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Synthesis, Characterization, DNA-binding and spectral properties of complex $[Ru(py)_4(dppz)]^{2+}$

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ABSTRACT

The new Ru (II) complex $[Ru(py)_4(dppz)]^{2+}$ (dppz = dipyrido phenazine) have been synthesized and characterized by different analytical techniques like elemental analysis, LC-MS, ^1H NMR , ^{13}C NMR. The interaction of this complex with calf thymus DNA has been explored by electronic absorption spectroscopy, Fluorescence measurements, viscosity measurements. Their photocleavage behaviour towards pBR 322 and anti microbial properties of this complex was also investigated. The experimental results show that the complex binds with DNA by intercalation mode.

Key words: Ru(II) complex, pyridine, DNA – binding.

INTRODUCTION

Ruthenium (II) complexes with polypyridine ligands, due to a combination of easily constructed rigid chiral structures spanning all three spatial dimensions and a rich photophysical properties have attracted considerable attention.[1-3] Recently ruthenium (II) polypyridyl complex ion $[Ru(phen)_2dppz]^{2+}$ was shown to be a remarkable luminescence light switch for DNA. In aqueous solutions this compound lacks luminescence but it shows intense luminescence in the presence of DNA.[4] Dipyridophenazine complexes of ruthenium (II) have shown interesting properties that could be used for the development of sensors of high sensitivity and selectivity. This quenching of luminescence in aqueous media is possibly due to the interaction of the phenazine nitrogens of the dipyridophenazine ligand with water via hydrogen -bonding or excited state proton transfer.[5,6]

Liang-Nian ji et al [7] reported the synthesis and characterization of a new polypyridyl ligand 2-(4'- phenoxy-phenyl)- imidazo phenanthroline (PPIP) and its ruthenium (II) complexes $[Ru(bpy)_2(PPIP)]^{2+}$. The DNA binding properties of the two Ru(II) complexes were explored by spectroscopic methods and viscosity measurements.Their photocleavage behaviors towards pBR 322 were also investigated. The preparation of polypyridyl ruthenium (II) complexes have received considerable attention during the last decade because of their potential use in novel probes of nucleic acid structure and probing photosensitized DNA cleavage.[8-16] The mixed ligand complexes $[Ru(phen)_2\ dppz]^{2+}$ and $[Ru(bipy)_2\ dppz]^{2+}$ have been proposed as luminescent DNA probes on the basis of their strong binding to double helical DNA.[17-20]

This study demonstrates that the ancillary ligands with hydrogen bonding potential supports the intercalative interaction of ligands with extended aromatic rings and enhances the DNA binding affinity.

Ru(II)-dppz complexes with bidentate ancillary ligands e.g., bpy, phen, dmb, dmp etc., have been largely reported for their interesting properties.[21,22] Changing substitutive group or substituent position on the intercalative ligand can also create some interesting differences in the DNA-binding properties, currently, a great deal of efforts are focused on modifying the main ligand dppz.[23-29] The complexes $[Ru\ (L)_4\ (dppz)]^{2+}$ (L= imidazole, 1-me imidazole) were early synthesized and characterized. [30] The interaction of these complexes with DNA were studied.

Now we are reporting the synthesis, characterization of $[Ru\ (py)_4\ (dppz)]\ (Clo_4)_2.2H_2O$ using different techniques. The binding of this complex with CT DNA was explored using electronic absorption measurements, fluorescence measurements, viscosity measurements. Their photo cleavage behaviour towards pBR 322 was studied. The antimicrobial property towards E.Coli bacteria was also studied.

EXPERIMENTAL SECTION

2.1 Materials

All the common chemicals utilized in this study were obtained from Sigma Aldrich. The solvents were purchased from Merck. DNA was purchased from Sigma Chemicals was sonicated and purified according to the previous literature.[31]

2.1a Physical measurements

Elemental analysis (C, H and N) was performed on Flash EA 1112 series Thermofinnigon, Molecular weight determinations were performed on LC-MS 2010A Shimadzu with Column- C-18, Detector-UV (254) with MS probe of ESI, ^1H and ^{13}C NMR Spectra were recorded on a Bruker ARX-300 NMR Spectrometer with DMSO as Solvent at COSIST, University of Hyderabad, Hyderabad. Electronic absorption spectra were recorded on a *Elico BL 198* Model spectrophotometer with temperature control.

