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Solid-phase synthesis, characterization and DNA binding properties of the first chloro(polypyridyl)ruthenium conjugated peptide complex

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A general method for the synthesis of chloro(polypyridyl)ruthenium conjugated peptide complexes *via* a solid-phase strategy is described. The method is applied to synthesize two positional isomers of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF_6). Even though the separation of the isomers was only partially achieved chromatographically, the isomers were unambiguously assigned by NMR spectroscopy. The interactions of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF_6) with CT-DNA and plasmid DNA, have been studied with various spectroscopic techniques, showing that (i) the complexes coordinatively bind to DNA preferring the bases guanine and cytosine over the bases thymine and adenine after hydrolysis of the coordinated chloride, (ii) electrostatic interactions between the complex cation and the polyanionic DNA chain assist this binding (iii) only in the case of one isomer the peptide does interact further with DNA as evidenced from ^{31}P NMR spectroscopy, (iv) DNA unwinding occurs in all cases with high binding ratio (Ru/base) values ($r > 0.3$).

1 Introduction

Transition-metal complexes conjugated with short peptides synthesized by various solid-state strategies[1–11] have recently been introduced for their DNA-binding properties.[2–5,7,8] One of the pioneering reports is a paper by Peek *et al.*[1], where some resin-bound amino acids and peptides have been tethered to the complex *rac*-[Ru(bpy)$_2$(4-CO_2H-4′-Mebpy)]$^{2+}$ (4-CO_2H-4′-Mebpy = 4′-methyl-2,2′-bipyridine-4-carboxylic acid), after activation of the carboxyl group of the 4-CO_2H-4′-Mebpy ligand. The same strategy has also been used in the cases of the Ru(II) complexes *rac*-[Ru(phen)(bpy′)(dppz)] (bpy′ = 4′-methyl-2,2′-bipyridine-4-butyric acid and dppz = dipyrido[3, 2-a: 2′,3′-c] phenazine)[2,4] and for the conjugation of Λ- and Δ-[Ru(bpy)$_2$(4-CO_2H-4′-Mebpy)]$^{2+}$[8] with various resin-bound peptides. The complex *rac*-[Ru(phen)(bpy′)(dppz)] was found to cross-link to DNA. The nature of the peptide affects both the DNA binding affinity and the cross-linking efficiency of the ruthenium-peptide conjugates.[2] Metallointercalator–peptide conjugates of Rh(III) complexes, such as [Rh(phi)$_2$(agphen)]$^{3+}$[5–7] (phi = phenanthrenoquinone diimine and agphen = 5-(amidoglutaryl)-1,10-phenanthroline) were prepared by a different solid-state approach. Thus, the resin-*N*-terminus deprotected peptide reacted with the activated agphen resulting in the resin-peptide-agphen, which, in turn, coordinates to the complex [Rh(phi)$_2$(dmf)$_2$]$^{3+}$. The final product was obtained by deprotection of *t*-Boc groups and cleavage of the complex from the resin. The complex [Rh(phi)$_2$(agphen)]$^{3+}$ binds to DNA by intercalation of one of the phi ligands in the major groove. Applying photoactivation, specific cleavage of DNA occurs due to the site recognition of the peptide.[5–7] The DNA site-specificity is seen to depend on the peptide side-chain functional groups. The same coordination strategy was also followed in the case of [Rh(phi)$_2$(bpy′)]$^{3+}$[3] where a 22-mer peptide was conjugated at the rhodium complex.[3] These metallointercalator–peptide conjugates cleave DNA under photoactivation conditions and the peptide conformation and its nature appears to affect the DNA recognition.[3] Hastings[7] and Sardesai[5] studying the complex [Rh(phi)$_2$(agphen)]$^{3+}$ have found the absolute requirement of the glutamate for the DNA recognition. The results have shown that the glutamate is required indirectly in folding the metal–peptide complex into a unique conformation and participates directly in interaction with the DNA bases.[5,7]

A chimeric metallopeptide that contains a 54-residue polypeptide conjugated on the [Ru(bpy)$_2$(phenIA)](PF_6)$_2$ (phenIA = *N*-iodoacetyl-5-amino-1,10-phenanthroline) was synthesized and characterized.[12] The chimeric compound does not produce photoinduced DNA damage probably due to the large distance between the ruthenium centre and the DNA bases.[12] A number of chimeric ruthenium compounds with polypeptides was synthesized and studied regarding their stereochemistry.[13–15] Photoinduced electron transfer between the metallopeptide [Ru(bpy)$_2$(phenam)-Cys-(Glu)$_5$-Gly]$^{3-}$ and ferricytochrome *c* was observed.[16,17]

It should be noted that in all the above cases all six coordination sites around the metal are occupied by nitrogen donor atoms, excluding any possibility for coordination interactions between the metal and the DNA. A series of peptides tethered to an ethylene diamine molecule, which acts as a chelating ligand to Pt(II)[9,10] have been synthesized and studied for their cytotoxic properties. Dinuclear platinum complexes have also been synthesized with the same strategy by coupling a bridged lysine or lysine peptide moiety with *trans*-(NH_3)$_2$$PtCl_2$.[11] The crucial difference between the rhodium or ruthenium complexes[1–8] to those of platinum[9–11] is that the peptide moiety in the latter remains uncoordinated to the metal, despite that platinum contains one,[11] or two,[9,10] potential coordination sites (initially containing chloride). This property makes the complexes biologically interesting to coordinate further with biomolecules, such as proteins or nucleic acids. In fact, the remarkable anticancer activity of such Pt(II) complexes depends on the nature of the conjugated peptide.[10] Since the chloro(polypyridyl)ruthenium complexes have shown notable cytotoxicity,[18] we report herein the synthesis of the chloro-complex of the tethered peptide [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF_6) **1** by a solid-state

