J Fluoresc (2010) 20:631–643
DOI 10.1007/s10895-009-0592-6

ORIGINAL PAPER

Fluorescence Labelling of DNA by Carboxylic Polypyridyl-Ru Complexes Containing bpy and DIP Ligands: A Study Revisited

Michèle Lamoureux · Olivier Seksek

Received: 28 October 2009 / Accepted: 22 December 2009 / Published online: 3 April 2010

Abstract The coordination complexes $(DIP)_2Ru(CH_3bpy\text{-}COOH)$ and $(DIP)_2Ru(COOHbpyCOOH)$, where DIP and bpy are diphenylphenanthroline and bispyridine, have been recently proposed as fluorescent markers of nuclear DNA (Musatkina et al., J. Inorg. Biochem. 101:1086–1089, 2007), but no DNA binding investigation and no quantitative fluorescence evaluations had been done. Both complexes, as well as the smaller ones with bpy's in place of DIP's, have been investigated here by spectroscopic DNA titrations (UV–vis absorption, fluorescence, circular dichroism) and by in vitro cellular studies (flow cytometry and fluorescence imaging). Contrary to previous reports, neither the carboxylic function nor the more extended DIP ligand ensures any appreciable binding to DNA. This is clearly illustrated by the appearance of an isosbestic point of a second kind and by the proportionality of the fluorescence maximum intensity to the absorbance at the excitation wavelength. Above all, the lack of enhanced fluorescence in the presence of DNA definitively rules out the use of such complexes as DNA markers. Moreover, there is no detectable nuclear uptake. However, the fluorescent complexes with the DIP ligands, especially $(DIP)_2Ru(CH_3bpyCOOH)$, are massively incorporated into the cytoplasm while preserving cell integrity, which could suggest other types of biological application.

Keywords Coordination complexes · Metal complexes · DNA fluorescent marker · Ru complex internalization

M. Lamoureux (✉) · O. Seksek
Laboratoire Acides Nucléiques et Biophotonique,
CNRS FRE 3207, Case courier 138,
Université Pierre et Marie Curie,
4 place Jussieu,
75252 Paris Cedex, France
e-mail: michele.lamoureux@upmc.fr

Introduction

The interaction of ruthenium polypyridyl complexes with DNA has attracted considerable interest during the last decades due to their optical properties [e.g. 1–3], and the biological activity of some ruthenium complexes was already investigated in the fifties [4]. The principal purpose of the present study is to revisit a pair of ruthenium coordination complexes, known as Ru-DIP's, that have recently been proposed [5] as potential cell DNA dyes because of an intense fluorescence with a high Stokes shift, low toxicity, presumed strong interaction with DNA, and promising microscopy images. These hexacoordinated chiral complexes contain extended π-systems and also carboxylic acid groups which are mostly deprotonated under physiological conditions. The Ru-DIP's incorporate two 4,7-diphenyl-1,10-phenanthroline (DIP) ligands, as well as a bispyridine (bpy) ligand which, in turn, bears one or two carboxylic acid groups. As illustrated in Fig. 1, Ru-DIP1, and Ru-DIP2 are akin to the Ru-bpy1 and Ru-bpy2 complexes studied earlier [6], except that all the ligands of the Ru-bpy's are small bispyridines. The association was there identified as being of 1:1 stoichiometry, and the association constant (K) of Ru-bpy1 with DNA was estimated from an NMR study to be a few 10^{+3} M^{-1}. Since

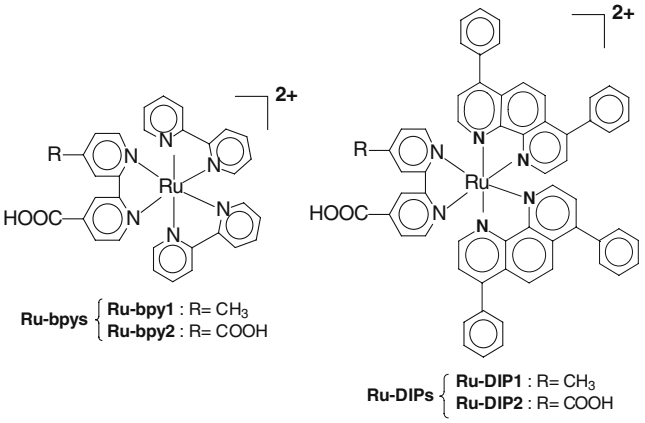


Fig. 1 Schematic structures and short names of the coordination complexes, Ru-bpy's and Ru-DIP's. The last character, 1 or 2, gives the number of COOH groups on the common bpy ligand. bpy = bispyridine; DIP = 4,7-diphenyl-1,10-phenanthroline. Ru-bpy1 = $bpy_2Ru(CH_3bpyCOOH)$ and Ru-bpy2 = $bpy_2Ru(COOHbpyCOOH)$. Ru-DIP1 = $DIP_2Ru(CH_3bpyCOOH)$ and Ru-DIP2 = $DIP_2Ru(COOHbpyCOOH)$

the non-substituted analogue $(bpy)_3Ru$ was described as inert [7], the carboxylic acid group was held responsible for the association of the substituted Ru-bpy's to DNA. Likewise, the fact that Ru-DIP's penetrate cells more easily than Ru-bpy's was attributed to the larger phenanthroline ligands [5]. At present, no *K* has been published for the Ru-DIP's. No fluorescence spectrum was ever given for any of these Ru-bpy and Ru-DIP complexes, except for Ru-bpy1 but then only in the absence of DNA [8]. Moreover, their cellular uptake has been evidenced only qualitatively, through preliminary fluorescence camera imaging with a moderate magnification [5].

In view of potential biological applications, a closer and comparative view of the properties of these complexes is desirable. To probe the strength and type of the association of coordination complexes with DNA, spectroscopic experiments are normally used first: electronic absorption in the UV–visible (UV–vis) range, fluorescence and circular dichroism (CD). Fluorescence results of the complexes exposed to DNA are all the more necessary in that the idea was to use them as DNA light switches. In the first part of this study, the experiments on the complexes with DNA in solution were performed under the same buffer conditions as in the earlier NMR study [6] on the Ru-bpy's, but at roughly a hundred times lower reactant concentrations, as accessible by and typical of the spectroscopic and biological experiments. This has the additional advantage of reducing the possible formation of dimers and higher polymers of the complexes [9] and of not modifying appreciably the biological properties of the cell cultures.

In the second part of this study, cytometric investigations were made to compare cellular uptake of the Ru-bpy and Ru-DIP complexes quantitatively. Membrane integrity and viability of the cells exposed to the Ru complexes were checked by use of the calcein AM indicator. Finally, microscopy images were obtained to determine the intracellular complex localization.

Experimental and spectra analysis section

Chemical reagents

The Ru-bpy's and Ru-DIP's had been synthesized as crystalline powders, as described earlier [10, 11]. Ethidium bromide (EtBr) and calf thymus DNA were from Sigma-Aldrich Chemical Company. A solution of DNA fragments was prepared by sonication of buffered DNA in a microcentrifuge tube over ice, by bursts of 10 s for a total time of 200 s. Stock solutions of complex and of DNA were prepared in an aqueous buffer (pH 7.0, ionic strength, 0.05 M). Under such conditions, the complexes are mostly deprotonated, so that Ru-bpy1 and Ru-DIP1 have a single positive net charge, and Ru-bpy2 and Ru-DIP2 are neutral. Further dilutions of the DNA stock solutions gave the ratio of the optical densities at 260 and 280 nm as 1.93, as expected for protein-free DNA. The concentration, expressed in base-pairs, was determined by absorption spectroscopy, using the molar extinction coefficient of 12,800 cm^{-1} M^{-1} at 260 nm for calf thymus. In cases where a cosolvent was needed, the buffer and both stock solutions contained the same volumic concentration of acetonitrile. The mixtures of complexes and DNA never required more than 10% *v*/*v* of acetonitrile. This proportion has already been used [e.g. 12], and does not perturb the association with DNA.

Cell lines and cell culture

MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection. The cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate and 50 U/ml streptomycin (all obtained from Life Technologies Inc.), at 37 °C in a humidified 5% CO_2 atmosphere. All in vitro cell experiments were carried out at 37 °C in a 5% CO_2 incubator.