2.1b Spectroscopic characterization

$[Ru(Py)_4(dppz)]\ (ClO_4)_2.2H_2O$ Complex was characterized by elemental analysis (Anal.Calc. for $C_{38}\ H_{34}\ N_8Cl_2\ O_{10}Ru$: C,50.2; H, 3.74; N, 12.32; found C, 49; H, 3.67; N, 12.12%) given in fig1 , ESI-MS m/z = 897 ($-2H_2O$) displays intense molecular peaks at 737, 656 and 488 given in fig 2. In pyridine scrambling of hydrogen occurs before fragmentation.[32] ^1H NMR (DMSO) $H_2(H_6)$ 7.65 ppm $H_3(H_5)$ 7.58 ppm and H_4 at 7.92 ppm for pyridine and dipyridophenazine 8.1(1H), 8.12(2H), 8.13(3H), 8.15(4H), 8.25(5H) given in fig 3, ^{13}C NMR spectrum dppz signals at C_1-150, C_2- 120.8, C_3-129.4, C_4-129.6, C_5-135.7, C_6-142.8, C_7- 129.6, C_8- 129.4 and Pyridine signals at 152.2ppm(C_2, C_6) 129.3ppm(C_3,C_5) 140 ppm(C_4) given in fig 4.

2.2 Synthesis: ligands like phen-dione and dipyridophenazine (dppz) were synthesized as per the reported procedures.[33,34]

2.2a Synthesis of Phen-dione: 1,10 Phenanthroline hydrate (1gm), Potassium bromide or Sodium bromide (5.95gm) were taken in a flask. conc.H_2SO_4 (20ml) was added through the ice cooled walls of flask drop by drop followed by conc. HNO_3 (10 ml). Reflux for 2 hrs, the mixture was taken in a 400ml of H_2O. The solution was neutralized with $NaHCO_3$ and then extracted with CH_2Cl_2. Removal of CH_2Cl_2 give 0.91gm of Phen-dione as yellow needles followed by recrystallization with ethanol or toluene.

2.2b Synthesis of Dipyrido phenazine(dppz) : A solution of Phendione (0.210 gm) and ortho phenylenediamine (OPDA 0.124 gm) in ethanol (20ml) was heated and refluxed for 4hrs. After cooling, the yellow precipitate was collected by filtration, washed with cold ethanol and vacuum dried.

2.2c Synthesis of [Ru(dppz)Cl$_4$] : Catherine J.Murphy et al [35] reported the synthesis of [Ru(dppz) Cl_4] via modification of the literature method for $[Ru(bpy)Cl_4]$ [36]. A 0.5572 gm amount of $RuCl_3.xH_2O$ and a 0.7583 gm amount of dipyridophenazine were added to 50ml of 1M HCl and stirred for about 30 min under nitrogen atmosphere and then allowed to sit under nitrogen for 10 days. The insoluble product was filtered, washed with water and dried in air.

2.2d Synthesis of [Ru(Py)$_4$(dppz)] (ClO$_4$)$_2$.2H$_2$O: According to the reported procedure of Liang- Nian Ji et al [37] $[Ru(L)_4\ (dppz)]^{2+}$ was synthesized. A mixture of $[Ru(dppz)Cl_4]$ (0.131 gm) Pyridine (0.34gm) and DMF (N,N-

dimethyl formamide) 20ml was refluxed under argon atmosphere for 8hrs to give a dark red solution. After being cooled to room temperature, the solution was filtered to remove a small amount of insoluble components. The filtrate was reduced to 5ml and then diluted with 15 ml water. Upon dropwise addition of saturated aqueous $NaClO_4$ solution, a lot of red solid was collected by filtration. It was purified by column chromatography on alumina using acetonotrile-toluene as eluent. The deep red product was further recrystallized with acetonitrile – ether and dried in vacuo.

2.3 DNA binding studies:

Concentration of CT DNA was measured by using its known extinction coefficient at 260 nm.[38] Buffer A (5mM tris, pH 7.1, 50mM NaCl) was used for absorption titration experiments and luminescence measurements, buffer C (1.5 mM Na_2HPO_4, 0.5mM NaH_2PO_4, 0.25mM Na_2EDTA, pH =7.0) was used for the viscometric titrations.

