru(1)-N(3)	2.068(3)	Ru(1)-N(6)	2.054(3)
Bond angles			
N(5)-Ru(1)-N(6)	78.62(11)	N(4)-Ru(1)-N(3)	79.64(12)
N(5)-Ru(1)-N(4)	96.59(11)	N(2)-Ru(1)-N(3)	92.48(12)
N(6)-Ru(1)-N(4)	91.76(11)	N(5)-Ru(1)-N(1)	95.17(11)
N(5)-Ru(1)-N(2)	91.50(11)	N(6)-Ru(1)-N(1)	171.00(11)
N(6)-Ru(1)-N(2)	93.88(12)	N(4)-Ru(1)-N(1)	95.46(11)
N(4)-Ru(1)-N(2)	170.92(11)	N(2)-Ru(1)-N(1)	79.66(12)
N(5)-Ru(1)-N(3)	175.30(11)	N(3)-Ru(1)-N(1)	88.02(12)
N(6)-Ru(1)-N(3)	98.59(12)		

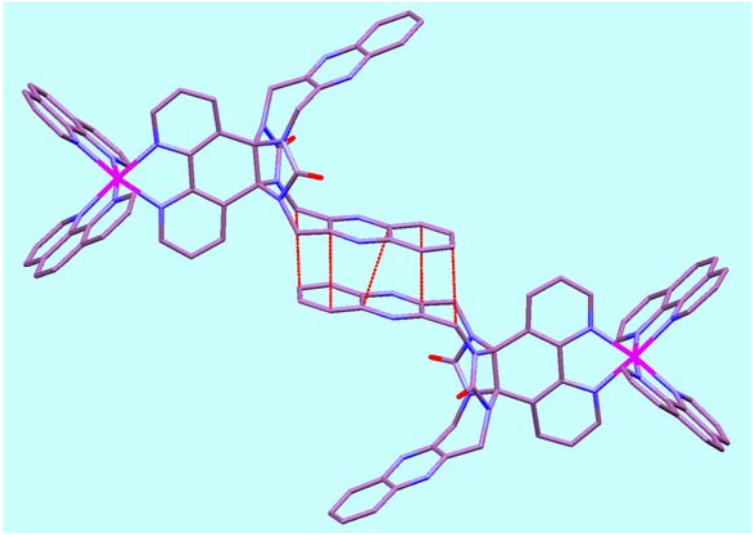
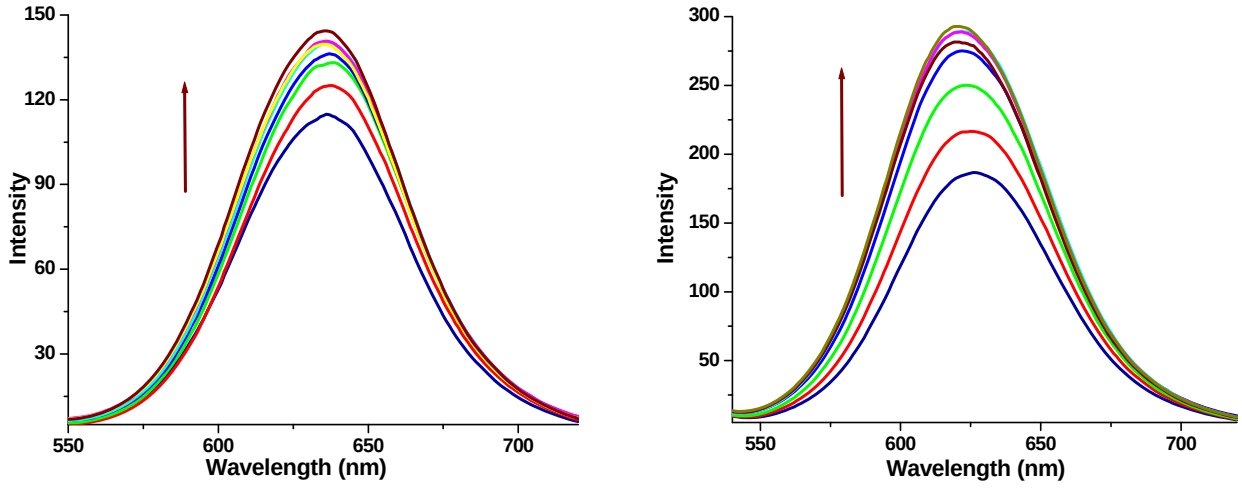


Fig. S1 π–π stacking interactions of the aromatic rings of the bqbg ligand in complex **3** (π–π stacking distances are 3.339-3.389 Å).



(A) (B)

Fig. S2 Emission spectra of Ru(II) complexes (A) **2** , (B) **3** (20μm) in phosphate buffer (pH 7.2) at 298 K with increasing [DNA]/[Ru] ratio from 0-30. Arrow shows the intensity changes upon increasing DNA concentrations.

