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Review

Photo-reduction of polyazaaromatic Ru(II) complexes by biomolecules and possible applications

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Contents

Abstract

An important property of the Ru(II) complexes that contain polyazaaromatic ligands such as TAP (1,4,5,8-tetraazaphenanthrene) and HAT (1,4,5,8,9,12-hexaazatriphenylene) is the very high oxidation potential of the ^{3}MLCT (Metal to Ligand Charge Transfer) state. Therefore, under illumination, they are able to abstract an electron even from rather poor electron donors. In this work, we show how it is possible to take advantage of this property for applications of photo-induced electron transfer between these excited complexes and biopolymers such as DNA, oligonucleotides and oligopeptides.

One of the consequences of the charge transfer processes is photocleavage of plasmid DNA, demonstrated some years ago by agarose gel electrophoresis and more recently detected and quantified by AFM. Another effect of the photo-induced electron transfer is the formation of photo-adduct(s) of the metal complex with the guanine units of oligonucleotides or with the tryptophan moieties of oligopeptides. In the photo-adducts with the guanine nucleobases, a covalent bond is formed between one of the polyazaaromatic ligands and the guanine base without destruction of the ligands sphere around the metal ion. Interestingly, the formation of these photo-adducts can be exploited in order to photo-crosslink two

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single-stranded oligonucleotides. Such photo-crosslinking via the metal complexes could lead to applications in biological diagnostic tests and possibly in vivo in the area of gene phototherapy.

Keywords: Polyazaaromatic Ru(II) complexes; Photo-induced electron transfer; Photo-crosslinking; Photo-adduct formation; DNA; Oligonucleotides

1. Introduction

Extensive research has been performed on Ru(II) polypyridyl complexes and these developments have led to many applications. With respect to the properties of their MLCT (Metal to Ligand Charge Transfer) triplet excited state, several Ru(II) polypyridyl complexes have been studied with the view of producing hydrogen from water splitting [1–5], for the development of new Light Emitting Devices (LED's) [6–8] or as sensitizers of wide band gap semiconductor electrodes in photoelectrochemical cells [9–15]. These research areas have been discussed in other reviews.

More recently, some polypyridyl complexes have also been developed as photoprobes and photoreagents for biomolecules, such as DNA or proteins. There is presently growing interest for novel probes and labels of biomaterials, and for the development of new metal containing therapeutic agents for various diseases [16–20]. Because the oxidising and reducing power of the Ru(II) complexes are considerably enhanced upon irradiation, these metal complexes can give rise to both oxidative and reductive photo-induced electron transfers with different donors or acceptors [21,22].

This review deals with the photochemistry and photophysics of Ru(II) complexes that behave more particularly as excellent oxidising agents versus genetic material and protein residues. Moreover, this work focuses on possible applications and exploitations of these photo-oxidising properties.

An easy way to increase the oxidation power of Ru(II) polypyridyl complexes is to chelate the metal centre to polyazaaromatic ligands that contain additional non-chelated nitrogen atoms. In Fig. 1 are depicted several polyazaaromatic ligands. Among them, solely the TAP and HAT ligands confer very high oxidising properties to the complex.

Therefore, these metal complexes are able to abstract an electron from rather poor electron donors, such as nucleotides in DNA and protein residues. A sufficiently high oxidation power is however reached only when the Ru(II) compounds are chelated to, at least, two oxidising polyazaaromatic ligands like TAP or HAT. The number of complexes discussed in this review is thus rather limited. They are displayed in Table 1 together with some non-oxidising complexes for comparison purposes. Their corresponding emission and electrochemical data are also given.

As it can be concluded from Table 1 the TAP and HAT complexes are indeed quite oxidising in the excited state and should be capable of abstracting electrons from biological electron donors such as the nucleobases of DNA or oligonucleotides and amino acid residues (vide infra for the corresponding electrochemical values).

2. Photo-induced electron transfer processes

2.1. Photo-oxidation of guanine moieties

On the basis of the oxidation potential of guanosine-5′-monophosphate (GMP—$E_{ox} \approx +1.05$ V versus SCE) [36–40], a favourable photo-electron transfer process is expected to occur from guanine to the ^{3}MLCT excited state of the TAP and HAT complexes of Table 1 according to reactions (1) and (2) (L = TAP or HAT, L′ = TAP, phen, bpy, dip, dppz or PHEHAT and R is the reducing agent, i.e. a guanine moiety in the present case):

$$[Ru(II)(L)_2(L')]^{2+} + h\nu \rightarrow [Ru(III)(L^{\bullet -})(L)(L')]^{2+*} \quad (1)$$

$$[Ru(III)(L^{\bullet -})(L)(L')]^{2+*} + R \rightarrow [Ru(II)(L^{\bullet -})(L)(L')]^{1+} + R^{\bullet +} \quad (2)$$

$$[Ru(II)(L^{\bullet -})(L)(L')]^{1+} + R^{\bullet +} \rightarrow [Ru(II)(L)_2(L')]^{2+} + R \quad (3)$$

The existence of process (2) was indeed demonstrated experimentally several years ago in the presence of the guanine mononucleotide by steady-state and time-resolved emission, transient absorption spectroscopy under pulsed illumination and spectroelectrochemistry [31,41,42]. For example, in the case of $[Ru(TAP)_2(phen)]^{2+}$, the transients recorded after a pulsed illumination in the presence of GMP, were attributed to the formation of the reduced Ru complex $[Ru(TAP)_2(phen)]^{1+}$ and oxidised guanine, and decay in a few hundred microseconds according to a second order process (in agreement with the back electron transfer, reaction (3)).