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coordination strategy, with one remaining coordination site available. This is the first example of a chloro(polypyridyl)-ruthenium complex synthesized *via* the solid state, leading to high yields (77%) and pure products and as it appears, this method is generally applicable. The chloro(polypyridyl)-ruthenium–peptide conjugates presented here can lead to a new class of anticancer agents as these complexes can easily hydrolyse and coordinate to DNA bases. Moreover, these ruthenium–peptide complexes can combine the possible peptide recognition for DNA site specificity with the cytotoxicity properties that the chloro(polypyridyl)ruthenium complexes have shown.[18]

2 Experimental

The infrared spectra of the complexes in the 4000–300 cm^{-1} range were recorded on a Perkin-Elmer Paragon 1000 FTIR spectrophotometer equipped with a Golden Gate Diamond ATR device, using the diffuse reflectance technique. C, H and N determinations were performed on a Perkin-Elmer 2400 Series II analyzer. UV-visible spectra were recorded on a Varian Cary 3-Bio with temperature controller. CD spectra were recorded on a Jobin Yvon CD-6 instrument at room temperature. For the electrophoretic mobility assay the samples were analyzed by gel electrophoresis on 0.8% (w/v) agarose gel at 10 V cm^{-1}. The bands were photographed with a digital camera and printed with a Sony video printer. Plasmid pUC9, 2665bp, was isolated and purified twice with CsCl gradient centrifugation. The plasmid isolation contained over 95% of supercoiled DNA with a faint relaxed DNA band. The restriction enzymes Dra*I* (three recognition sites of TTTAAA at position 1073, 1765 and 1784 and Sma*I* (recognition site GGGCCC at position 260) (Sigma Chemical Corporation) were used in this work for the digestion of the supercoiled DNA.

^{1}H NMR, ^{23}Na NMR and ^{31}P NMR measurements were performed on a Bruker 300 DPX spectrometer operating at 300.13 MHz, at 121.49 MHz and at 79.39 MHz for ^{1}H, ^{31}P and ^{23}Na NMR, respectively. 1-D and 2-D spectra were recorded in MeOD-d_4 and D_2O with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard. Temperature was kept constant at 298 K. MALDI-TOF-MS was performed on a Vision 2000 instrument (Finnigan MAT). The instrument operated with a nitrogen laser at 337 nm, while 2,5-dihydroxybenzoic acid (DHB) was used as matrix (Aldrich Chemie). 2,2′:6′,2″-Terpyridine was purchased from Aldrich Chemical Company and used without further purification. Calf thymus DNA, agarose, ethidium bromide and PIPES (piperazine acid *N*,*N*′-bis(2-ethanesolfonic)) were purchased from Sigma Chemical Company. The DNA concentration, expressed as moles of nucleotides per liter, [P], was determined on a Pharmacia LKB-Biochrom 4060 UV-visible spectrophotometer from the absorbance at 260 nm ($\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$, $T = 298$ K). The sonication of the calf thymus DNA was performed according to the literature.[19] The length of the fragments after the sonication, that used for the NMR and CD experiments, was checked with size exclusion chromatography which found a dispersion of DNA lengths from 70bp to 300bp. The complex Ru(terpy)Cl_3[20] and the ligand 4-carboxy-4′-methyl-2,2′-bipyridine (4-CO_2H-4′-Mebpy)[1] were prepared according to literature procedures.

Synthesis of the peptide GHK (3)

The elongation of the Fmoc-protected Rink Amide resin **2** with the commercially available protected amino acids Fmoc-Lys-(Boc)-OH, Fmoc-His(Trt)-OH and Fmoc-Gly-OH was performed with a standard Fmoc protocol.[21] The couplings were achieved using 1*H*-benzotriazolium 1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate(1−), 3-oxide (HCTU, 1 equiv.) and diisopropylethylamine (DIPEA, 2 equiv.) as coupling agents. The deprotection of the Fmoc group was performed using a 20% solution of piperidine in *N*-methyl-pyrrolidone (NMP). The purity and the mass of the peptide, after cleavage from the resin with TFA/H_2O (95/5, v/v), was checked with ^{1}H NMR and LC-MS spectrometry. ESI-MS: *m*/*z*: 340.2 $[M]^+$. Yield: (∼40%).

Synthesis of the 4-CO_2H-4′-Mebpy-GHK (4)

500 mg (0.11 mmol, resin substitution 0.22 mmol/g) of resin bound peptide **3** was treated with 20% piperidine solution in NMP to deprotect the Fmoc group of the amino acid glycine. The conjugation of the ligand 4-CO_2H-4′-Mebpy (0.5 mmol) to the resin-bound tripeptide was achieved with the coupling agents benzotriazol-1-yl-oxytris(pyrrolidino)phosphanium hexafluorophosphate (PyBOP, 0.75 mmol) and diisopropylethylamine (DIPEA, 1 mmol). The purity of the ligand 4-CO_2H-4′-Mebpy-GHK, after the cleavage from the resin with TFA/H_2O (95/5, v/v), was checked with ^{1}H NMR and LC-MS spectrometry. ESI-MS: *m*/*z*: 536.3 $[M]^+$.