Spectroscopic instruments

Electronic absorbance spectra were recorded on a Varian Cary 1E spectrometer. Fluorescence spectra were recorded on a SLM Aminco-Bowman Series 2 spectrofluorimeter between 500 and 750 nm with excitation at 456 nm unless otherwise stated. Both excitation and emission bandwidths

were 4 nm. After a warm-up period of nearly an hour, the lamp reaches a stable regime within ±2% as repeatedly checked by measuring the fluorescence of a rhodamine B standard. CD spectra were recorded on a Jasco model J-810 spectropolarimeter with a thermoelectrically controled cell-holder. Quartz Suprasil cuvettes were from Hellma. The optical path length (l) used for the absorption measurements was 0.1 cm, 0.4 cm or 1 cm depending on the solution and wavelength, to ensure optical densities between 0.05 and 2.0, that is, in the domain of linear response of optical density vs. concentration. Nearly all the fluorescence measurements were made at l=0.4 cm in the direction of the excitation beam.

All measurements were made in an air-conditioned room at 21 °C. In addition, two of the three spectrometers have a temperature-controled cell-holder which was regulated at 21 °C.

Spectroscopy measurements and analysis

When DNA is added to a solution of metal complexes, e.g. of Ru complexes, strong association with DNA, especially through intercalation, is well known to be revealed by the following spectroscopic signatures [e.g. 13–17]:

i) reduced absorption, $\varepsilon_b/\varepsilon_f$, of 10–25% at the metal to ligand charge transfer (MLCT) band often accompanied by a bathochromic shift;
ii) fluorescence enhancement, f_b/f_f, of 2 to 100 or even 1,000, with a frequent red shift of the fluorescence maximum;
iii) circular dichroism modifications specifically helpful to probe the enantioselectivity, here the propensity of the Δ enantiomers to bind canonical right-handed DNA more strongly than the Λ counterparts when intercalation is the dominant binding process.

Note that most of these spectroscopic modifications are produced by intercalation, but also generally appear as soon as the association with DNA is tight. For example, though the well known Hoechst markers of cellular DNA [e.g. 18] principally associate with groove binding, the fluorescence enhancement is high and K reaches values of a few 10^{+6}–10^{+7} M^{-1}. Needless to say, the essential requisite of a fluorescent DNA marker is property ii) above.

Absorption and fluorescence titrations have been extensively exploited for decades to determine association constants through various data analyses [e.g. 15, 19–22]. For coordination complexes strongly associated with DNA, complex concentrations of about 10 μM are typically involved [e.g. 17]. Since the K of Ru-bpy's [6] are at least two orders of magnitude smaller than for current markers, it is expected for this case that the reactant concentrations have to be increased by about an order of magnitude to produce some observable effects, and this leads to less straightforward experiments. For example, insolubility and condensation problems may arise, and acetonitrile may have to be added to ensure the solubility of the Ru-DIP's. Because of the larger concentrations involved, the fluorescence may no longer be linear with complex concentration, and a preliminary calibration needs to be done. The solubility of DNA is also limited, and the large DNA concentrations required in the titration solutions lead to aliquots whose volume cannot be neglected. Even with these adaptations, K may remain inaccessible by spectroscopic determination, and the only possibility is then to propose an overestimate. As a last point, two contrasting situations when the spectra of a titration series cross each other at a single point will be discussed.

In the following, for any solution investigated, the subscripts b and f denote the bound and free species, respectively. The total concentration of Ru complex is $R=R_f+R_b$. The total concentration of the DNA titrant in base-pairs is $D=D_f+D_b$, with D being increased from 0 to D_{max} in the course of the titration. For any data point of a standard titration, a volume—or cumulated volume—v from a stock DNA solution at $D°$ is added to the volume V of a solution of free ruthenium complex initially at the concentration R^{in}. In as much as v remains completely negligible versus V, there is no dilution effect, and R is the same throughout the titration. Otherwise, R is reduced over the course of a standard titration. To ensure a constant R for various values of D, separate solutions with appropriate buffer volumes have then to be prepared. This longer approach avoids dilution effects, but may cause some slight irreproducibility in R and consumes more material.

The absorbance (A) and the fluorescence intensity (F) were measured for all solutions, and circular dichroism only in a few cases. In the visible range, roughly for λ>350 nm, the absorption spectrum is essentially that of the Ru complex, DNA not absorbing in this range. Therefore, both $A/(Rl)$ and F/R are just the extinction coefficient (ε) and the normalized fluorescence intensity (f) of the Ru complex increasingly exposed to DNA. In the case of a two-state model, ε and f for a fixed λ (conveniently taken at or near the spectrum maximum) follow a very regular monotonic evolution with D from the initial free state towards the fully bound one. For the 1:1 stoichiometry proposed for the Ru-bpy's [6] and tentatively expected for the Ru-DIP's, Fig. 2 gives simulated b and f/f_f curves for the initial value of R^{in}= 0.088 mM, and a wide variety of K. Note that a quick K estimate is provided at mid-course with $1/K = D_{1/2} - 1/2R_{1/2}$ since by definition $K = b/(1-b)/(D-bR)$. All curves in Fig. 2 are initially rectilinear, but the asymptotic behavior at large D is of higher order. K is thus more

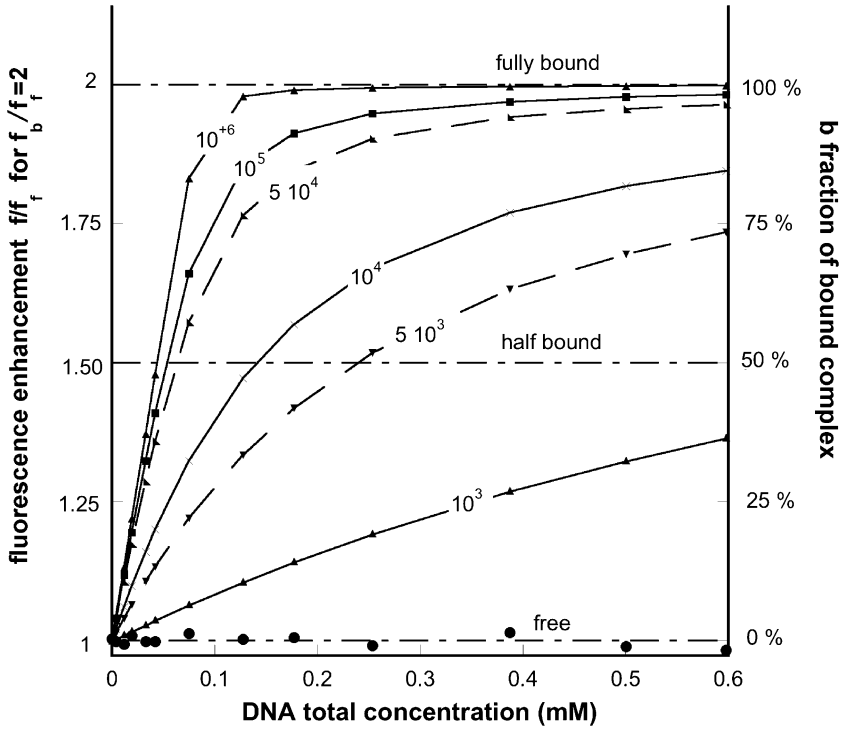


Fig. 2 Modeled fluorescence enhancement and proportion of bound complex in a two-state and 1:1 stoichiometry model, for various K in M^{-1}. Example for f_b/f_f=2. R^{in}=0.088 mM, and DNA concentrations as in titration of Figs. 5 and 6. The *bottom full circles* data points are the measured f/f_f for that titration of Ru-DIP2 by DNA

precisely determined when the asymptotic behavior is already reached, and it is always recommended to have $D_{max} >> 1/K$ [e.g. 15]. This probes the assumption of a two-state reaction. It, moreover, ensures that the bound limit can be safely extrapolated, and that the most curved section of the titration plot is covered by the titration. Conversely, if no marked concavity has yet been reached for D_{max}, one could suggest that $D_{1/2}$ is greater than $D_{max}/4$, and thus $1/K$ bigger than $D_{max}/4$. This tentative rule of the thumb can give a conservative and safe overestimation of the association constant (smaller than $4/D_{max}$) even when the reaction is far from complete.

The titration experiments are particularly easy to interpret when D can be increased at fixed R and when the association is strong [15, 17, 22]. The hallmark of a simple two-state reaction of 1:1 stoichiometry is that the absorption spectra cross each other at a few precise wavelengths, namely at the isosbestic points. This happens in the visible region where the DNA absorption is negligible and at the λ where the free and bound complexes have the same extinction coefficient:

$$A/l = R_f\varepsilon_f + R_b\varepsilon_b = R\varepsilon = \text{cst} \quad \text{when } \varepsilon_b = \varepsilon_f = \varepsilon \qquad (1)$$

The isosbestic point on the lower λ side of the MLCT band appears at 375–400 nm for Ru complexes [e.g. 2, 9, 23]. With weaker associations, common sense calls for larger reactant concentrations, and D_{max} has to be increased with larger $1/K$. Since the solubility of DNA, and therefore $D°$, is limited, v may have to be increased to such a point that dilution cannot be neglected in the course of the titration. Thus, even in the simple two-state model, the standard isosbestic crossing point then becomes blurred.