2.3a Electronic absorption measurements: The application of electronic absorption spectroscopy in DNA-binding studies is one of the most useful techniques.[39] Absorption titration experiment was performed by maintainng the metal complex concentration constant (10μM)and varying the concentration of DNA from 20- 200 μM. While measuring the absorption spectra, equal amount of DNA was added to both complex solution and the reference solution to eliminate the absorbance of DNA itself. From the absorption data, the intrinsic binding constant K_b was determined from the plot of [DNA]/($\varepsilon_a - \varepsilon_f$) v_s [DNA], where [DNA] is the concentration of DNA in base pairs, ε_a, the apparent extinction coefficient obtained by calculating A_{obsd}/ [complex] and ε_f corresponds to the extinction coefficient of the complex in its free form. where ε_b refers to the extinction coefficient of the complex in the fully bound form.

$$[DNA] / (\varepsilon_a - \varepsilon_f) = [DNA] / (\varepsilon_b - \varepsilon_f) + 1/(K (\varepsilon_b - \varepsilon_f) \quad (1)$$

Each set of data, when fitted to the above equation, gave a straight line with a slope of 1/ K_b ($\varepsilon_a - \varepsilon_f$). K_b was determined from the ratio of the slope to intercept. The binding constants indicate that the complex binds more strongly.

2.3b Fluorescence Measurements: Fluorescence quenching experiments [40] were carried out at 20^0C by addition of microlitre aliquots of 0.02M $K_4[Fe(CN)_6]$ to 2ml samples of the complex. For the quenching experiments, samples were excited at 559 nm and emission was monitored. In experiments ionic strength was maintained, KCl was added along with $K_4[Fe(CN)_6]$ such that the final, total concentration of K^+ was constant. Emission was recorded in the absence and presence of 20- 200 μM CT-DNA.

$F_o/F = 1 + K_{sv}[K_4Fe(CN)_6]$, where F_o is the intensity of fluorescence in the absence of quencher, F is the fluorescence intensity in the presence of quencher and K_{sv} is the Stern- Volmer quenching constant. Quenching curves were analyzed by linear and nonlinear least squares methods. [41]

2.3c Viscometry: Viscosity experiments were carried out using an Ubblehode viscometer maintained at a constant temperature at 30.0 ± 0.1^0C in a thermostatic waterbath. Each compound was introduced into the degassed DNA solution. Mixing of the complex and DNA was done by bubbling with nitrogen. Flow time was measured with a digital stop watch and each sample was measured three times and an average flow time was calculated. Reduced specific viscosity was calculated according to Cohen and Eisenberg. [42] Plots of $[\eta / \eta_o]^{1/3}$ (η and η_o are the reduced specific viscosities of DNA in the presence and absence of the complex.) versus [complex]/[DNA] were constructed. Plot of $[\eta/ \eta_o]^{1/3}$ versus [DNA] was found to be similar to that reported in the literature.[43]

2.3d DNA Photocleavage: Irradiation of sample containing pBR 322 DNA and the complex were carried out for 1hr. The cleavage reaction on plasmid DNA was monitored by agarose gel electophoresis. When circular plasmid DNA was subjected to gel electrophoresis, relatively fast migration will be observed for the intact supercoiled form (Form 1). If scission occurs on one strand (nicking), the supercoils will relax to generate a slower- moving open circular form (Form II) [44]. If both strands are cleaved, a linear form (Form III) will be generated that migrates between Form I and II.

2.3e Anti microbial activity: The prescence of metal ions in biological matter created great interest and curiosity among chemists as well as biologists, due to their antimicrobial activities. It may be anticipated that a stable metal

chelate will produce its effect either by structural or functional activation or inactivation of a susceptible biological site. Attempts have been made to correlate the stability of the metal drug chelates with their antimicrobial activity.