Synthesis of [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF$_6$). (1)

At this stage, the resin bound 4-CO_2H-4′-Mebpy-GHK **4** (0.11 mmol) was refluxed with an excess of Ru(terpy)Cl_3 (3 equiv., 0.33 mmol), LiCl (0.36 mmol, ∼15 mg) and Et_3N (0.25 ml), in DMF/EtOH (3 : 1) for 7 h. The resin with the immobilized complex **5** was carefully washed with DMF (5 × 5 min) afterwards, to remove the excess of Ru(terpy)Cl_3 and possible side products and then with CH_2Cl_2 (3 × 5 min) for drying the resin. The last step includes the cleavage of the complex and the protecting groups using TFA/H_2O (95/5, v/v). The crude complex was obtained by precipitation in diethyl ether. The product was dissolved in 1 mL of methanol and added to a saturated aqueous solution of NH_4PF_6. Methanol was removed by evaporation and the complex **1** was precipitated, as evidenced from MALDI–TOF-MS. Yield: (77%). [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF$_6$). Anal. Calcd. for C, 46.88; H, 4.22; N, 16.00%, Found: C, 47.23; H, 4.67; N, 15.32. ESI-MS: *m*/*z* 905, [Ru(terpy)(4-CO_2H-4′-Mebpy-GHK)Cl]$^+$.

Preparation of ruthenium adducts with DNA for CD spectroscopy

The required volume of a freshly prepared solution of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)-Cl](PF$_6$) dissolved in 1 mM PIPES and 20 mM aqueous sodium chloride was added to calf thymus DNA solutions (100 μM), incubating at 25 °C for 24 h. Samples were prepared in such a way as to have final ruthenium/DNA base pair ratios (*r*) of 0.1, 0.3, 0.5. CD spectra were recorded at room temperature.

Preparation of ruthenium adducts with DNA for electrophoretic mobility assays

Adducts with pUC9 plasmid DNA were prepared by adding the required volume of a freshly prepared solution of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)-Cl](PF$_6$) using 10 mM PIPES and 4 mM aqueous solution of sodium chloride. The concentration of pUC9 DNA in the reaction mixture was 38 ng ml^{-1}, while the concentration of the complex was varied to give different metal-to-base pair stoichiometries (0.1, 0.3, 0.5). The supercoiled plasmid DNA was incubated for 2 h at RT and each sample was purified using GFX DNA and Gel Band Purification Kit (Amersham 27-9602-01) to remove excess metal complex not bound to DNA.

The mobility of the complex-treated pUC9 samples was analyzed by agarose gel electrophoresis at RT for 2 h in Tris-acetate/EDTA buffer, and then the gel was stained for 1 h in 0.5 mg ml^{-1} (w/v) ethidium bromide.

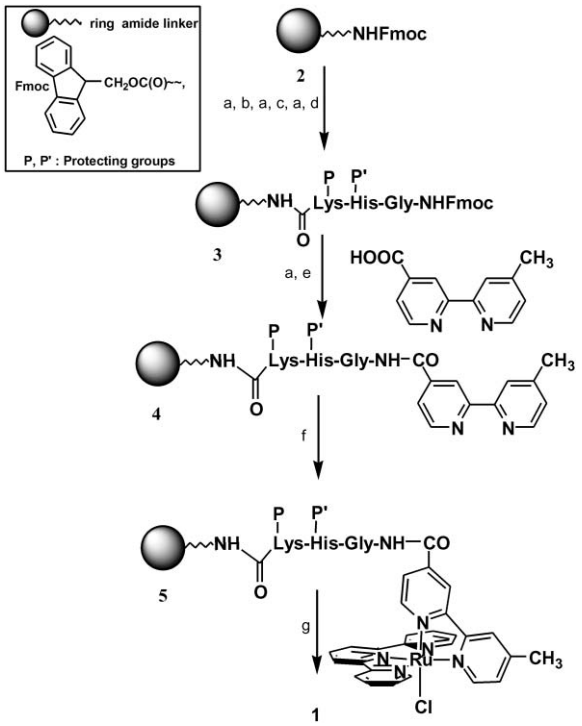
Digestion of plasmid DNA by restriction enzymes

Enzymic digestions were carried out by incubating the untreated and pUC9 treated samples of [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) (at $r = 0.1, 0.3$) with Dra*I* and Sma*I*, respectively. 200 ng of each sample were incubated with the restriction enzymes at 37 °C for 1 h in the appropriate buffer recommended by the manufacturer. Then DNA restriction fragments were loaded on 0.8% (w/v) agarose gel and after running the gel at 10 V cm^{-1} the gel was stained for 1 h in 0.5 mg ml^{-1} (w/v) ethidium bromide. The bands were photographed and analyzed as above.

3 Results and discussion

3.1 Synthesis and characterization of the complexes

The coordination strategy is presented in Scheme 1.



Scheme 1 (a) Piperidine (b) Fmoc-Lysine(Boc)-OH, HCTU, DIPEA, NMP (c) Fmoc-Histidine(Trt)OH, HCTU, DIPEA, NMP (d) Fmoc-Glycine-OH, HCTU, DIPEA, NMP (e) pyBOP, DIPEA, NMP (f) Ru(terpy)Cl_3, LiCl, Et_3N, DMF/EtOH (3 : 1), reflux 7 h, (g) TFA/H_2O (95/5, v/v), 2 h, HCTU = 1*H*-Benzotriazolium 1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate(1−), 3-oxide, DIPEA = diisopropylethylamine, NMP = *N*-methylpyrrolidone, PyBOP = benzotriazol-1-yloxytris(pyrrolidino)phosphanium hexafluorophosphate.