For very weak associations, the spectra of a titration series may again cross each other in a clear-cut manner, this time in a misleading way, and only as an artefact caused by dilution. If the two reactants do not interact with each other, A reduces to a linear sum involving the extinction coefficients and the concentrations of the two free reactants. For a precise value of λ, A can be independent of D, i.e. of v, as follows:

$$A/l = \left(\varepsilon_f R^{in} V + \varepsilon_f{}^{DNA} D° v\right)/(v + V)$$
$$= \text{cst} = \varepsilon_f R^{in}, \text{ when: } \varepsilon_f R^{in} = \varepsilon_f{}^{DNA} D° \qquad (2)$$

In other words, the reduction of absorption by the Ru complex caused by its dilution is exactly balanced by the onset of the absorption by the added DNA. Contrary to the well-known isosbestic point of Eq. 1, this isosbestic point of a second kind appears in the UV region where DNA also absorbs, and the corresponding λ depends on the ratio of the stock concentrations. Far from demonstrating an important association and a two-state model, as in the previous paragraph for constant R, this sort of isosbestic point proves that the association of DNA to the complex is very weak. It also shows that no insolubility or condensation problem comes into play in the course of the titration.

The interpretation in the case of weak associations is more sensitive to minor imprecision in the reproducibility of the solutions and to various artefacts. In general,

different concentrations and cuvettes are used for absorption and fluorescence, since the latter process is much more sensitive to association. Here, on the contrary, care was taken to record both spectra throughout a titration with the same 0.4 cm×1.0 cm cuvette. The other important advantage is that the same equilibrium resulting from the same processes is being studied by absorption and fluorescence. The concentrations of the free complex were kept small enough for A with l=1 cm to remain below 1.5 at the MLCT band, but this condition is not sufficient to ensure the linearity of the fluorescence with concentration, even if the smaller optical length along the excitation beam l=0.4 cm is chosen. The observed fluorescence (F_{obs}) cannot be taken at its face value, and the partial absorption of the excitation beam by the solution has to be taken into account. The correction is expected to correspond roughly to one half the optical length, i.e. to be close to $10^{A/2}$, where A is the absorption for l=0.4 cm. A precise calibration was made. Finally, in order to eliminate small uncertainties in the concentrations, it appeared more appropriate to study the F vs. A curve than only A and F versus concentration. This representation is also a better test, because DNA binding tends to diminish A and increase F. In brief, if the association is too weak to produce any spectroscopic modification, the linearity of F versus A reveals this fact more clearly.

Flow cytometry and fluorescence microscopy

Cells were seeded on Petri dishes (diameter, 30 mm; density, 50,000 cells per dish), grown for 24 h and treated for 2 or 4 h with various Ru complex concentrations. Thirty minutes before doing flow cytometry experiments, 10 μM of calcein AM (Interchim, Montluçon, France) were added. Cells were then washed twice in phosphate buffer saline (PBS). Treated and untreated cells were harvested by trypsinization (500 μl Trypsin-EDTA). Cell suspensions were analyzed with a Beckton Dickinson FACSCalibur 3C (argon laser wavelength at 488 nm) flow cytometer, with emission filters set at BP530/30 and LP670 nm for calcein AM and Ru complexes, respectively, and with the appropriate compensation factor set-up. Ten thousand cells per sample were measured for forward-angle light scattering (FSC), side scattering (SSC) and fluorescence. Fluorescence internalization histogram data were obtained using light scatter (FSC vs. SSC) and fluorescence (related to calcein AM) gates to exclude debris and dead cells (i.e. non-calcein AM labeled cells).

For fluorescence microscopy, MDA-MB-231 cells were washed twice in PBS. Microscopic observation was carried out in 2 ml of PBS with a Nikon Optiphot-2 to which was added a coaxial-confocal module with a Nipkow wheel (Technical Instruments, model K2 BIO). The optimal depth resolution was 0.5 mm. The excitation light source was a high-pressure mercury lamp. Cells were observed in PBS with a Zeis 63× water immersion objective. Appropriate fluorescence emission filters were used for calcein AM (FITC filter set: 450–490, FT510, 520–570) and ruthenium complexes (rhodamine filter set: BP546, FT590, LP600). Images were collected using a cooled CCD camera (Micromax, Princeton Instruments, Evry, France). Display and analysis were performed with IPLab software (Scanalytics, Fairfax, VA).

Results and discussion

Spectroscopic characteristics of the free Ru coordination complexes and fluorescence normalization

The absorption characteristics are first given for the free Ru coordination complexes. The results are similar for the complexes bearing one or two carboxylic acid groups. The MLCT absorption maximum (A_{max}) is located at around λ_{max}=460 nm, as typical for such complexes, more precisely at 457 nm for the Ru-bpy's and at 462 nm for the Ru-DIP's. For the Ru-bpy's, a good linear regression of A_{max} vs. R ranging from 0.005 mM up to 0.100 mM leads to ε_f=11,000 cm^{-1} M^{-1}. As it should be for enantiomers, the absorption and fluorescence spectra are the same for Ru-bpy1Λ and Ru-bpy1Δ. For the DIP compounds, the extinction coefficient is roughly twice as great, with ε_f= 20,000 cm^{-1} M^{-1}. The presence of 5 to 20% v/v acetonitrile cosolvent, necessary for the highest concentration, does not appreciably modify ε_f.

All these Ru complexes exhibit typical fluorescence in the 600 nm region, with maxima (F_{max}) at λ_{fluo}=620 nm for the Ru-bpy's and Ru-DIP1, and λ_{fluo}=634 nm for Ru-DIP2. As expected, the values of λ_{max}, ε_f and λ_{fluo} for the Ru-bpy's are very close to those published for deprotonated Ru-bpy1 [8]. The large Stokes shift of 170 nm makes these complexes suitable for flux cytometry. When an excitation wavelength (λ_{exc}) of 457 nm is used, the f_f^{457} values are 89 arb. un. for the Ru-bpy's and 262 arb. un. for the Ru-DIP's. The solutions were also illuminated at the laser wavelength of the cytometer 488 nm, and gives $f_f^{488} = 33$ arb. un. for the Ru-bpy's and 154 arb. un. for the Ru-DIP's.

The fluorescence response was empirically calibrated. For free Ru-bpy1, the fluorescence maxima at λ_{fluo}= 620 nm are plotted vs. R and A in Fig. 3 with R increasing from 0.005 mM to 0.200 mM, that is, A from 0.022 to 0.88 for an absorption depth of l=0.4 cm. The fit to $F=F_{obs}$ $10^{(A/a)}$ leads to a=2.66, as expected, not far from 2. The same parameter was obtained when a l=1 cm cuvette was used over a wider concentration range, 0.005–0.500 mM of Ru-bpy1, as well as from EtBr solutions.

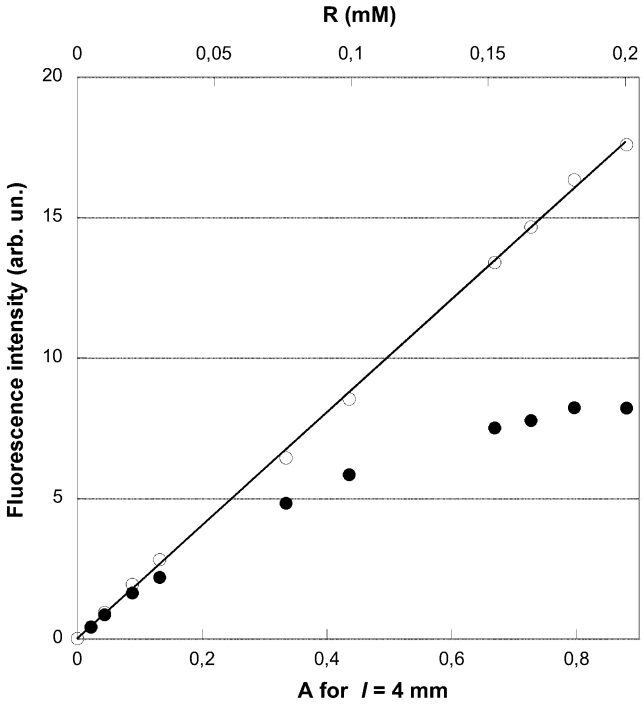


Fig. 3 Fluorescence maximum intensities F_{max} at 622 nm, vs. R and A (with l=0.4 cm) before (●) and after (○) the absorption of the exciting photons is taken into account. Example of free Ru-bpy1Λ with λ_{exc}= 457 nm