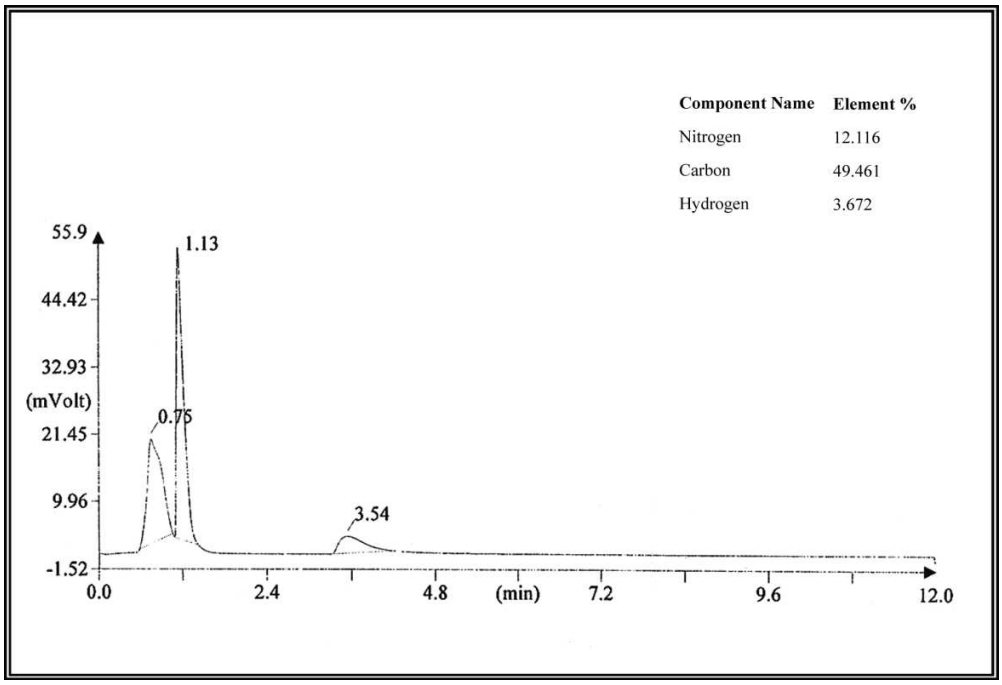

Fig 1: CHNS report of [Ru (Py)$_4$ (dppz)] (ClO$_4$)$_2$

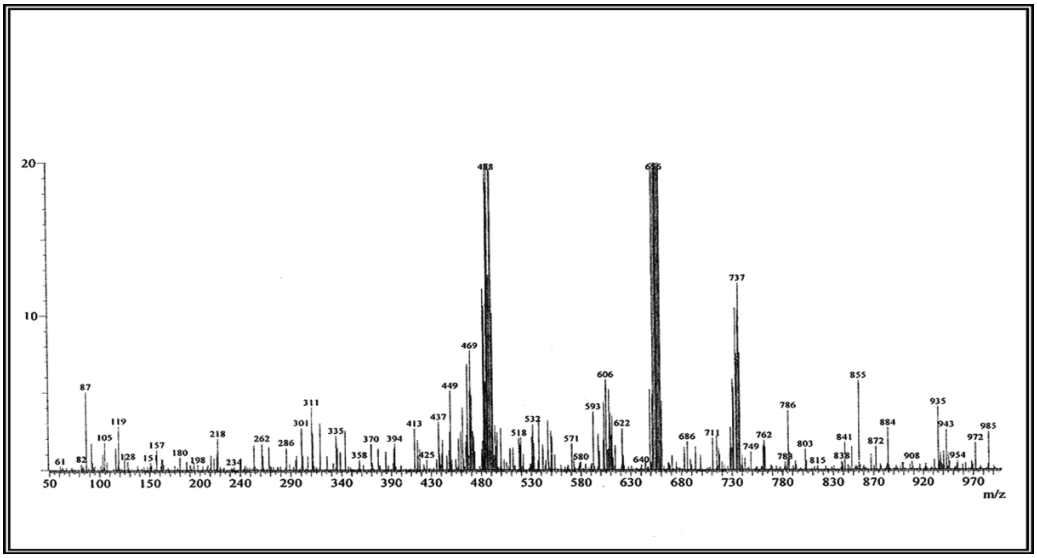

Fig 2: LC-MS spectrum of [Ru (Py)$_4$ (dppz)] (ClO$_4$)$_2$

RESULTS AND DISCUSSION

3.1 The electronic absorption measurement:

The electronic absorption spectroscopy is the most common way to investigate the interactions of metal complexes with DNA [45-50]. A metal complex binding to DNA through intercalation usually results in hypochromism and bathochromism, due to the intercalation mode involving a strong π–π^* stacking ineraction between an aromatic chromophore and the base pairs of DNA. It seems to be generally accepted that the extent of the hypochromism in