The resin bound tripeptide Gly-His-Lys **3** was synthesized by the standard Fmoc protocol.[21] Then, the glycine-deprotected amino group was coupled with the activated carboxyl group of the 4-CO_2H-4′-Mebpy to afford derivative **4**. Subsequently the resin was treated with 3 equiv. (based on initial resin loading) of Ru(terpy)Cl_3 in a mixture of DMF/EtOH 3 : 1 at 80 C for 7 h. The brown solution became slightly decolorized over 7 h, consistent with the coordination of the ligand. After successive washing of the resin bound complex with DMF and CH_2Cl_2, complex **1** was cleaved from the resin and the protecting groups removed with TFA/H_2O 95/5. The mass spectra (ESI and MALDI TOF) of **1** were found consistent with the identified formula (Fig. 1).

The ^{1}H NMR spectrum of the crude complex shows a double set of resonance signals for all complex protons. This behaviour can be explained with the formation of two positional isomers differing in the orientation of the peptide towards coordination to ruthenium[22] (Fig. 2). The separation of the isomers was partially achieved chromatographically in a Sephadex LH-20 column based on their solubility differences in acetonitrile. After successive applications of the above procedure (4 times) the isomers were sufficiently separated, as observed from their ^{1}H NMR spectra where the resonances of the major isomer predominate in each case. Assignments of proton signals were assisted by ^{1}H^{1}H-COSY and ^{1}H^{1}H-TOCSY experiments. The ^{1}H NMR spectra of both isomers (Table 1) show distinct proton signals for each of the two pyridine rings of the ligand 4-CO_2H-4′-Mebpy-GHK. Double resonances were also observed in the spectrum of the free ligand arising from the different substitution (4-COGHK and 4′-Me) of the two pyridine rings. The lowest field doublets at δ 8.96 and 8.67 ppm are assigned to the H6 and H6′ protons of the ligand correspondingly on the basis that carbonyl groups typically induce lower field shifts than alkyl substitutes. Also, a crosspeak correlating the methyl protons signal of the 4′-methyl substituted pyridine ring and the H6′ was observed in the TOCSY (mixing time 80 ms) spectrum of the ligand. Since the terpyridine coordinates to the ruthenium octahedron in a meridional fashion, the bidentate ligand 4-CO_2H-4′-Mebpy-GHK occupies one axial and the remaining meridional site. In the resulting complex there are two possible isomers with respect to the location of the two pyridyls of the ligand 4-CO_2H-4′-Mebpy-GHK. In isomer **I** the 4′-Me substituted ring coordinates to the meridional plane of the Ru(II) octahedron while in the case of isomer **II** it coordinates the pyridine ring with the substitution of 4-COGHK (Fig. 2). In the ^{1}H NMR spectrum of the isomer **I** the lowest field doublet at $\delta = 9.72$ ppm assigned to H6 of the 4-COGHK substituted pyridine ring, since no correlation was observed with the 4′-methyl protons signal in the TOCSY (mixing time 80 ms) spectrum. In contrast, in the case of the isomer **II** the lowest field doublet at δ = 9.34 ppm correlates with the methyl protons signal, indicating that this proton belongs to the 4′-methyl substituted pyridine ring system. In general such high downfield shifts for most pyridyl protons such as H6 have been reported in many cases of ruthenium complexes containing a coordinated chloride.[22,23] When all the coordination sites of the Ru(II) are occupied by nitrogen donor atoms, such as in the case of the complexes Λ- and Δ-[Ru(bpy)$_2$(4-CO_2H-4′-Mebpy-GHK)]$^{2+}$, the lowest field doublets for the ligand 4-CO_2H-4′-Mebpy-GHK were observed at δ 8.76 and 8.39 ppm.[8] The orientation of the ligand 4-CO_2H-4′-Mebpy-GHK also strongly affects the shift of its methyl group, as this is shifted downfield by $\Delta\delta$ −0.27 ppm in isomer **II** and upfield by $\Delta\delta$ +0.73 ppm in isomer **I**. Relatively small differences between the chemical shifts of the peptide protons of the two isomers were observed, probably due to their large separation from the ruthenium center. The highest value is observed for the α-Gly protons ($\Delta\delta$ 0.18 ppm), which are nearer to the poly-pyridine ring system. It is worth mentioning that in both isomers the peptide proton resonances were hardly shifted compared with the free ligand 4-CO_2H-4′-Mebpy-GHK, except for the H2 of the imidazole. The latter shifts upfield by about 0.6 ppm in both isomers in comparison with the free ligand. This behaviour could be explained by considering that the pH of the solutions is close to the pK_a value of imidazole (N1–H pK_a = 6.04). Similar shifts have been reported in the case of the diastereomeric complexes Λ- and Δ-[Ru(bpy)$_2$(4-CO_2H-4′-Mebpy-GHK)]$^{2+}$ where all the coordination sites of Ru octahedron are occupied and coordination to the peptide is impossible[8] Finally, the ligand terpy shows similar shifts for both isomers indicating that the influence of the asymmetric nature of the ligand 4-CO_2H-4′-Mebpy-GHK does not affect the meridionally coordinated terpyridine.

The complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) (**1**) and particularly its chloride salt are very soluble in water. The conversion to the chloride was achieved by dissolving 0.1 mmol of the [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) in saturated LiCl

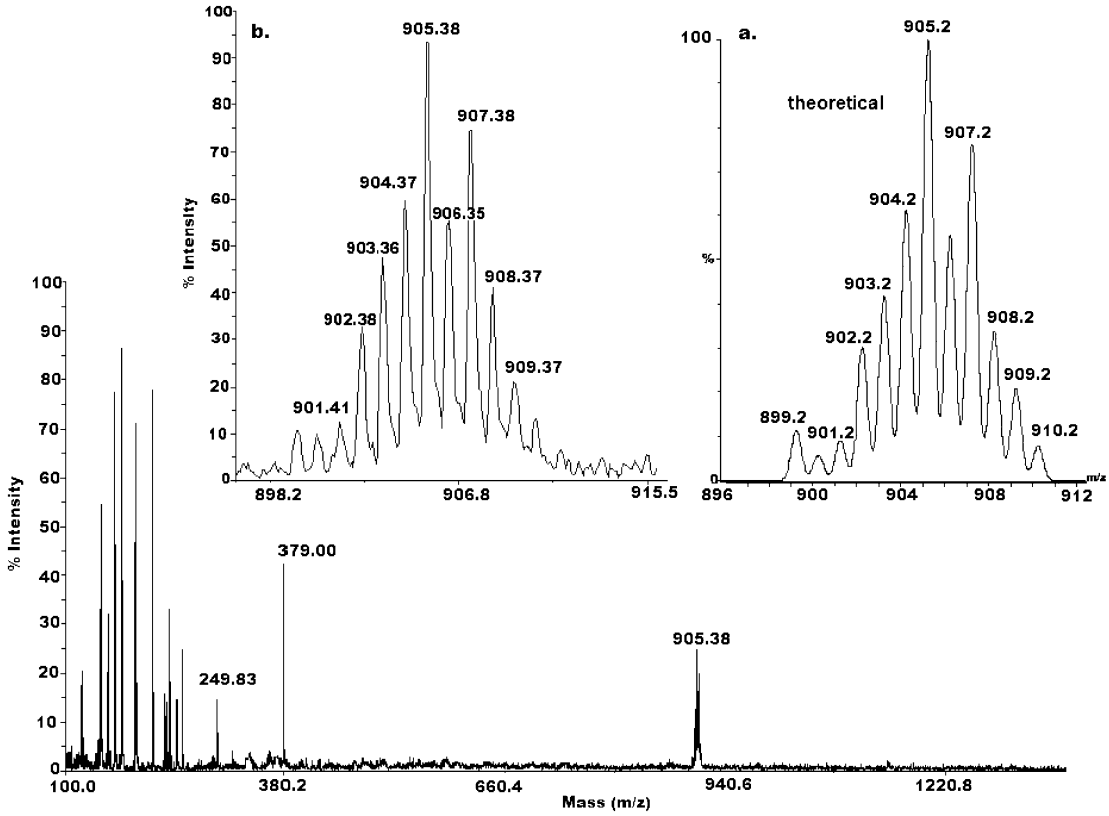


Fig. 1 MALDI-TOF MS of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl]. Inset, (a) the calculated spectrum and (b) the experimental.

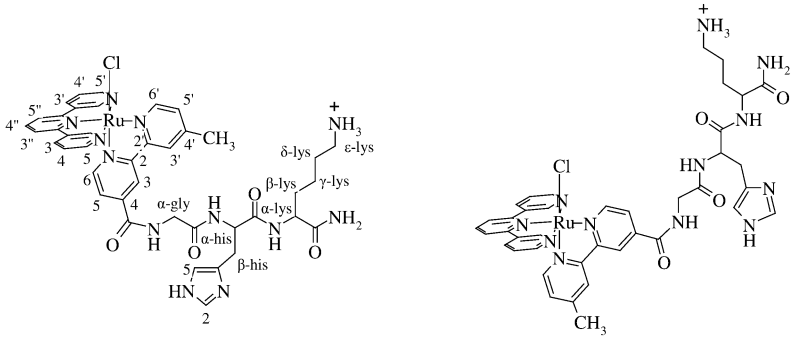


Fig. 2 Structures of the positional isomers of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl]$^+$ with atom numbering.

acetonic solution. The [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl]Cl precipitated immediately.

The complete substitution of the coordinated chloride with a molecule of water occurs after 12 h, producing the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)-(H_2O)](PF_6) as confirmed from the ESI-MS spectra after dissolving complex **1** in water. The hydrolysis reaction was monitored by ^{1}H NMR spectroscopy. In the spectrum of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)-Cl](PF_6) (**1**) in D_2O, new peaks started to appear a few minutes after dissolution indicating that the hydrolysis begins. The easy hydrolysis of the complex is an important factor for its reactivity with biological molecules. For example, covalent binding to the DNA bases requires the hydrolysis of the coordinated chloride.

3.2 DNA binding studies

3.2.1 Circular dichroism. The interaction of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)-Cl](PF_6) with CT-DNA was studied with circular dichroism, since this is a sensitive spectroscopic technique that gives information on the conformational changes and destabilization of the DNA helix. The CD spectra of calf thymus DNA after addition of complex (**1**) at ratio's $r = 0.1$, $r = 0.3$ and $r = 0.5$ (Ru/base) are shown in Fig. 3. At a ratio $r = 0.1$ the compound produces a strong alteration of the characteristic CD bands of the B-type DNA at 278 and 245 nm. The changes in the positive band at 278 nm (UV: λ_{max} 260 nm) can be explained by the alteration at the base stacking and at the negative band at 245 nm due to the changes of the helicity of B-DNA. As expected the coordination of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF_6) (**1**) to DNA has a strong effect in the DNA conformation. Upon increasing the ratio to $r = 0.3$ and $r = 0.5$ these changes become stronger, indicating that the complex can continue to interact with the DNA bases. Moreover, a new band at 325 nm appeared in place of the higher energy band at 295 nm This red shift of about 30 nm could reflect the interaction of the conjugated peptide with the DNA.