Spectroscopic results for Ru coordination complexes with DNA

Spectroscopic results for Ru-bpy's with DNA

For Ru-bpy1 and Ru-bpy2, the titrations were performed at constant R, at either a usual value of a few tens of μM [15–17], or at a much higher one. The easily soluble Ru-bpy's were available in large quantities, and the mixtures were prepared separately for each D datum to ensure a constant R. Absorption spectra and fluorescence spectra (data not shown) were recorded for R=0.020 mM and D=0–30 R, and for R=0.500 mM and D=0–6 R. In both cases, the spectra were unchanged by the presence of DNA. D_{max} being 3 mM, one may infer that K is smaller than about $1.3\times 10^{+3}$ M^{-1}. However, since ε and f never appreciably depart from ε_f and f_f, it is more justified to refer to simulated data. In fact, even with a f_b/f_f of only 2, simulations in the two-state 1:1 stoichiometry model confirm that the fluorescence enhancement upon binding should be easily detectable for this binding strength. For bpy1Λ, bpy1Δ and bpy2Δ detailed titrations were carried out. For the other compounds available, i.e. bpy2Λ and racemic bpy2, only the solutions with D=0 and with the highest D/R ratio were studied. Ru-bpy1 was also studied for the smaller ionic strength of 0.01 mM, at which tighter associations could be expected. Again, no spectral change was observed.

The upper limit tentatively set to K is not contrary to the NMR determinations found for Ru-bpy1 solutions at R^{in}= 3 mM and with decreasing R [6]. In this respect, note that NMR titrations have been used only very rarely to determine K for the association of DNA with coordination complexes, and then also as a secondary determination [24]. In this field, NMR experiments are normally performed to determine the binding regions of both the complex and DNA [e.g. 24–27]. The K was deduced in [6] by analyzing the chemical shift (δ) of the Ru-bpy1 proton whose δ value is modified the most upon addition of DNA, the overall change (Δδ) being 0.13 ppm. This proton, labeled H3, is attached to the carbon adjacent to that carrying COOH and faces the methyl group. The authors, therefore, inferred that the DNA helix interacts with the complex through the substituted bpy ligand. In the same vein, a higher Δδ=0.8 ppm, though for a smaller R of 0.5 mM, was observed for a proton belonging to the dppz ligand of $(phen)_2Ru(dppz)$, and this was interpreted as indicative of the region linked with DNA [25]. The K determined earlier by standard spectroscopic approaches was in that case of the order of 10^{+6} M^{-1}. The K reported for bpy1Δ and bpy1Λ are only $0.9\times 10^{+3}$ and $2.2\times 10^{+3}$ M^{-1}, respectively [6]. The fact that K is twice as great for the Λ enantiomer as for the Δ one was taken as evidence against the intercalation mode. However, the difference between the association constants is moderate, and there is a kind of discontinuity in the δ curves, especially for Ru-bpy1Λ. The authors rejected the intercalation hypothesis more convincingly by noting that the melting temperature of DNA is not changed by Ru-bpy1Δ. A subsequent SERRS study confirms the implication of the same complex region in the interaction with DNA, but this time suggests intercalation as the binding mode [28].

In order to contribute to this discussion on the dominant mode of association, a circular dichroism study was carried out for the Δ enantiomer, in principle the more favorable to intercalation. Figure 4 shows CD results for a constant R, and regularly spaced D/R ratios. For D=0, the curve is as published [10] but for the erratum indicating the chiral notations [29]. Above 320 nm, the optical activity of the Ru complex is dominant, and would be strongly affected upon intercalation into DNA, as clearly observed for a K much higher than 10^{+4} M^{-1} [e.g. 16, 17]. In parallel, the CD of DNA at lower λ would also be changed, since base stacking and helicity are affected [17, 23]. No effect is here detectable for Ru-bpy1Δ exposed to DNA. For λ< 280 nm, the insert in Fig. 4 highlights two crossing points at 228 and 257 nm, precisely where free calf thymus DNA is optically inactive. The fact that the CDs of both DNA and Ru-bpy1Δ are unchanged confirms that no significant intercalation occurs. As a last remark, the behavior at λ= 249 nm, where free Ru-bpy1Δ is optically inactive, can

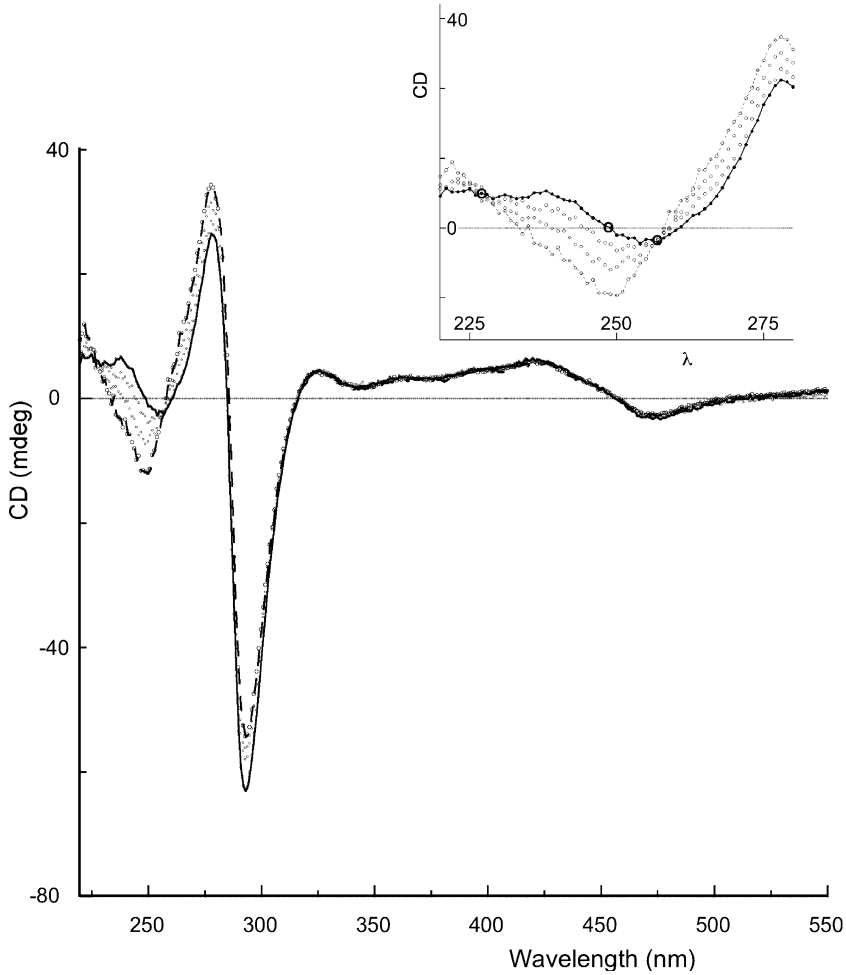


Fig. 4 Circular dichroism spectra for solutions of constant Ru-bpy1Δ concentration R=0.10 mM in the absence (*solid line*) and with increasing DNA concentrations (*dotted lines*): D=0.20, 0.40 and 0.60 mM. Ellipticity in millidegrees. l=1 cm

easily be interpreted in a similar way. This wavelength happens to correspond to the strong activity of DNA signalling the right-hand helicity. In the absence of any intercalation, the CD signal remains exclusively due to DNA and should then be proportional to D. Indeed, the linear regression (not shown) using the data points of the insert, together with further ones obtained for intermediate D and/or another l, has an excellent correlation coefficient of 0.998. In brief, be it in the visible or the UV spectral regions, the optical activities of Ru-bpy1Δ and DNA are unchanged by each other. The same conclusion was obtained for Ru-bpyΛ.

In summary, no intercalation and no strong association can be detected for any of the Ru-bpy complexes investigated by the three standard spectroscopic approaches, and there is no fluorescence enhancement upon exposure to DNA.