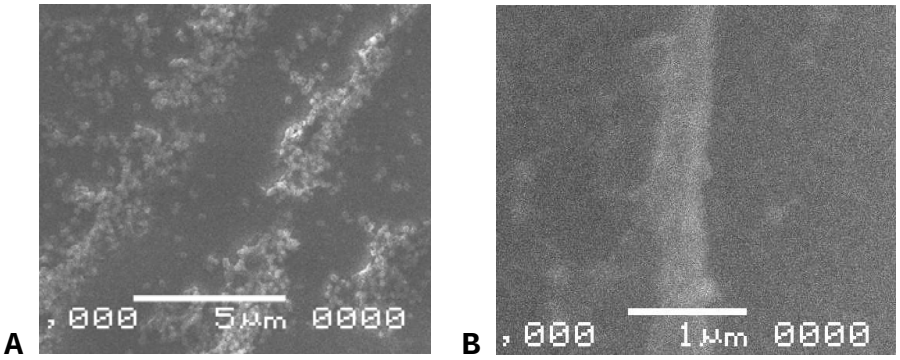


Fig. S3 Scanning electron microscopy images of DNA-condensates incubation time 10min (A) **2,** (B) **3**.

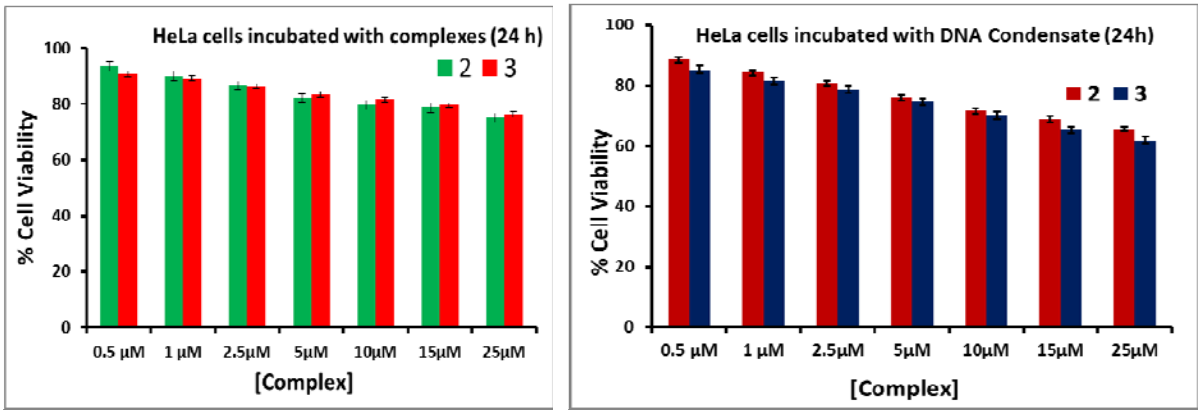


Fig. S4 Cytotoxicity evaluation of (A) complexes (B) DNA condensates of complexes against HeLa. The cell viability was measured after 24h by MTT assay. Each data point represents the mean for three separate experiments.

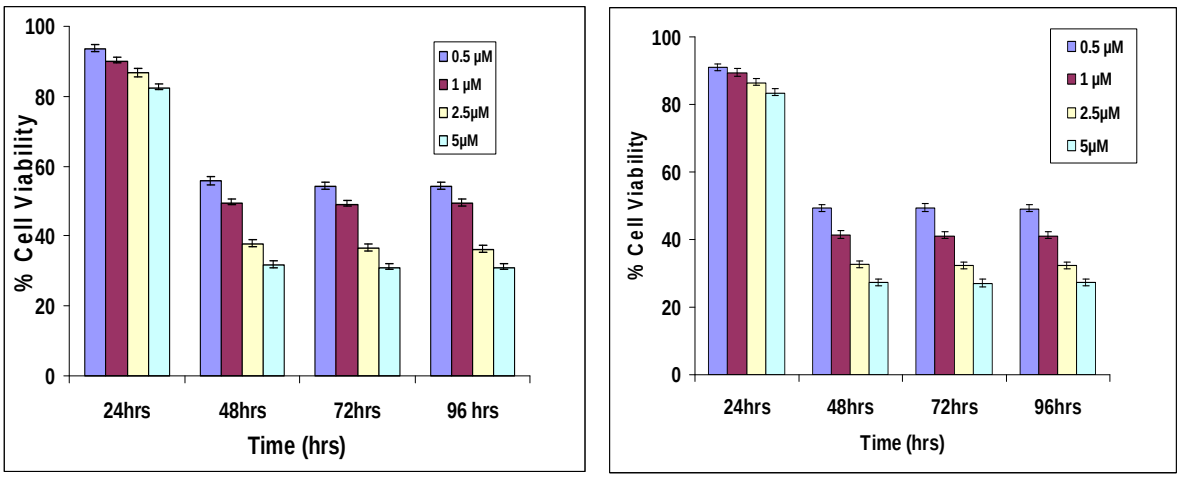


Fig. S5 Cytotoxicity evaluation of DNA condensates of complex **2** (Left) and complex **3** (Right) against HeLa cell lines. The cell viability was measured after 24hrs, 48hrs, 72hrs, and 96hrs by MTT assay. Each data point represents the mean for three separate experiments.

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