the UV- Visible band is consistent with the strength of intercalative interaction. [51-54] The absorption spectra of complex $[Ru(py)_4(dppz)](ClO_4)_2$ in the absence and presence of CT-DNA (at a constant concentration of complexes, [Ru] = 20μM) are measured. The absorption spectra of complex shows three bands with comparable intensity as in fig-5, lying in the range of 200- 600nm. The first one is a strong and broad absorption band centered at 360 nm, which is generally assigned to a singlet metal to ligand transfer (MLCT). There are two narrow separated bands within the range of 265 - 255 nm. And one strong and narrow band centered at 220nm, both of them are characteristic of the intraligand (IL) π–π^* transitions. These assignments of absorption bands can be confirmed and analyzed in detail by TDDFT calculations in aqueous solution. With the increase in concentration of DNA, the absorption band of the complex display clear hypochromism. The spectral characteristics suggest that the Ru(II) complex interact with DNA.

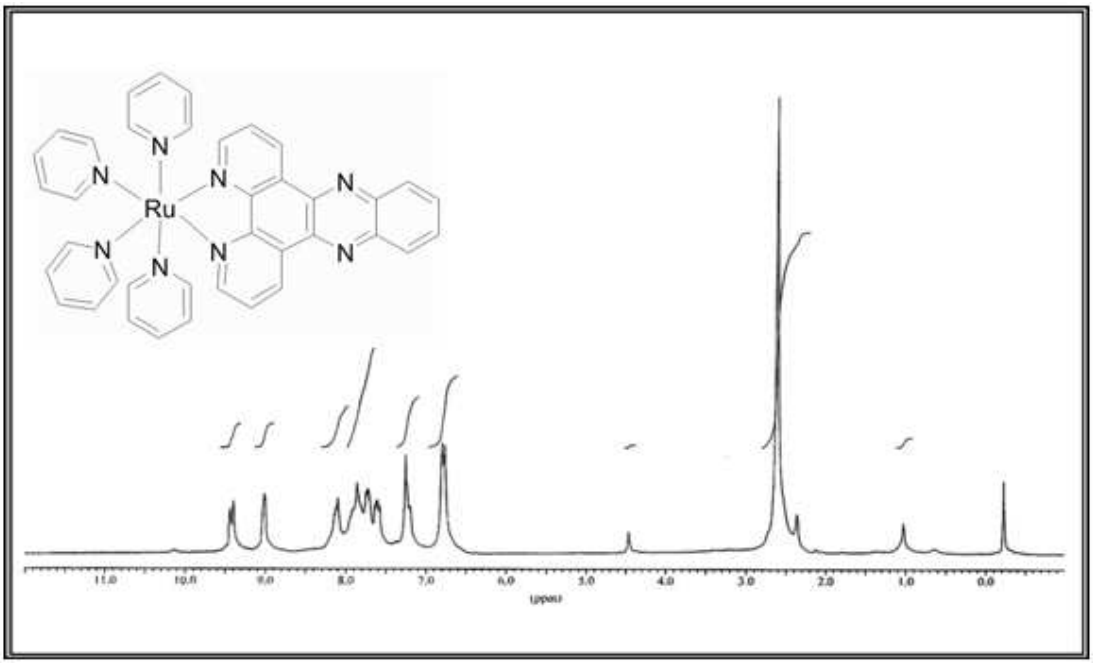

Fig 3: ^{1}H NMR spectrum of [Ru (Py)$_4$ (dppz)] (ClO$_4$)$_2$

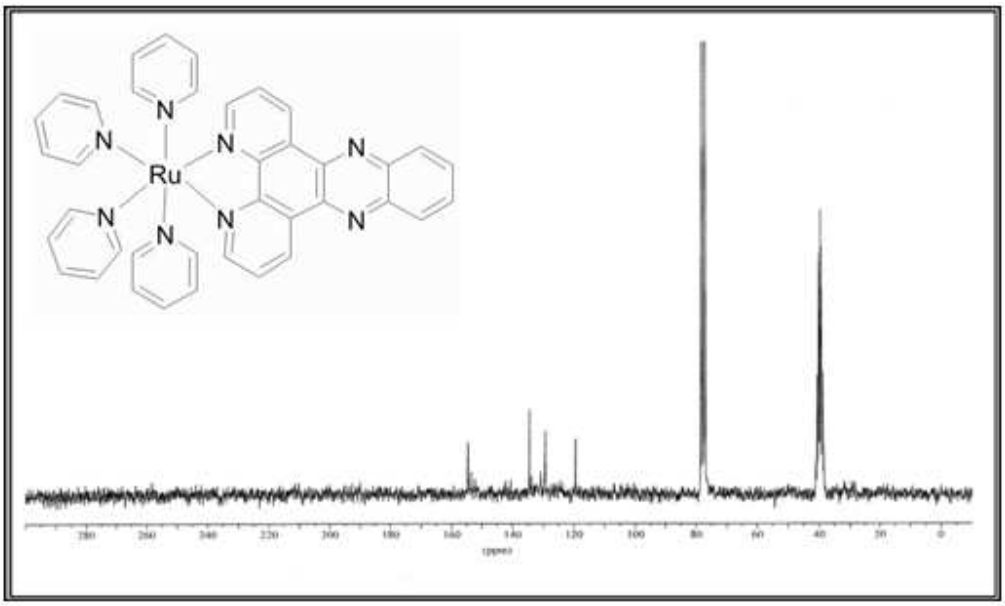

Fig 4: ^{13}C NMR Spectrum of [Ru (Py)$_4$ (dppz)] (ClO$_4$)$_2$

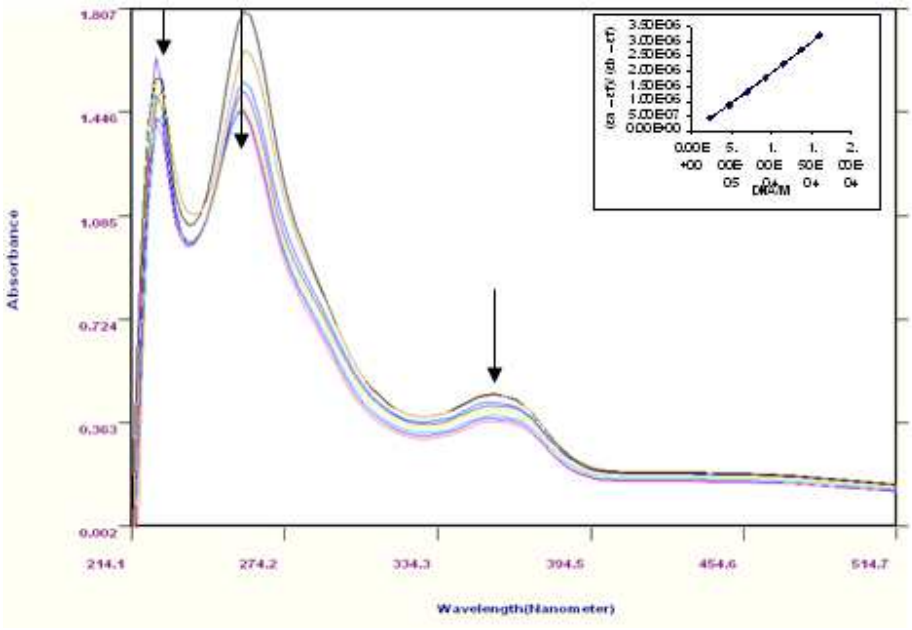

Fig 5 –UV-Visible spectra of [Ru(py)$_4$(dppz)](ClO$_4$)$_2$

Tris- HCl buffer upon addition of CT DNA in absence (top) and presence of CT DNA (bottom) the [Ru] = 10 μM; [DNA] = 0-126 μM. Insert: plots of [DNA] / (Σ_a - Σ_f) vs [DNA] for the titration of DNA with complex

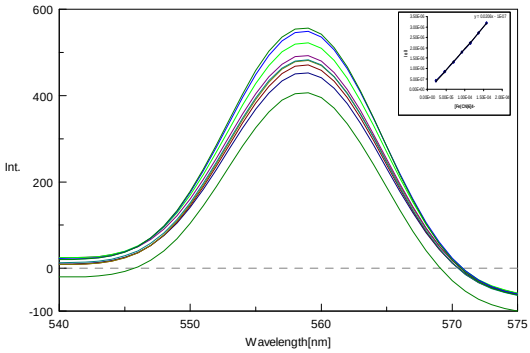

Fig 6 – Fluorescente emisión spectra of complex [Ru(py)$_4$(dppz)]ClO$_4$.2H$_2$O in aqueous buffer

Tris 5mM, NaCl 50mM, pH 7.0) in the presence of CT DNA, [Ru] = 20μM,
[DNA] / [Ru] 0,5,10,15,20 (The arrow shows the intensity changes upon increasing concentration)
Insert: Plots of relative integrated emission intensity vs [DNA] / [Ru].