Spectroscopic results for Ru-DIP's with DNA

Spectroscopic results are given for the Ru-DIP's, which have been considered as more promising for biological applications, on the grounds that they correspond to an increase in size and hydrophobicity. In particular, it was taken for granted that their association with DNA would be tighter because of the more extended DIP ligands presumably amenable to base stacking [5]. In contrast to the Ru-bpy's, the Ru-DIP's were available only as racemic mixtures, in small quantities. The titrations were performed at decreasing R. A first titration for Ru-DIP2 and Ru-DIP1 with R^{in}=0.020 mM did not reveal any detectable modification other than the dilution effect. So as to favor association, a second study was carried out with R^{in}= 0.088 mM, that is, a significantly higher concentration, but still low enough to avoid precipitation or condensation if there is 10% acetonitrile.

Figures 5 and 6 show the absorption and fluorescence spectra for Ru-DIP2. There is no shift of λ_{max} or λ_{fluo} but both the absorption in the MLCT band and the fluorescence peaks are lowered by the addition of DNA. For absorption, the reduction has nothing to do with the hypochromic effet revealing tight associations, as could be mistakenly assumed at first sight. It is entirely caused by dilution, and the plot of A_{max} versus R is a straight line, as shown in the

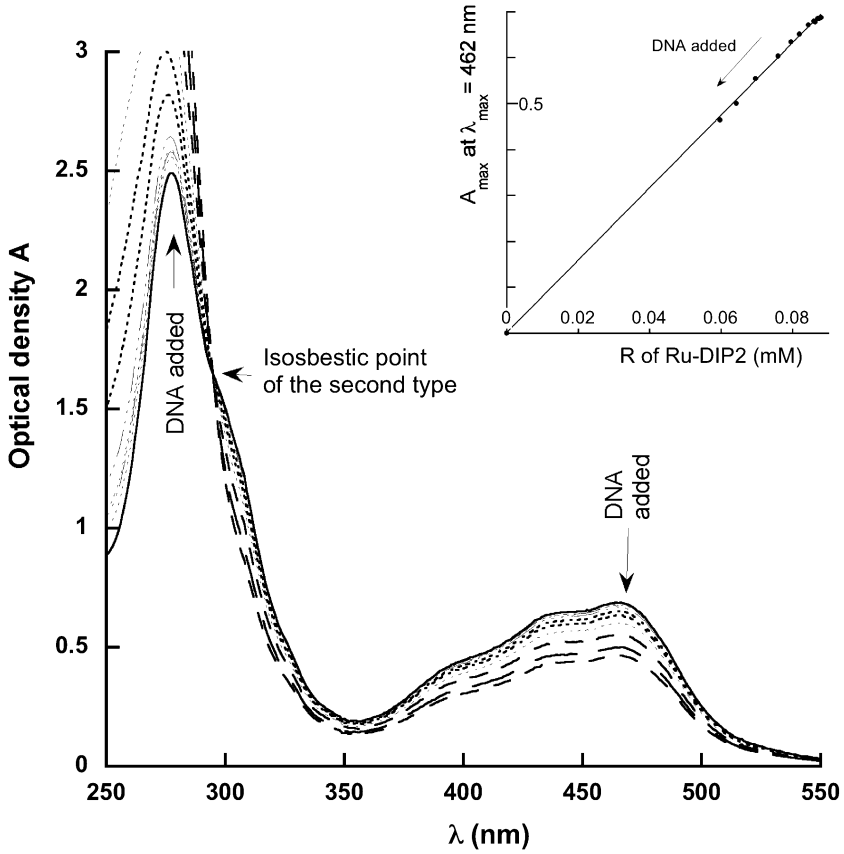


Fig. 5 Absorption spectra of Ru-DIP2, with l=0.4 cm. The DNA concentration D/R is increased from 0 to 10, at which point the aliquots have reduced R from R^{in}=0.088 mM to R=0.060 mM. The cumulative aliquots v (0, 2, 10, 17, 22, 40, 70, 100, 150, 250, 350 and 450 μL) of DNA solution are added into a volume V=950 μL of buffered Ru-DIP2 initially at 0.088 mM. The lowering of the maximum in the visible region simply corresponds to this dilution, as confirmed by the insert. Note the isosbestic point of the second kind at 295 nm

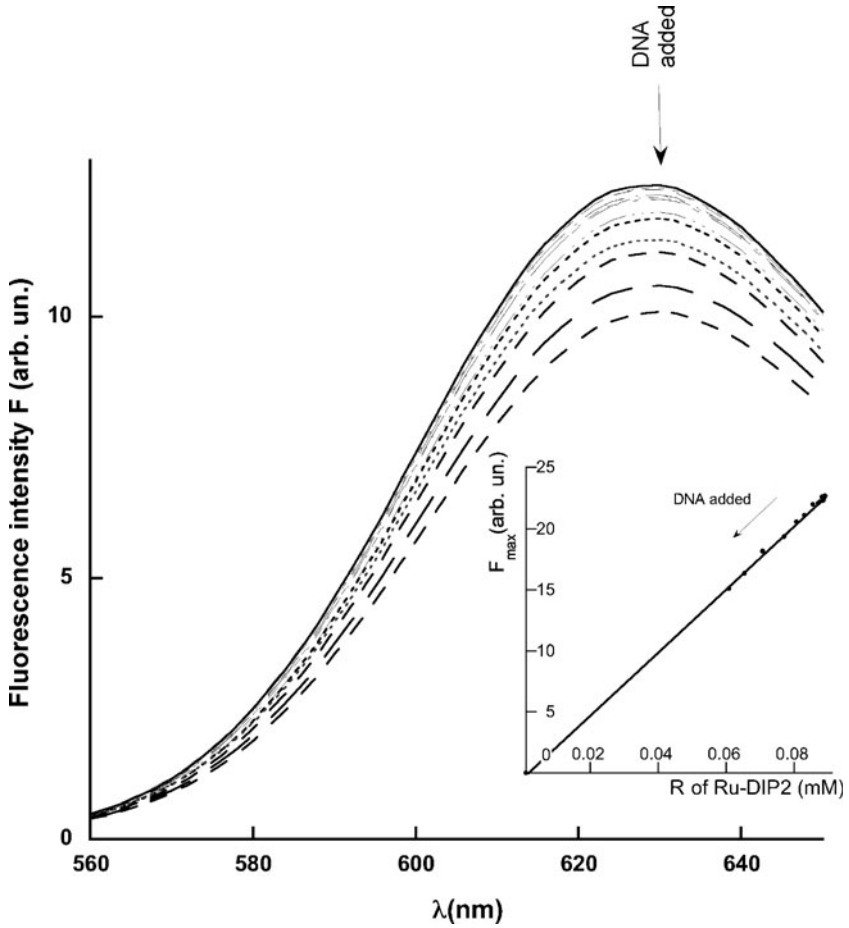
insert of Fig. 5. Note that the spectra cross each other at 295 nm. This isosbestic point of the second type, expressed by Eq. 2, confirms that absorption is not modified and checks that no precipitation or condensation has occurred. This is useful to have checked that point before discussing the fluorescence spectra.

The reduction of F_{obs} in Fig. 6 is similarly caused by dilution, though mitigated by a diminishing absorption of the exciting photons with decreasing R. When both effects are taken into account, F_{max} is also linear with R, as illustrated in the insert of Fig. 6. At any point, that is, at any D/A ratio ranging from 0 to 10, the values of $\varepsilon = A/lR$ and $f = F/R$ remain the ε_f and f_f of the free complex. As for the Ru-bpy's, and whatever the R^{in} value, there is no spectroscopic evidence of intercalation or strong association of Ru-DIP with DNA. Since the highest D involved was 0.6 mM, one may overestimate K with $7\times10^{+3}$ M^{-1} before confronting the data with simulated plots. This would be higher than for the Ru-bpy's but still too low for acceptable DNA markers. Those considered to perform adequately have K larger than 10^{+5} M^{-1} and a f_b/f_f much larger than 2. The modelled fluorescence curves in Fig. 2 correspond to the concentrations involved here, and show that the data points by no means satisfy these combined requirements.