Further increasing of the ratio ($r = 0.1$ to $r = 0.5$) resulted in significant intensity changes observed in both positive and

Table 1 ^{1}H NMR (300 MHz) chemical shifts (δ with respect to TMS) for the isomeric complexes and its components at 298 K

	GHKCONH$_2$	Bpy-GHK	A Isomer I	B Isomer II
4-CO$_2$H-4′-Mebpy				
H$_6$		8.96 (d)	9.72 (d)	8.58 (d)
H$_5$		7.95 (d)	8.29 (d)	8.24 (d)
H$_3$		8.54 (s)	8.96 (s)	8.62 (s)
H$_{6'}$		8.67 (d)	8.58 (d)	9.34 (d)
H$_{5'}$		7.91 (d)	8.26 (d)	7.94 (d)
H$_{3'}$		8.48 (s)	8.62 (s)	8.62 (s)
4-Me		2.73 (s)	3.00 (s)	2.02 (s)
Terpy				
H$_3$			8.44 (d)	8.44 (d)
H$_{3'}$			8.44 (d)	8.44 (d)
H$_4$			7.98 (t)	7.98 (t)
H$_{4'}$			7.98 (t)	7.98 (t)
H$_{5,5'}$			7.32 (t)	7.32 (t)
H$_{6,6'}$			7.76 (d)	7.76 (d)
H$_{3''}$			8.60 (d)	8.60 (d)
H$_{4''}$			8.19 (m)	8.19 (m)
H$_{5''}$			8.60 (d)	8.60 (d)
GHK				
His-H$_2$	8.58 (s)	8.62 (s)	8.03 (s)	7.99 (s)
His-H$_5$	7.29 (s)	7.31 (s)	7.18 (s)	7.16 (s)
α-His	4.62 (d)	4.64 (d)	4.73 (d)	4.69 (d)
β-His	3.21 (t)	3.23 (t)	3.26 (t)	3.17 (d)
α-Gly	3.83 (s)	3.85 (s)	4.03 (s)	3.86 (s)
α-Lys	4.28 (d)	4.31 (d)	4.29 (d)	4.26 (d)
β-Lys	1.75 (m)	1.79 (m)	1.82 (m)	1.79 (m)
γ-Lys	1.42 (m)	1.43 (m)	1.49 (m)	1.43 (m)
δ-Lys	1.66 (m)	1.69 (m)	1.74 (m)	1.67 (m)
ε-Lys	2.98 (t)	2.96 (t)	2.99 (t)	2.98 (m)

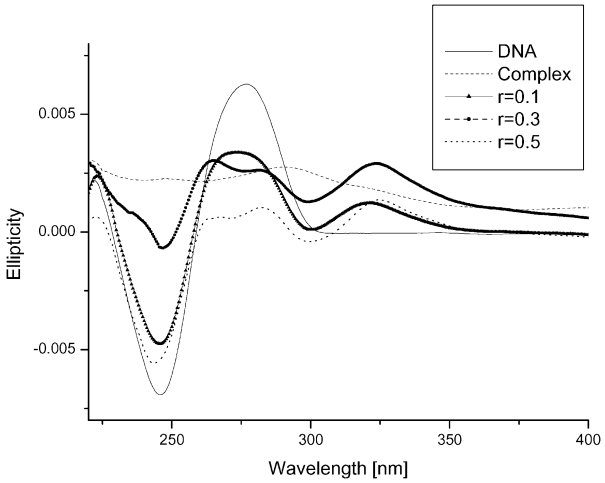


Fig. 3 Circular Dichroism spectra of CT DNA following the addition of the isomeric mixture of the complex [Ru(terpy)(4-CO$_2$H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF$_6$).

negative CD features which are similar to those previously observed for monofunctional platinum complexes.[24] In the reaction between octahedral ruthenium *cis*-dichloro-complexes and DNA it has been reported that similar changes occur in the CD spectrum of DNA.[24] Even though these complexes have two potentially reactive sites, their bifunctional binding appears to be sterically forbidden.[25] The covalent binding to DNA of the complexes NAMI and RAP caused similar alterations of the characteristic CD bands of B-type DNA.[26] In that case, the unaffected band at 245 nm indicates that the helicity remains in the B-type. The decrease of the intensity of the CD bands with increasing r values, suggests the unwinding of the DNA helix and the loss of its helicity.[27,28]

3.2.2 ^{23}Na and ^{31}P NMR spectroscopy. ^{31}P NMR spectroscopy is sensitive to conformational changes[29] and interactions of metal complexes with DNA[30] because of the potential sensitivity of ^{31}P chemical shifts to phosphate bonds.