For detailed results and discussion concerning these charge transfer processes with GMP, the reader can refer to other reviews [21,43–45].

The TAP and HAT complexes were also examined under pulsed illumination in the presence of guanine units of polynucleotides [46]. Interestingly, in this case, especially when the complex contains an extended planar ligand, such as the dppz, able to intercalate between the stacking of bases of the polynucleotide double helix, the back electron transfer (reaction (3)) is considerably faster than a few hundred microseconds as found with GMP. Indeed, process (3) takes place in this case, in the ns time domain [32,47].

There has been a problem with the oxidation potential of guanine first determined electrochemically with GMP, for evaluation of the driving forces of the direct photo-electron transfer process (reaction (2)). Indeed, because of the irreversible character of the oxidation wave of GMP, the potential values did

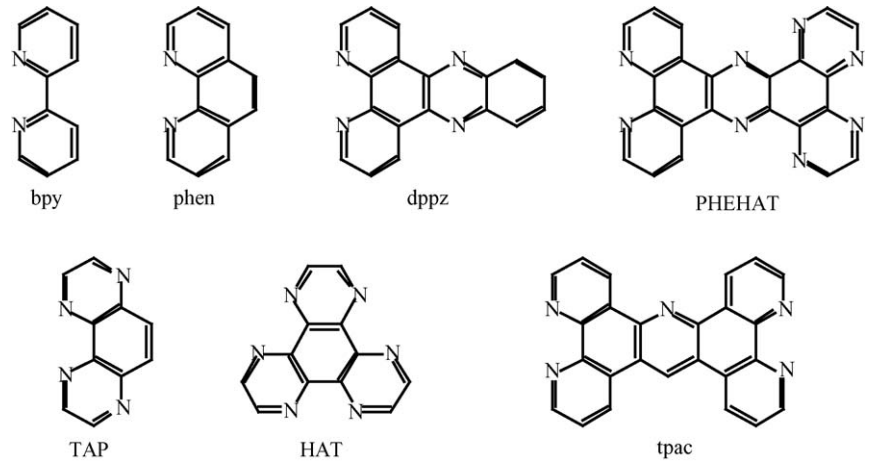

Fig. 1. Structure of different polyazaaromatic ligands: bpy = 2,2′-bipyridine; phen = 1,10-phenanthroline; TAP = 1,4,5,8-tetraazaphenanthrene [23,24]; HAT = 1,4,5,8,9,12-hexaazatriphenylene [25]; dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine [26]; PHEHAT = 1,10-phenanthrolino-[5,6-*b*]-1,4,5,8,9,12-hexaazatriphenylene [27]; TPAC = tetrapyrido[3,2-*a*:2′,3′-*c*:3″,2″-*h*:2‴,3‴-*j*]acridine [28].

not correspond to the thermodynamic data. It is now generally accepted that the oxidation potential determined by pulse radiolysis is the most reliable [48]. If this value is used (+1.35 V versus SCE) for GMP rather than the electrochemical potential, reaction (2) becomes endergonic for most of the oxidising complexes of Table 1, since the reduction potential of the excited state (E^*_{red}, Table 1) is estimated to be in the range of +1.10/+1.49 V versus SCE. It has recently been proposed [32,47] that a proton-coupled electron transfer (PCET) rather than a simple electron transfer (ET) would occur. Indeed, if simultaneously to the electron transfer, a deprotonation of the guanine radical cation ($pK_a = 3.9$) [49,50] takes place, reaction (2) is expected to be weakly exergonic. Isotopic effects H/D obtained with the complex $[Ru(TAP)_2(dppz)]^{2+}$ ($E^*_{red} = +1.20$ versus SCE) illuminated in the presence of a guanine unit of either GMP or [poly(dG-dC)]$_2$ [32,47,51] indicate that indeed both direct (process (2)) and back (process (3)) ET proceed by a PCET.

The adenine species (in adenosine-5′-monophosphate (AMP) or polynucleotides) can also be photo-oxidised, but only by the stronger oxidising complexes (such as $[Ru(HAT)_3]^{2+}$ or $[Ru(TAP)_3]^{2+}$) [31,41].