Ru-bpy's and Ru-DIP's cannot be used as DNA light switches

Figure 7 decisively illustrates the invariability of the absorption and fluorescence characteristics upon DNA addition, and this both for the Ru-bpy's and the Ru-DIP's. These complexes would thus be ranked with the least effective ones, such as $(bpy)_3Ru$, in a comparative list of Ru complexes that also possess bpy or DIP ligands [30]. Racemic $(DIP)_3Ru$ exposed to calf thymus DNA and λ_{exc}= 482 nm yields a luminescence enhancement of 1.6 [31]. The experiments were performed with R=0.005 mM. This result led us to study again the Ru-DIP's at a smaller R= 0.009 mM than in the preceding paragraph and with less than 4% acetonitrile. Another modification is that the samples were excited at λ_{exc}=488 nm, which is also the wavelength of the cytometer laser. Again, no fluorescence enhancement was observed when DNA was added (Fig. 8). Contrary to the expectations [5, 6], the substituted bpy ligand does not increase the response of the Ru-bpy's relative to that of $(bpy)_3Ru$, nor the reactivity of the Ru-DIP's compared to that of $(DIP)_3Ru$. It thus appears that the reduction of the net positive charge brought by the deprotonated COOH has a dominant effect and weakens the association. As for the DIP ligands, they are more

Fig. 6 Same as in Fig. 5, but for the fluorescence spectra. Samples were excited at 456 nm



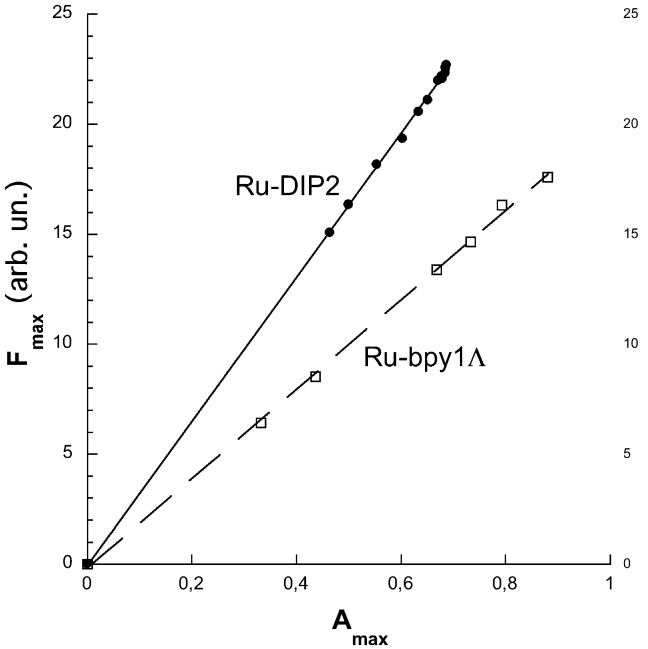


Fig. 7 Comparison of Ru-bpy and Ru-DIP complexes in the F_{max} vs. A_{max} representation, for different R values obtained by dilution with DNA aliquots. The linearity of both plots clearly indicates that neither absorption nor fluorescence is modified by DNA

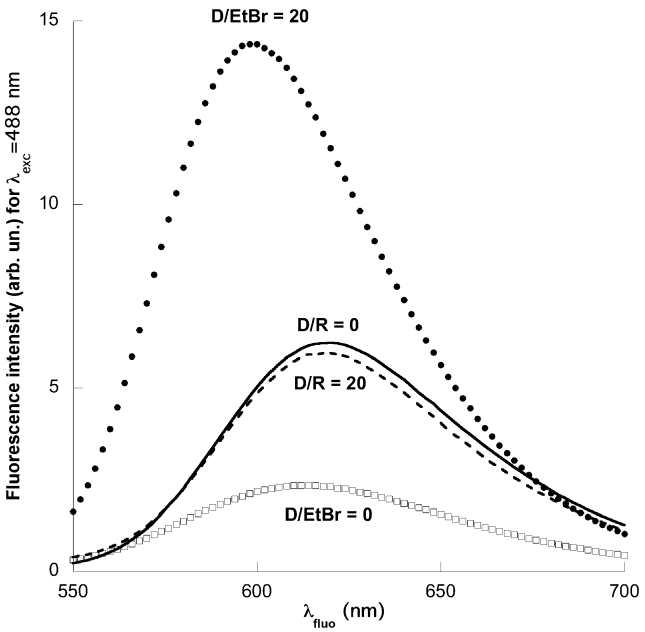


Fig. 8 Invariability of the fluorescence spectra of Ru-DIP exposed to DNA, in contrast with the enhancement found for the standard DNA marker, EtBr, with similar concentrations. Samples were excited at 488 nm. R^{in}=0.009 mM, and $D°$=1.6 mM

extended than the bpy ligands, but too bulky to permit intercalation. The four complexes investigated here thus do not bind DNA better than the simple complex $(bpy)_3Ru$ itself.

As a last point of comparison, results using EtBr at similar *D* and *R* were obtained for R^{in}=0.007 mM. As a test of its possible hindrance to DNA binding, 10% of acetonitrile was added to see if this maximum value could hinder attachment to DNA. In Fig. 8, the invariability of the Ru-DIP curve, except for a small drop due to dilution, stands in sharp contrast to the strong response of the EtBr curve, where the dilution effect is completely overwhelmed by the fluorescence enhancement. However, EtBr is a only a DNA marker of moderate efficiency with a *K* of a few 10^{+5} M^{-1}. A variety of more recent DNA markers produce even stronger associations of a few 10^{+6} or 10^{+7} M^{-1} for Hoechst [18] and DAPI [32]. Other Ru complexes show similarly high K. Most of them possess a dipyridophenazine (dppz) ligand [e.g. 30, 33]. This very elongated ligand ensures deep π-stacking between the DNA base-pairs and tight DNA binding. In the case of $bpy_2Rudppz$, the luminescence enhancement upon binding to DNA even exceeds 10^{+4} [30]. Most of the latest developments on specific DNA recognition involve this dppz ligand attached to a Ru or Cr atom, and the ancilliary ligands are designed for the desired DNA sites [3].

Ru complex internalization

Beside the interactions between DNA and Ru complexes in solution, the potential internalization of such complexes into cells was also investigated. The fluorescence properties of these molecules allow two assays: flow cytometry and microscopy imaging. The flow cytometry approach has become a standard way of quantifying internalizations of various types [34] and has recently been applied to some Ru complexes [e.g. 35–37]. MDA-MB-231 cells were here treated for various incubation times and with different concentrations of Ru-complexes, calcein-AM being added for some of the cytometry experiments. Since this latter molecule is a cell viability staining, the possible incorporation of Ru-complexes into non calcein-fluorescent cells could then be discarded (fluorescence emission gating performed after data acquisition).

Very little internalization of the Ru-bpy complexes was observed either by microscopy (non-fluorescent cells, data not shown) or flow cytometry at the analytical concentrations of a few μM. However, at the concentration of 20 μM, a significantly shifted histogram was obtained. In Fig. 9, a typical flow cytometry histogram for Ru-bpy1Δ after 2 or 4 h of incubation indicates that the cells were only slightly labeled as compared to the untreated cells. The longer incubation does not intensify the luminescence but

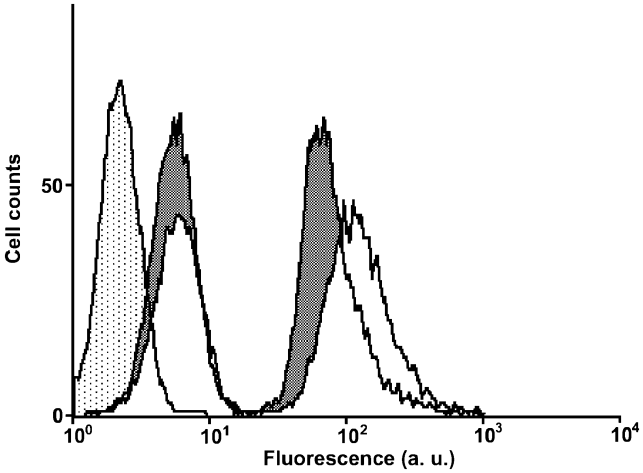


Fig. 9 Flow cytometry analysis of MDA-MB-231 cells incubated with 0.020 mM Ru complexes. From left to right, cell counts vs. fluorescence histograms obtained for: non-treated cells, Ru-bpy1Δ (*top*: incubation time: 2 h; *bottom*: 4 h), Ru-DIP1 (*top*: incubation time: 2 h; *bottom*: 4 h)

only reduces the population of marked cells, implying a slight toxic effect at these large concentrations and after lengthy incubation. On the contrary and in the same figure, a significant and massive internalization of the Ru-DIP1 complex was found. The longer incubation also reduces the population of marked cells, but the general effect is a more intense luminescence.