In the present work, ^{23}Na and ^{31}P NMR spectra of sonicated DNA, dissolved in PIPES buffer, were recorded at 37 °C, while keeping the ionic strength constant, as ionic strength may have an effect on chemical shifts. The ratio between the DNA and the complex (**1**) was increased from 0.1 to 0.3 and 0.5. ^{23}Na NMR measurements have been performed for the study of the local ion exchange Na$^+$ and (**1**) in the vicinity of B-DNA. The electrostatic interaction between complex (**1**) and DNA was verified by increasing the ratio from $r = 0.1$ to $r = 0.5$ (Fig. 4a) the linewidth narrowing changed from $\Delta\nu_{1/2} = 23$ Hz at initial DNA sample ($r = 0$) to $\Delta\nu_{1/2} = 13$ Hz at $r = 0.1$ and $\Delta\nu_{1/2} = 10$ Hz at $r = 0.5$. The positively charged complex interacting covalently

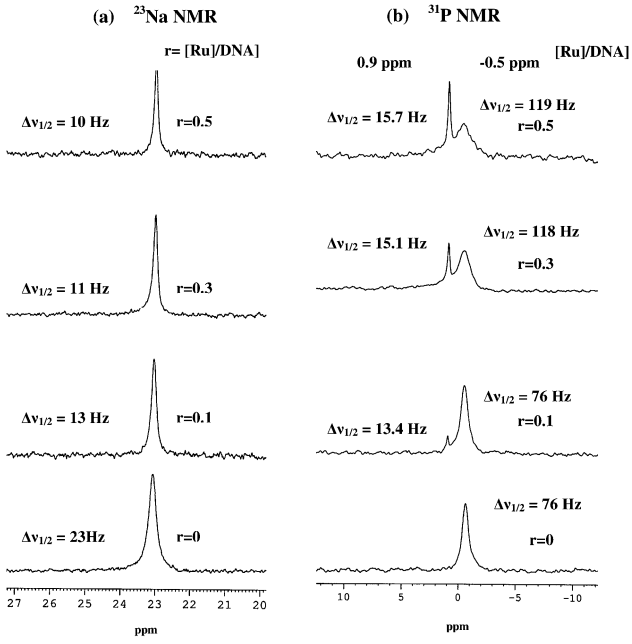


Fig. 4 ^{23}Na and ^{31}P NMR spectra of the sonicated DNA following the addition of the of the isomeric mixture of the complex [Ru(terpy)(4-CO$_2$H-4′-Mebpy-Gly-L-His-L-LysCONH$_2$)Cl](PF$_6$) in various ratios.

caused irreversible modification in the total charge of the DNA polyanion, apparently replacing the Na^+ ions from interacting with the phosphates. This binding causes a narrowing in the sodium peak, while the ratio increased. Similar observations were found for platinum compounds such as *cis*-DDP and *trans*-DDP.[31]

In the ^{31}P NMR spectra a new peak appears at δ 0.9 ppm ($\Delta\delta = -1.4$ ppm), which increases when the ratio Ru/base increases, corresponding to an alternating DNA structure (Fig. 4b). Simultaneously the initial peak was slightly shifted upfield (0.1 ppm) with a significant broadening in the ^{31}P linewidth (from $\Delta\nu_{1/2} = 76$ Hz $r = 0$ to $\Delta\nu_{1/2} = 119$ Hz $r = 0.5$). The Ru complex not containing the peptide on the other hand, [Ru(terpy)(4-CO_2H-4′-Mebpy)Cl](PF_6)[22] produces a significant broadening in the ^{31}P NMR DNA signal upon increasing the ratio (Fig. 5). Therefore, the appearance of two signals upon increasing the ratio [1]/[DNA] containing the peptide is obviously due to its presence. The broadening of the peak could be due to an increased dispersion of ^{31}P chemical shifts as a result of neighbouring effects of the binding of the complex to DNA. The reduction of the DNA mobility due to the complex binding is a less likely alternative explanation.

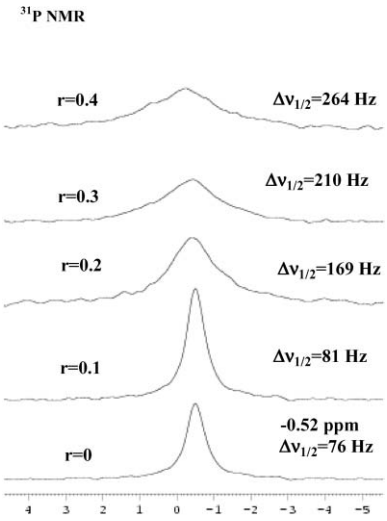


Fig. 5 ^{31}P NMR spectra of the sonicated DNA following the addition of the isomeric mixture of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy)Cl](PF_6) in various ratios.

The new ^{31}P peak is extremely narrow ($r = 0.5$ $\Delta\nu_{1/2}$ =15 Hz) indicating an insignificant dispersion of ^{31}P chemical shifts or that an increase in the molecular size (*e.g.* unwinding) has resulted, which would lead to a more rapid molecular tumbling.[32] A transition from B-form to A-form is also possible as this is known to reduce the linewidth of the ^{31}P signal up to 40%.[33] The downfield shift of about 1.4 ppm is also consistent with unwinding of the double helix[33] and distortion of the phosphate backbone that occurs due to the formation of the complex.[34] A modest downfield shift was also observed with the binding of cisplatin to CT-DNA.[30] On the other hand strong interactions with the phosphates whith pyridine nucleotides 5′-CMP and 5′-UMP, such as that of the Et_2SnCl_2 in acidic media, do cause strong downfield ^{31}P shifts of about 2 to 3 ppm.[35,36] In our case the downfield shift is probably originating from the coordination of the complex or possibly unwinding of the DNA helix.