Table 1
Potentials of the first reduction wave (vs. SCE) determined by cyclic voltammetry in acetonitrile for the oxidising and non-oxidising Ru(II) complexes discussed in this work

Complex	E_{red} (V/SCE)	$\lambda_{em,max,MeCN,nm}$	E^*_{red} (V/SCE)
Oxidising complexes			
$[Ru(HAT)_3]^{2+}$ [29]	−0.62	587	+1.49
$[Ru(TAP)_3]^{2+}$ [30]	−0.75	604	+1.30
$[Ru(HAT)_2(phen)]^{2+}$ [31]	−0.66	650	+1.25
$[Ru(HAT)_2(bpy)]^{2+}$ [29]	−0.76	642	+1.17
$[Ru(TAP)_2(dppz)]^{2+}$ [32]	−0.80	621	+1.20
$[Ru(TAP)_2(phen)]^{2+}$ [32]	−0.83	626	+1.15
$[Ru(TAP)_2(bpy)]^{2+}$ [30]	−0.83	641	+1.10
$[Ru(TAP)_2(dip)]^{2+}$ [33]	−0.82	633	+1.14
Non-oxidising complexes			
$[Ru(bpy)_2(HAT)]^{2+}$ [29]	−0.84	703	+0.92
$[Ru(bpy)_2(TAP)]^{2+}$ [30]	−0.88	679	+0.95
$[Ru(bpy)_2(phen)]^{2+}$ [34]	−1.35	601	+0.71
$[Ru(phen)_3]^{2+}$ [35]	−1.35	604	+0.70
$[Ru(bpy)_3]^{2+}$ [30]	−1.35	621	+0.65

The corresponding reduction potentials in the excited state (vs. SCE), estimated from the reduction potential in the ground state and the energy of the emission maximum in acetonitrile are also given ($E^*_{red} = E_{red} + \Delta E_{00} \approx E_{red} + \Delta E_{\lambda_{max}}$).

2.2. Photo-oxidation of amino acids

More recently, the photo-induced ET process was investigated with amino acids. The excited ^{3}MLCT states of $[Ru(TAP)_2(phen)]^{2+}$ or $[Ru(TAP)_3]^{2+}$ can be reduced by tyrosine (Tyr) or tryptophan (Trp) (E_{ox} Tyr = +0.85 V versus SCE, E_{ox} Trp = +0.78 V versus SCE) [52,53] (Fig. 2). With Trp more particularly, the photo-production of the reduced metal complexes and oxidised Trp has clearly been demonstrated by luminescence quenching and laser flash photolysis transient absorption experiments [54].

In this system, the presence of oxygen in the medium plays an important role. Indeed, the singlet oxygen photosensitised by the Ru(II) triplet excited state reacts with Trp to form oxidised derivatives of Trp such as *N*-formylkynurenine (*N*-FK) and 3*a*-hydroxypyrrolidinoindole (HPI) with a rate constant of $7 \times 10^7\ M^{-1}\ s^{-1}$ [54]. These side reactions make the study of the electron transfer more difficult.

In contrast to the PCET process demonstrated with the guanine units, the electron transfer process with Trp or Tyr is clearly exergonic even for the less oxidising Ru(II) complex such as $[Ru(TAP)_2(phen)]^{2+}$ (ΔG°_{ET} $[Ru(TAP)_2(phen)]^{2+*}$/Tyr = −0.30 eV; ΔG°_{ET} $[Ru(TAP)_2(phen)]^{2+*}$/Trp = −0.37 eV). However, it is likely that in this case too, the process is coupled to a proton transfer. Indeed, several authors have reported that the ET reaction between a $[Ru(III)(bpy)_3]^{3+}$ species (generated in situ by laser flash photolysis in the presence of methyl viologen dichloride—MV^{2+} or hexaamminoruthenium(III)—$[Ru(III)(NH_3)_6]^{3+}$) and a tyrosine or tryptophan moiety chemically anchored to one of the ancillary ligand of the metallic dye [55,56], corresponds to

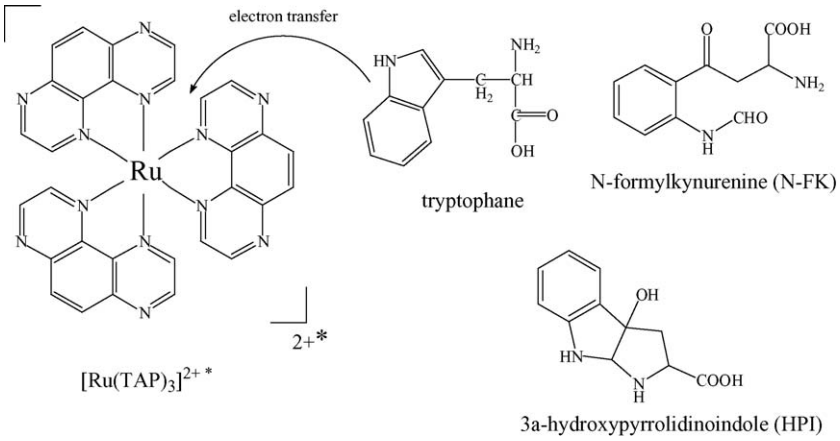

Fig. 2. Photo-induced ET process with excited $[Ru(TAP)_3]^{2+}$ and tryptophane (Trp), and structure of oxidised derivatives *N*-FK and HPI.

an intramolecular PCET reaction. Therefore, in the case of $[Ru(TAP)_2(phen)]^{2+}$, a PCET process would probably also take place when the complex is irradiated in the presence of Trp or Tyr.