The intrinsic binding constants K_b of complexes was measured to be 2.06 x 10^5 M^{-1} respectively. The K_b values are in the same order of those bidentate $[Ru(L)_2(dppz)]^{2+}$ complexes. Where L= bpy, phen, dmb, dmp. This suggest that the complex most likely intercalatively bind to DNA, involving a strong stacking interaction between the aromatic chromophore (dppz) and the base pairs of the DNA. It indicates that the ancillary ligands Pyridine enhance the DNA – binding affinity. The DNA- binding constant of complex shows that it has larger hypochromism value but smaller K_b value.

3.1a Fluorescence emission studies:

In the absence of CT- DNA, complex [Ru $(Py)_4$ (dppz)] $(ClO_4)_2$ emit luminescence in tris buffer at ambient temperature, with a maximum appearing at 559nm. Upon addition of CT DNA the emission intensities of this complex increase and it implies that complex interacted strongly with DNA and was protected efficiently. The hydrophobic environment inside the DNA helix restricts the mobility of the complex at the binding site. Emission

quenching experiments using $[Fe(CN)_6]^{4-}$ as quencher may provide further information about the binding of complexes with DNA, the emission of complex was efficiently quenched by $[Fe(CN)_6]^{4-}$ as in fig-6 & 7 and the plot was not linear which imply that the quenching process is both dynamic and static.[55] However, in the presence of DNA. the Stern – Volmer plots are changed drastically and the emission intensity was hardly affected by the addition of anionic quencher. This may be explained by repulsion of the highly negative $[Fe(CN)_6]^{4-}$ from the DNA polyanion backbone which hinders access of $[Fe(CN)_6]^{4-}$ to the DNA bound complexes. [56] The plot for the DNA bound complexes become linear, indicative of only dynamic quenching , which was consistent with the strong DNA – binding affinity of complex. The curvature reflects degrees of protection or relative accessibility of bound cations, a larger slope for the Stern – Volmer curve parallels poorer protection and low binding. From the results, it was suggested that the complex has a stronger DNA- binding affinity

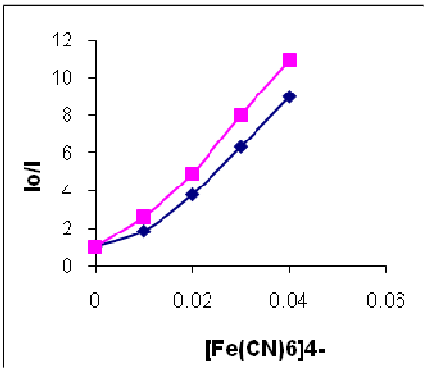

Fig-7: Emission quenching of [Ru (Py)$_4$ (dppz)] (ClO$_4$)$_2$ with increasing $[Fe(CN)_6]^{4-}$ in the presence and absence of DNA

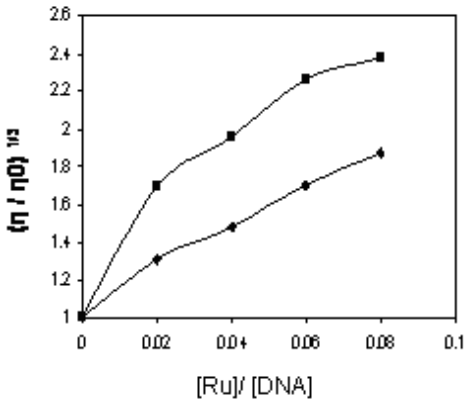

Fig 8: Effect of increasing amount of complexes ([Ru(py)$_4$(dppz)]ClO$_4$.2H$_2$O & [Ru(4-Et-py)$_4$(dppz)]$^{2+}$)on the relative viscosity of CT DNA at 25± 0.1^{0}C.