3.2.3 Electrophoretic mobility studies. The effect of the binding of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) on plasmid DNA was determined by the ability of this compound to produce changes in the electrophoretic mobility of the supercoiled form. Fig. 6 shows an electrophoresis gel in which increasing amounts of the ruthenium complex were bound to supercoiled pUC9 DNA. The plasmid pUC9 gives a single major electrophoretic band

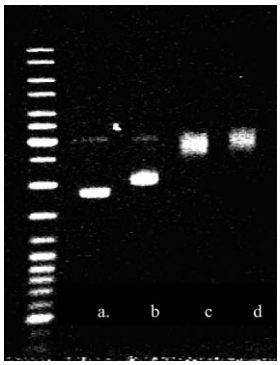


Fig. 6 Electrophoresis of the plasmid DNA (a) pUC9, and with the addition of the of the isomeric mixture of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) at ratios (b) $r = 0.1$, (c) $r = 0.3$, (d) $r = 0.5$ (r = [Ru]/[DNA]).

corresponding to the supercoiled form and a weaker band corresponding to relaxed DNA.[37]

Upon addition of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) to the supercoiled pUC9 a remarkable decrease in the mobility of the bands, at ratio $r = 0.1$, $r = 0.3$ and $r = 0.5$ (Ru/base) was observed (Fig. 6). At $r = 0.3$ the band is starting to be diffused and most likely the plasmid is already partially in a single strand. At $r = 0.5$ probably the DNA of the plasmid is still closed and supercoiled, but some of the nucleobases are not paired anymore. No bands are visible and there is no evidence whether the DNA is relaxed or linear. In all these procedures no substantial discrimination in the DNA binding between isomers **I** and **II** were observed.

The unwinding of the DNA has been observed in many cases where metal complexes interact covalently such as the interaction of the antitumor ruthenium complexes Na[*trans*-$RuCl_4$-(DMSO)(Im)] (NAMI) and dichloro(1,2-propylendiaminetetraacetate) ruthenium(III) (RAP) with supercoiled DNA.[26] Also, cisplatin causes a large decrease in the mobility of the supercoiled form as the amount of the added complex increases.[38]

The sequence binding selectivity of the ruthenium complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)-Cl](PF_6) to DNA was also studied by measuring the inhibition of two selected restriction enzymes which differ in their target sequence. The enzymes *Dra*I, which recognizes the base sequence

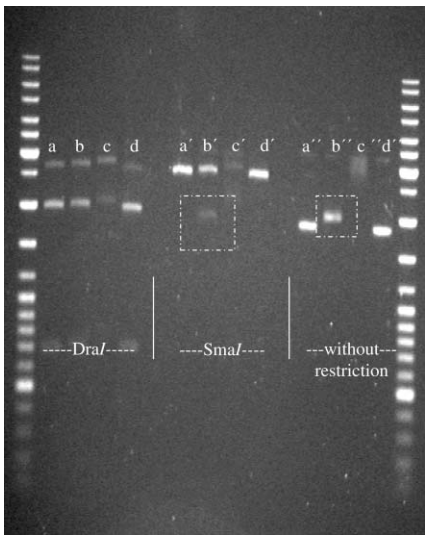


Fig. 7 Electrophoresis of the plasmid DNA digested by the restriction enzymes *Dra*I and *Sma*I. (a, a′, a″): DNA alone ($r = 0$) (b, b′, b″): $r = 0.1$, (c, c′, c″): $r = 0.3$, (d, d′, d″): fresh DNA (r = [isomeric mixture of (**1**)]/[base pair]). Inside the square are the bands at ratio $r = 0.1$, where a weak band was observed in the same position when no enzyme was used.

-TTTAAA-, and *Sma*I, that recognizes the base sequence -CCCGGG-, were chosen and their ability to cleave ruthenated plasmid pUC9 samples was tested. This analysis was performed on the concentrations $r = 0.1$ and $r = 0.3$ and the results were compared with the cleavage of a fresh sample of plasmid pUC9 and samples at the same concentrations without enzyme treatment. Testing the inhibition of the enzyme *Sma*I, a weak band was observed (lane b′, $r = 0.1$) in the same position as when no restriction enzyme was used (lane b″, $r = 0.1$). This observation is consistent with a binding of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) to the bases guanine and cytosine and consequently the enzyme can no longer recognize the restriction site. Inhibition of the enzyme by the ruthenium complex can be excluded due to the observed cleavage of the DNA in other uncomplexed sites in low ratio (Fig. 7). The cleavage of the plasmid pUC9 samples using the enzyme *Dra*I, was performed and no inhibition of the enzyme action was observed. These results suggested a preference of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) for the bases guanine and cytosine over the bases thymine and adenine.

4 Conclusions

A generally applicable method to synthesize chloro(polypyridyl)ruthenium conjugated peptide complexes *via* solid-phase strategy is described herein for the first time. The method is used to synthesize the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) as a mixture of two positional isomers. Even though, the separation of the isomers was only partially achieved chromatographically the isomers were assigned by NMR spectroscopy. The interactions of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys$CONH_2$)Cl](PF_6) with CT-DNA and plasmid DNA, were studied with various spectroscopic techniques showing that (i) the complex coordinatively binds to DNA preferring the bases guanine and cytosine over the bases thymine and adenine after hydrolysis of the coordinated chloride, (ii) electrostatic interactions between the complex cation and the polyanionic DNA chain assist this binding (iii) only in the case of isomer **I** the peptide interacts further with DNA as evidenced from ^{31}P NMR spectroscopy, (iv) DNA unwinding occurs in all cases with high binding ratio (Ru/base) values ($r > 0.3$).

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