3. The different effects induced by the photo-electron transfer

In this section and Section 4, we highlight the different consequences of the photo-electron transfer process when it takes place with biopolymers such as DNA or polynucleotides and with small polypeptides.

The ET step, triggered by visible light absorption, is clearly correlated with two types of reactions: (i) single-strand cleavage of plasmid DNA and (ii) photo-adduct formation of the metal complex with either the guanine units of polynucleotides or the tryptophan residues of polypeptides.

3.1. Photo-induced cleavage in plasmid DNA

Single-strand breaks are very easily detected and quantified with plasmid DNA. Indeed a single nick of a strand transforms the supercoiled covalently closed circular form (ccc) into an open circular form (oc) of the plasmid (Fig. 3). This change of conformation can be monitored by agarose gel electrophoresis. Staining the gel with ethidium bromide reveals the resulting DNA bands of the ccc and oc forms.

Most of the Ru(II) complexes under illumination in the presence of supercoiled plasmid DNA (ccc form) give rise to single-strand breaks that can be detected by the appearance of the oc form of the plasmid. These photocleavage have been reported for $[Ru(phen)_3]^{2+}$ and $[Ru(bpy)_3]^{2+}$, non-oxidising complexes of Table 1 [57,58]. Both the ionic strength and oxygen concentration in the medium are crucial for an efficient reaction. Oxygen is indeed important because this photo-cleavage originate from singlet oxygen photosensitisation by the metal complexes. However, the efficiency of this cleavage is very low [59]. In contrast, higher yields are observed with the photo-oxidising Ru(II) complexes of Table 1 [21,31]. The increased yield can be attributed to another cleavage mechanism. Indeed, the photocleaving ability correlates fairly well with the oxidation power of the excited state of the complex (Table 1 and Fig. 4).

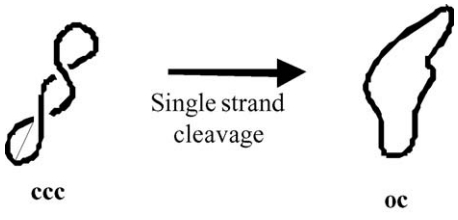

Fig. 3. Schematic representation of the ccc (covalently closed circular form) and oc (open circular form) of the double stranded plasmid.

This means that in addition to singlet oxygen photosensitisation, the oxidising complexes undergo an one electron reduction by the nucleobases. Following this charge transfer process, the nucleobase radical cations produced (mainly the guanine radicals) or their deprotonated derivatives abstract an H-atom from nearby riboses which, after several reactions, give rise to the final strand breaks [60]. This mechanism (type I photo-oxidation), different from the singlet oxygen photosensitisation (type II photo-oxidation), explains the higher percentage of transformation of the ccc form into the oc form.

More recently, these topological modifications of plasmid DNA have been examined by Atomic Force Microscopy (AFM) [61–63]. The idea was to monitor directly the change of topology induced by photocleavage in the presence of the photo-oxidising Ru-TAP complexes. The AFM allows the visualisation and counting of the ccc and oc forms when adsorbed onto a mica modified surface. The possibility of observing directly, at the single molecular level, the topology of a plasmid DNA represents an important breakthrough in this area. Indeed, these topological variations play crucial roles in the functions of DNA and in its interactions with several enzymes [64,65]. The observation of the plasmid recognition by an enzyme at the single molecular level would be highly interesting.

The problem of deposition of the plasmid DNA onto a flat mica surface had however first to be solved. Indeed, the adsorption induces a transition from a three-dimensional structure to a two-dimensional one, which can modify the conformation of the