Ru-DIP incorporation was then studied for an incubation time of 2 h at various usual concentrations ranging from 2.5 to 10 μM. All Ru-DIP fluorescent and non-fluorescent (control) cell populations were equally labeled by calcein AM (fluorescence histogram not shown), indicating that these Ru complexes are not toxic under these conditions. Unlike some other Ru complexes [35], the Ru-DIP

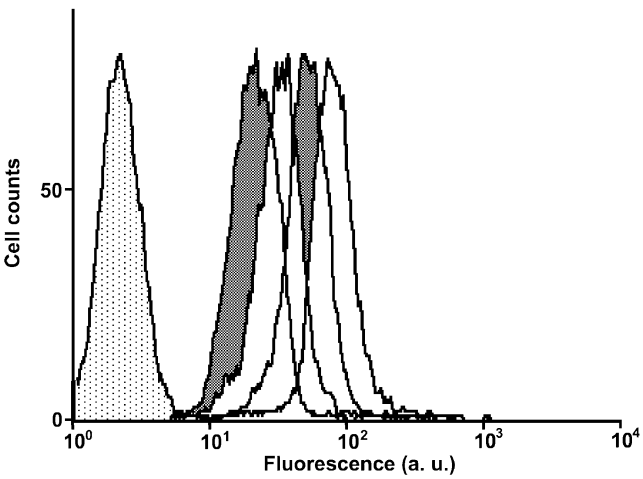


Fig. 10 Flow cytometry analysis of MDA-MB-231 cells incubated with Ru complexes. From left to right, cell counts vs. fluorescence histograms obtained for: non-treated cells, 3 μM Ru-DIP2, 3 μM Ru-DIP1, 10 μM Ru-DIP2 and 10 μM Ru-DIP1

complexes seem to accumulate mainly in living cells. Figure 10 displays a similar dose-dependence for the uptake of Ru-DIP1 and of Ru-DIP2. The median luminescences of the treated cell populations at 3 μM and 10 μM were 32 and 77 for Ru-DIP1, and 21 and 50 for Ru-DIP2, compared to the blank population at 2.15. For both complexes, the ratio of the fluorescence values is slightly smaller than the ratio of the corresponding concentrations, because of a saturation effect. In fact, the luminescence is linear only with doses below about 7 μM. Contrary to what was first suggested [5] on the basis of microscopy photographs with relatively low magnification, the present data (see again Fig. 10) show that Ru-DIP1 with the single positive net charge penetrates the cells more easily than neutral Ru-DIP2. In the region of linear concentration response, for example at 5 μM, the luminescence intensifications relative to the blank value are 15 and 25 for Ru-DIP2 and Ru-DIP1, respectively. Note that the second value, 25 for Ru-DIP1 [= $DIP_2Ru(CH_3bpyCOOH)$], is very similar to the factor of 26 obtained for $DIP_2Rudppz$ at the same concentration. This factor is much higher than for any other complex bearing the dppz ligand in conjunction with other ligands: bpy, CO_2Etbpy, mcbpy, phen, or even positively charged mcbpy [36]. Therefore, the DIP ligands facilitate complex uptake.

Microscopy fluorescence imaging performed on cells incubated with Ru-DIP1 (Fig. 11) and Ru-DIP2 showed an intense punctate fluorescence pattern in the cytoplasm but a weak labeling in the nucleus compartment. Contrary to the first impressions obtained at lower magnification [5], the Ru-DIP's do not target the nucleus. This is in line with the small attachment to DNA itself. It is also coherent with the data obtained for $DIP_2Rudppz$ [36, 37]. Though this Ru complex is an excellent DNA marker in vitro, it is also mostly staining the cytoplasm. Due to their lipophilicity, the DIP ligands facilitate the uptake of the complexes which are likely to passively pass through the cell membrane in response to the membrane potential, as discussed for $DIP_2Rudppz$. The punctate patterns seen here inside the cytoplasm are similar to those found for $DIP_2Rudppz$. The Ru complexes appear to accumulate into acidic compartments such as lysosomes or endosomes. The better penetration of Ru-DIP1 with the single positive charge, as compared to the neutral Ru-DIP2, is coherent with the passive uptake process.

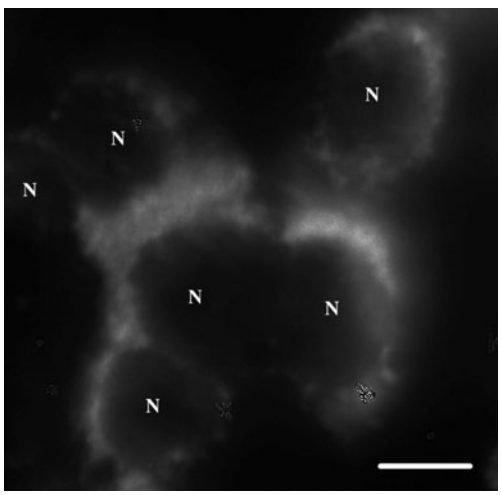


Fig. 11 Fluorescence micrograph of typical MDA-MB-231 cells treated for 2 h with 20 μM Ru-DIP1. N = nucleus. Bar=10 μm

Conclusion

In order to check the strength of their association with DNA and to probe their use as cellular DNA fluorescent markers, formerly proposed in [5], $DIP_2Ru(CH_3bpyCOOH)$ and $DIP_2Ru(COOHbpyCOOH)$ coordination complexes, together with the smaller $bpy_2Ru(CH_3bpyCOOH)$ and $bpy_2Ru(COOHbpyCOOH)$, were investigated by the appropriate systematic approaches: UV–vis absorption, fluorescence, as well as circular dichroism whenever the enantiomers were available. Under conditions typical for biological applications (pH, ionic strength, concentration, ...), and even at somewhat higher concentrations, no evidence was ever found for an association large enough for DNA marker candidates. In the absorption titrations carried out at constant complex concentration, no hypochromism in the MLCT band and no isosbestic point of the first kind was observed in the visible region. For the alternative titrations where the complex concentration was reduced over the course of the titration, a clear-cut isosbestic point of a second kind, this time in the UV region, proves that the spectra are modified only by dilution and not by association. In all cases, the spectroscopic data are merely a combination of the characteristics of the free reactants, weighted by their respective concentrations. As regards $bpy_2Ru(CH_3bpyCOOH)$, the NMR study [6] lead to K values of a few 10^{+3} M^{-1}, which cannot be confirmed or refuted here. In any case, this association would be too weak by at least two orders of magnitude for the biological application considered. For $DIP_2Ru(CH_3bpyCOOH)$ and $DIP_2Ru(COOHbpyCOOH)$, and contrary to what was assumed [5], the association is also insufficient. The bpy ligands are too small to enable appreciable stacking, and the larger DIP ligands too bulky to permit intercalation or close contacts; the deprotonated COOH unfavourably reduces the net charge of the complex. The major practical conclusion of this spectroscopic study is that the absence of fluorescence enhancement upon exposure to DNA under physiological conditions ruins the chance of using these complexes to mark cell DNA. In the Ru complexes bearing two bpy

ligands, a third elongated dppz ligand is unquestionably the key for excellent association with DNA [3, 30].

The present internalization study confirms some of the previous conclusions [5], namely that all four complexes preserve cell integrity and that only the complexes with DIP ligands naturally penetrate the cells. However, the microscopy photographs here recorded at high magnification indicate that the fluorescent complexes are principally contained inside the cytoplasm and do not significantly permeate the nucleus envelope. This is a second obstacle to using them as cell DNA dyes. This microscopy observation is coherent with recent results on $DIP_2Rudppz$ [3, 36, 37]. Thus, the dppz ligand, which is so essential for DNA association, is of no help in crossing the nucleus membranes, but the DIP ligand promotes passive diffusion. In conclusion, of the four complexes investigated here, the uptake is the highest for $DIP_2Ru(CH_3bpyCOOH)$, with the single positive net charge and the higher lipophilic character conferred by the DIP ligands. This complex is a good candidate for applications other than nuclear DNA marking, for example, to stain living cells.

Acknowledgments We are thankful to Dr. C. Cordier for having aroused our interest in these complexes. We are also very grateful to Dr. C. Cordier and Dr. H. Amouri for having provided us with the ruthenium complexes that had been synthesized by H. Amouri for their previous investigations. We thank Prof. J. Bolard for his advice on the spectroscopic approaches and Prof. J. Aubard for his encouragement. We are deeply grateful to Dr. J. Lomas for continuous support and fruitful discussions throughout this work.