Fig 9: Photo cleavage studies of [Ru(py)$_4$(dppz)]$^{2+}$

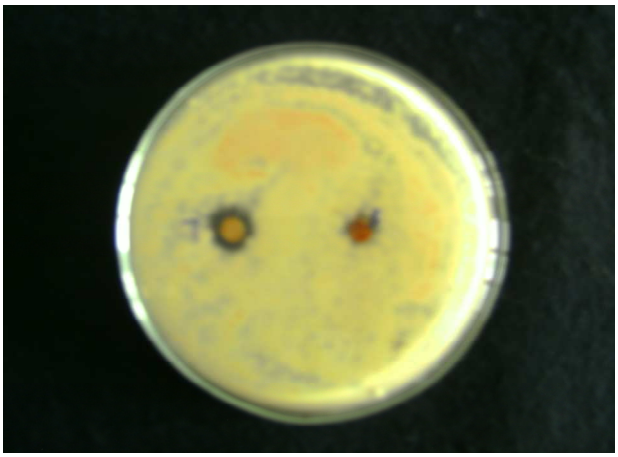

Fig 10: Anti microbial activity of [Ru (Py)$_4$ (dppz)] (ClO_4)$_2$

3.1b Viscosity studies:

Viscosity measurements were carried out to elucidate the binding mode of the complex, by keeping [DNA] = 0.4mM and varying the concentration of the complex. The changes in relative viscosity of rod- like CT-DNA in the presence of [$Ru(py)_4(dppz)]^{2+}$ were observed.. From the figure it was observed that with the addition of increasing amounts of complex to the DNA the relative viscosity of DNA increases steadily. The increased degree of viscosity, which inturn depend on the binding affinity to DNA. These results suggests that the complex intercalate between the base pairs of DNA and the binding affinity of complex is also more, which is consistent with the above results inserted in fig 8

3.1c Photoactivated cleavage of pBR 322 DNA:

There was substantial and continuing interest in DNA endonucleolytic cleavage reactions that were activated by metal ions [57,58] The cleavage reaction on plasmid DNA can be monitored by agarose gel electrophoresis. When circular plasmid DNA was subjected to electrophoresis, relatively fast migration was observed for the intact supercoil form (Form I). If scission occurs on one strand (nicking), the supercoil will relax to generate a slower moving open circular form (Form II). If both strands were cleaved, a linear form (Form III) that migrates between Form I and II will be generated. [59] Fig.9 shows gel electrophoresis separation of pBR 322 DNA after incubation with the complex and irradiation at 365nm. No DNA cleavage was observed for controls in which complex was absent (lane 0), or incubation of the plasmid with the Ru (II) complex in dark. With increasing concentrations of the Ru(II) complexes (lanes 1-8), the amount of Form I of of pBR 322 DNA diminish gradually, whereas Form II increases and Form III was also produced (lane 9). Under comparable experimental conditions, the complex exhibits more effective DNA cleavage activity.

3.1d Anti microbial activity:

The antimicrobial screening effects of these complexes were tested against *Escherichia coli* bacteria by the well diffusion method [60], using agar nutrient as the medium. In a typical procedure the agar medium was inoculated with microorganisms. The well was filled with the test solution using a micropipette and the plate was incubated, 24 h for bacteria at 35°C. During this period, the test solution was diffused and the growth of the inoculated microorganisms was affected. The inhibition zone was developed, at which the concentration was noted. The increase in the delocalization of π- electrons over the whole chelate ring enhances the penetration of the complexes blocking of the metal binding sites in the enzymes of microorganisms.[61] This complex also disturbs the respiration process of the cell and thus block the further growth of the organisms. [62]

$[Ru(Py)_4(dppz)]$ $(ClO_4)_2$ is active in inhibiting the growth of bacteria as shown in fig 10.

CONCLUSION

The study has provided an apparently novel method for $Ru(py)_4(dppz)]^{2+}$ to bind with DNA intercalatively. These complexes inhibit the growth of bacteria showing the antimicrobial activity and also cleave the PBr322 DNA. Hence this complex can be used as an antitumour and anticancer agent. Complexes with ethylene diamine also has been studied to show an antitumour and anticancer properties. [63,65]

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