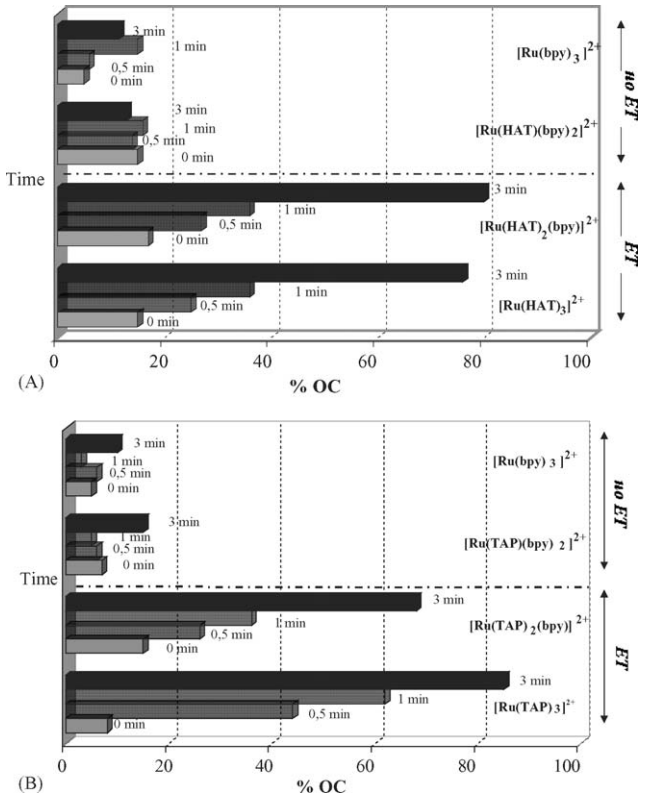

Fig. 4. Percentage of oc form of plasmid pBR322 vs. irradiation time (0, 30, 60 and 180 s; λ_{exc} = 436 nm) determined by agarose gel electrophoresis in the presence of: (A) different HAT complexes and (B) different TAP complexes. Experiments performed with a constant concentration of complex (3.6×10^{-6} M) and plasmid (3.6×10^{-5} M) in 10 mM phosphate buffered solution (pH 7).

material studied [66,67]. Appropriate deposition and adhesion methods had thus to be found in order to keep the original equilibrium conformation after transition towards a two-dimensional state. The best immobilization strategies were obtained when weak immobilization conditions were used, i.e. a mica surface treated with low concentrations of Mg^{2+}. These conditions allow the plasmid DNA to remain relaxed into its intrinsic conformation after adsorption onto the mica [63].

Once these experimental conditions were found, the plasmid DNA was irradiated in solution in the presence of $[Ru(TAP)_3]^{2+}$ for different times and, after deposition onto the mica, the conformational changes were monitored with the AFM technique. Fig. 5 shows AFM images of the ccc and oc forms.

The number of both forms were counted by AFM from different spots on the mica surface and the resulting percentage of each form was compared to that determined by agarose gel electrophoresis before and as a function of the illumination time (Ar-ion laser, 458 nm) of $[Ru(TAP)_3]^{2+}$ with the plasmid. As

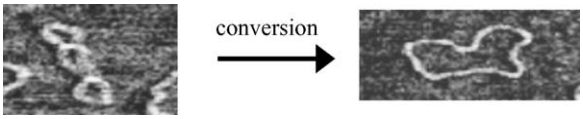

Fig. 5. Atomic Force Microscopy images of pUC18 plasmid DNA undergoing the conversion from the ccc to the oc form. Samples were immobilized onto a mica surface treated with Mg^{2+}.

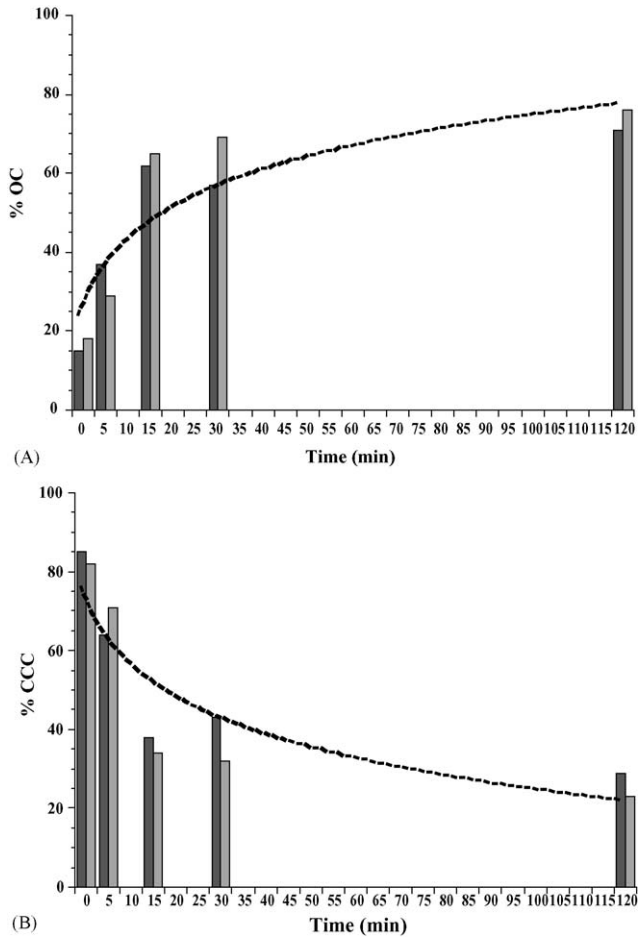

Fig. 6. Relative amounts of: (A) oc and (B) ccc forms of pUC18 plasmid DNA in the presence of $[Ru(TAP)_3]^{2+}$ as a function of irradiation time, obtained by AFM (onto mica modified with Mg^{2+} (5 mM)—black rectangles) and by agarose gel electrophoresis (grey rectangles).

expected (Fig. 6), the percentage of oc form increases at the expense of the ccc form. Very interestingly, the same relative amount of ccc form versus oc form is obtained by AFM (black rectangles) and by agarose gel electrophoresis (grey rectangles). This clearly confirms the reliability of the AFM technique to visualize at a single molecular level the conformational changes induced by a single-strand break in the plasmid.