References

1. Barton JK (1986) Metals and DNA: molecular left-handed complements. Science 233(4765):727–734
2. Jing B, Zhang M, Shen T (2004) Ruthenium(II)$(bpy)_2L]^{2+}$, where L are imidazo[f]-1, 10-phenanthrolines: synthesis, photophysics and binding with DNA. Spectrochim Acta A Mol Biomol Spectrosc 60(1):2635–2641
3. Zeglis BM, Pierre VC, Barton JK (2007) Metallo-intercalators and metallo-insertors. Chem Commun 44:4565–4579
4. Dwyer FP, Gyarfas EC, Rogers WP, Koch JH (1952) Biological activity of complex ions. Nature 170(4318):190–191
5. Musatkina E, Amouri H, Lamoureux M, Chepurnykh T, Cordier C (2007) Mono- and dicarboxylic polypyridyl-Ru complexes as potential cell DNA dyes and transfection agents. J Inorg Biochem 101(7):1086–1089
6. Caspar R, Musatkina L, Tatosyan A, Amouri H, Gruselle M, Guyard-Duhayon C, Duval R, Cordier C (2004) Efficient DNA binding by optically Pure Ruthenium Tris(bipyridyl) complexes incorporating carboxylic functionalities. Solution and structural analysis. Inorg Chem 43(25):7986–7993
7. Kumar CV, Barton JK, Turro NJ (1985) Photophysics of ruthenium complexes bound to double helical DNA. J Am Chem Soc 107:5518–5523
8. Belousoff NMJ, Bhatt AI, Bond AM, Deacon GB, Gasser G, Spiccia L (2007) Synthesis, structure, spectroscopic properties, and electrochemical oxidation of ruthenium(II) complexes Incorporating monocarboxylate bipyridine ligands. Inorg Chem 46 (21):8638–8651
9. Barnard PJ, Vagg RS (2005) A spectroscopic investigation of the self-association and DNA binding properties of a series of ternary ruthenium(II) complexes. J Inorg Biochem 99(5):1009–1017
10. Caspar R, Amouri H, Gruselle M, Cordier C, Malézieux B, Duval R, Lévêque H (2003) Efficient asymmetric synthesis of Δ- and Λ-enantiomers of (bipyridyl) ruthenium complexes and crystallographic analysis of Δ-bis(2, 2′-bipyridine-4, 4′-dicarboxylato) ruthenium: diastereoselective homo- and hetro ion pairing revisited. Eur J Inorg Chem 2003(3):499–505
11. Caspar R, Cordier C, Waern JB, Guyard-Duhayon C, Gruselle M, Le Floch P, Amouri H (2006) A new family of mono- and dicarboxylic ruthenium complexes $[Ru(DIP)_2(L2)]^{2+}$ (DIP = 4, 7-diphenyl-1, 10-phenanthroline): synthesis, solution behavior, and X-ray molecular structure of trans-$[Ru(DIP)_2(MeOH)_2][OTf]_2$. Inorg Chem 45(10):4071–4078
12. Srinivasan S, Annaraj J, Athappan PR (2005) Spectral and redox studies on mixed ligand complexes of cobalt(III) phenanthroline/bipyridyl and benzoylhydrazones, their DNA binding and antimicrobial activity. J Inorg Biochem 99(3):876–882
13. Long EC, Barton JK (1990) On demonstrating DNA intercalation. Acc Chem Res 23(9):271–273
14. Suh D, Chaires JB (1995) Criteria for the mode of binding of DNA binding agents. Bioorg Med Chem 3(6):723–728
15. Jenkins TC (1997) In: Fox KR (ed) Methods in molecular biology, vol. 90: drug-DNA interaction protocols. Humana, Totowa, pp 195–218
16. Murphy-Poulton SF, Vagg RS, Vickery KA, Williams PA (1998) DNA binding of some chiral metallointercalators derived from 9, 10-phenanthrenediamine. Met Based Drugs 5(4):225–231
17. Xi PX, Xu ZH, Liu XH, Chen FJ, Zeng ZZ, Zhang XW, Liu Y (2009) Synthesis, characterization, antioxidant activity and DNA-binding studies of three rare earth (III) complexes with 1-(4-aminoantipyrine)-3-tosylurea ligand. J Fluoresc 19:63–72
18. Guan Y, Shi R, Li X, Zhao M, Li Y (2007) Multiple binding modes for dicationic hoechst 33258 to DNA. J Phys Chem B 111 (25):7336–7344
19. McGhee JD, von Hippel PH (1974) Theoretical aspects of DNA-protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice. J Mol Biol 86(2):469–489
20. van Holde KE, Johnson WC, Ho PS (2006) In: Carlson G (ed) Principles of physical biochemistry. Pearson, Upper Saddle River, pp 615–654
21. Fielding L (2000) Determination of association constants (K_a) from solution NMR data. Tetrahedron 56(34):6151–6170
22. Graves DE (2001) In: Osheroff N, Bjornsti MA (eds) Methods in molecular biology, vol. 95: DNA topoisomerase protocols, part II: enzymology and drugs. Humana, Totowa, pp 161–169
23. Chen LM, Liu J, Chen JC, Tan CP, Shi S, Zheng KC, Ji LN (2008) Synthesis, characterization, DNA-binding and spectral properties of complexes $[Ru(L)_4(dppz)]^{2+}$ (L = Im and MeIm). J Inorg Biochem 102(2):330–341
24. Morgan JL, Buck DP, Turley AG, Collins JG, Keene FR (2006) Selectivity at a three-base bulge site in the DNA binding of ΔΔ-$[\{Ru(phen)_2\}_2(\mu\text{-dppm})]^{4+}$ [dppm is 4, 6-bis(2-pyridyl)pyrimidine; phen is 1, 10-phenanthroline]. J Biol Inorg Chem 11(7):824–834
25. Dupureur C, Barton JK (1997) Structural studies of Λ- and Δ-$[Ru(phen)_2d\ ppz]^{2+}$ bound to $d(GTCGAC)_2$: characterization of enantioselective intercalation. Inorg Chem 36(1):33–43
26. Bhattacharya PK, Lawson HJ, Barton JK (2003) ^1H NMR studies of nickel(II) complexes bound to oligonucleotides: a novel

technique for distinguishing the binding locations of metal complexes in DNA. Inorg Chem 42(26):8811–8817
27. Myari A, Hadjiliadis N, Garoufis A (2005) Synthesis and characterization of the diastereomers Λ- and Δ-[Ru(bpy)$_2$(m-bpy-L-Arg-Gly-L-Asn-L-Ala-L-His-L-Glu-L-Arg)]Cl$_2$ ^{1}H NMR studies on their interactions with the deoxynucleotide duplex d[(5′-GCGCTTAAGCGC-3′)$_2$] and d[(5′-CGCGATCGCG-3′)$_2$]. J Inorg Biochem 99(2):616–626
28. Gaudry E, Aubard J, Amouri H, Levi G, Cordier C (2006) SERRS study of the DNA binding by Ru(II) tris-(Bipyridyl) complexes bearing one carboxylic group. Biopolymers 82 (4):399–404
29. Caspar R, Amouri H, Gruselle M, Cordier C, Malézieux B, Duval R, Lévêque H (2003) CORRECTION Efficient asymmetric synthesis of D- and L-enantiomers of (bipyridyl) ruthenium complexes and crystallographic analysis of D-bis(2,2'-bipyridine-4,4' –dicarboxylato) ruthenium: diastereoselective homo- and hetro ion pairing revisited. Eur J Inorg Chem 2003(16):3055
30. Friedman AE, Kumar CV, Turro NJ, Barton JK (1991) Luminescence of ruthenium(II) polypyridyls: evidence for intercalative binding to Z-DNA. Nucleic Acids Res 19(10):2595–2602
31. Barton JK, Basile LA, Danishefsky A, Alexandrescu A (1984) Chiral probes for the handedness of DNA helices: enantiomers of tris(4, 7-diphenylphenanthroline)ruthenium(II). Proc Natl Acad Sci USA 81(7):1961–1965
32. Kapuscinski J (1995) DAPI: a DNA-specific fluorescent probe. Biotech Histochem 70(5):220–233
33. Nair RB, Teng ES, Kirkland SL, Murphy CJ (1998) Synthesis and DNA-binding properties of [Ru(NH$_3$)$_4$dppz]$^{2+}$. Inorg Chem 37 (1):139–141
34. Shapiro HM (2003) Practical flow cytometry. Wiley, Hokoben
35. Jiménez-Hernandez ME, Orellana G, Montero F, Portolès MT (2000) A ruthenium probe for cell viability measurement using flow cytometry, confocal microscopy and time-resolved luminescence. Photochem Photobiol 72(1):28–34
36. Puckett CA, Barton JK (2007) Methods to explore cellular uptake of ruthenium complexes. J Am Chem Soc 129(1):46–47
37. Puckett CA, Barton JK (2008) Mechanism of cellular uptake of a ruthenium polypyridyl complex. Biochemistry 47(45):11711–11716