In conclusion, this efficient plasmid photocleavage according to type I guanine oxidations (i.e. by electron transfer), constitutes the first illustration of the consequences of electron transfer photo-induced with oxidising Ru-TAP complexes.

3.2. Photo-induced adduct formation

Another important effect of the photo-induced electron transfer process is the subsequent formation of an adduct between the biomaterial and the metal complex. The reaction has been extensively studied with guanine moieties, i.e. first with GMP and afterwards with DNA, and more recently with amino acid residues. As described below, these photo-adducts are quite different from the rhodium photo-adducts [68–72] or platinum dark adducts [17,19,73–75] previously studied in the literature.

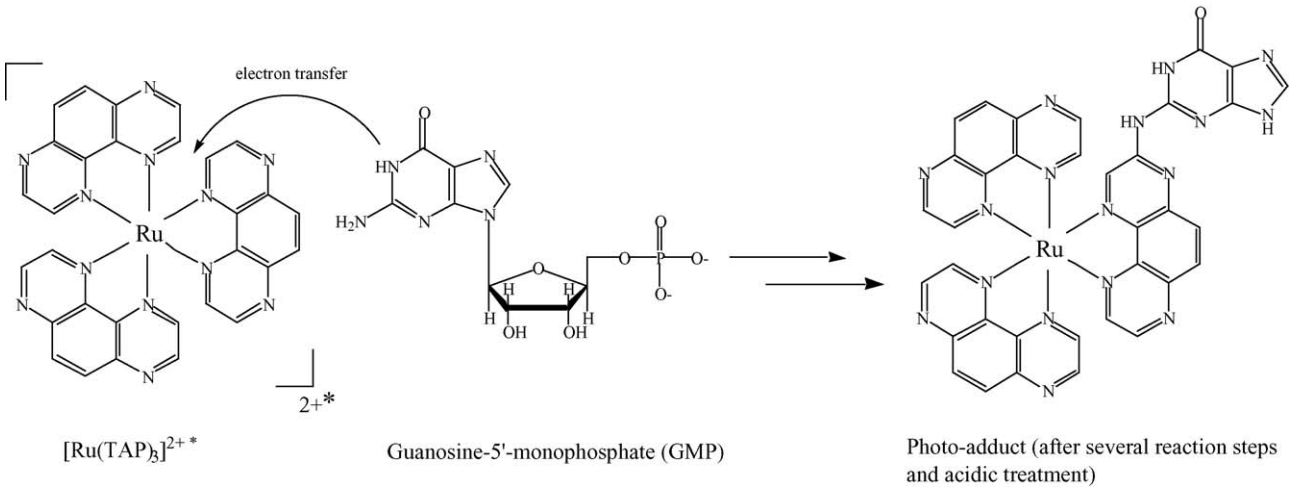

Fig. 7. Structure of the photo-adduct formed by the irradiation of $[Ru(TAP)_3]^{2+}$ in the presence of guanosine-5′-monophosphate (GMP), after acidic treatment (HCl) to remove the ribose phosphate.

3.2.1. Photo-adduct with GMP or DNA/oligonucleotides

Electron transfer from a guanine residue to an excited oxidising metal complex of Table 1 is followed by a series of reactions, which lead to the formation of photo-products. These light induced chemical processes, which do not take place with the non-oxidising compounds of Table 1, have clearly been demonstrated with the TAP and HAT complexes thanks to different types of experiments. Denaturing polyacrylamide gel electrophoresis of illuminated solutions of the complexes in the presence of a 5′-^{32}P-labelled oligonucleotides, showed the presence of unexpected bands of lower mobility than the starting 5′-^{32}P-labelled oligomer. These bands were attributed to the formation of covalently bound adducts of the metal complex with the oligonucleotide. The covalent nature of the bond between the complex and the oligomer has also been demonstrated by dialysis experiments, in which photo-products were retained in the dialysis bag [31,76]. Further evidence for photo-adduct formation was obtained by UV–vis absorption measurements. These spectroscopic investigations as a function of the illumination times, allow easy monitoring of the formation of photo-adducts with GMP, DNA or polynucleotides through occurrence of a strong hypsochromic band with a hyperchromic effect in the 350–400 nm region. This is in contrast to the 500 nm absorption due to the appearance of dechelation/substitution photo-products as previously observed for the $[Ru(TAP)_3]^{2+}$ in the presence of adenosine-5′-monophosphate (AMP) [41].

In the case of $[Ru(TAP)_3]^{2+}$ and GMP, several studies were conducted to determine the structure of the photo-adduct. After its isolation from the irradiated medium and purification, the structure of the resulting compound (Fig. 7) was determined by ^{1}H NMR and electrospray mass spectrometry (ESMS). Guanine is covalently linked to the complex by the exocylic amino group of the base, which substitutes one H-atom of the azaaromatic TAP ring next to the α-position of a non-chelated nitrogen [77].

The irradiation of $[Ru(TAP)_2(bpy)]^{2+}$ in the presence of a guanine moiety (GMP or DNA, treated afterwards by digestion enzymes) induces the formation of two photo-isomers due to the asymmetry of the starting complex. These two isomers result also from the formation of a covalent bond between the exocyclic amino group of the guanine with one of the TAP ligands [78].

At physiological pH, the proposed mechanism for the adduct formation consists in a succession of steps [76], in which the adduct results from the recombination of the monoreduced protonated complex, thus a radical metal complexes with the deprotonated guanine radical ($pK_{a_{\text{guanine radical cation}}} \sim 4$) [49,50]. These different steps are described in Eqs. (4)–(8), where L′ stands for TAP, phen or bpy, and G is a guanine residue from GMP or DNA.

$$[Ru(TAP)_2(L')]^{2+} + h\nu \rightarrow [Ru(III)(TAP^{\bullet -})(TAP)(L')]^{2+*} \quad (4)$$

$$[Ru(III)(TAP^{\bullet -})(TAP)(L')]^{2+*} + G \rightarrow [Ru(II)(TAP^{\bullet -})(TAP)(L')]^{1+} + G^{\bullet +} \quad (5)$$

As explained (vide supra), (5) could correspond to a proton-coupled electron transfer (PCET-deprotonation of the guanine radical cation (6) and protonation of the reduced complex (7)).

$$G^{\bullet +} \rightarrow G(-H)^{\bullet} + H^{+} \quad (6)$$

$$[Ru(II)(TAP^{\bullet -})(TAP)(L')]^{1+} + H^{+} \rightarrow [Ru(II)(TAP + H)^{\bullet}(TAP)(L')]^{2+} \quad (7)$$

$$[Ru(II)(TAP + H)^{\bullet}(TAP)(L')]^{2+} + G(-H)^{\bullet} \rightarrow \text{photo-product} \quad (8)$$

In the case of $[Ru(HAT)_2(phen)]^{2+}$, ESMS analyses of the isolated photo-products with GMP confirm the addition of one GMP on one HAT ligand, without any dechelation [79]. ^{1}H NMR analyses have also demonstrated the presence of two geometrical isomers for the photo-adduct, as shown in Fig. 8. In this case, it is the exocyclic oxygen of the guanine, which has been involved in the covalent bond. The origin of this difference with the TAP complexes is not known. It might be attributed to a different

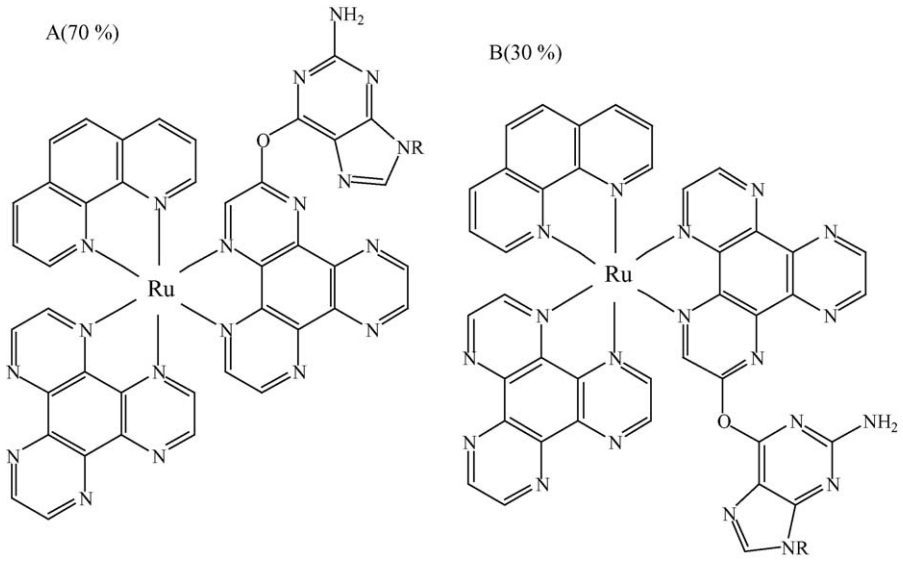

Fig. 8. Proposed structure for the two photo-adduct isomers (A and B) between $[Ru(HAT)_2(phen)]^{2+}$ and GMP. R stands for the sugar-phosphate group of the GMP moiety.

geometry of interaction of the reduced complex with DNA as compared to that of the TAP complex.

Moreover, with this HAT compound, two other minor photo-products were also detected: (i) adducts between $[Ru(HAT)_2(phen)]^{2+}$ and an oxidised guanine unit and (ii) formation of biadducts between the complex and two guanine residues. Production of such a bi-adduct could lead to the possibility of photo-crosslinking irreversibly two oligonucleotides via their guanine units to one complex, as each HAT ligand of the complex could form, under illumination, an adduct with a guanine residue of each of two complementary oligonucleotidic strands.

In conclusion, since the bis-TAP and bis-HAT complexes can form photo-adducts with G-containing polynucleotides, in Section 4, we explain how these properties can be exploited.

3.2.2. *Photo-adduct with an amino acid residue: the tryptophan*

As mentioned above, laser flash photolysis experiments with transient absorption detection carried out with the TAP complexes and tryptophan have demonstrated the occurrence of a reduced TAP compound and oxidised tryptophan. Further investigations with this system have also shown that, after this charge transfer process, an adduct is formed [54]. The ESMS analyses give a mass for the isolated photo-product which corresponds to the mass of the complex plus that of tryptophan minus 2H-atoms. This result is in full agreement with an adduct formation although its structure has not yet been determined by ^{1}H NMR. As in the case of the guanine unit, it is likely that the adduct between $[Ru(TAP)_2(phen)]^{2+}$ or $[Ru(TAP)_3]^{2+}$ and tryptophan is also formed after several steps initiated by the photo-induced electron transfer. Further investigation is still needed in order to clarify the exact mechanism of production and the structure of the photo-adduct. Nevertheless, this new type of photo-product is promising for future applications in the proteomic research area.

4. Exploitation of photo-adduct formation

The photochemical properties of the oxidising Ru(II) complexes of Table 1, make them attractive candidates for applications as molecular tools or probes for DNA studies. In this section, we show how to exploit the formation of adducts initiated by visible light absorption as described above for gene-silencing with specific or non-specific targets.

4.1. *In vitro inhibition of gene transcription from DNA templates*

(Photo)damage to DNA has dramatic effects on the DNA functions [80–84]. One of the different origins of these perturbations is the difficulty for the enzymes involved in gene transcription to bind to the DNA template at the level of the damage. Therefore, the normal progression of these enzymes along the template is prevented and transcription is inhibited. Thus, the DNA photo-damage caused by the TAP and HAT complexes could be exploited in gene-silencing (inhibition of gene expression). However, it will not be possible in the case of these complexes to target a specific gene because they do not exhibit specificity of interaction with specific DNA sites or sequences.

The possible photo-activity of $[Ru(TAP)_2(phen)]^{2+}$ in an in vitro system of transcription of a DNA template by an RNA-polymerase enzyme, has been tested and compared to that of a non-photo-oxidising complex, namely $[Ru(bpy)_2(phen)]^{2+}$. With this latter species, no photo-electron transfer process takes place with the genetic material and consequently there is no photo-adduct formation. The effects of these two complexes under illumination, at the level of the gene transcription of a plasmid vector used as template by the bacteriophage RNA-polymerase, have therefore been examined and compared [85].

A solution containing the template DNA and either $[Ru(TAP)_2(phen)]^{2+}$ or $[Ru(bpy)_2(phen)]^{2+}$ was irradiated (under air) in the visible region, before the transcription experi-

ments. Afterwards, RNA-polymerase was added to the medium to initiate the transcription process. If a RNA production of 100% (i.e. 100% transcription) is considered without irradiation or addition of complex, i.e. in the absence of any photo-process, the relative amount of transcribed messenger RNA is then reduced to about 50% with irradiated $[Ru(TAP)_2(phen)]^{2+}$ and 20% with illuminated $[Ru(bpy)_2(phen)]^{2+}$. Moreover, since no inhibition occurs in the dark, these reduced rates for RNA synthesis clearly demonstrate that the irradiation of the metal complexes inhibits DNA transcription. This inhibition obviously originates from damage of the plasmid DNA. As $[Ru(bpy)_2(phen)]^{2+}$ is a poor oxidising agent in its excited state and is unable to abstract an electron from a guanine unit (see Table 1), the damage in that case can only be induced by singlet oxygen photosensitisation (see above). In contrast, in the presence of $[Ru(TAP)_2(phen)]^{2+}$ it is likely that the enhanced percentage of inhibition of transcription stems from the photo-induced electron transfer process and also in part from the photo-adduct formation.

In conclusion, this strong inhibition of transcription induced by oxidising complexes such as $[Ru(TAP)_2(phen)]^{2+}$ under illumination, makes them potential candidates in phototherapy, with irradiation in the blue, i.e. for superficial treatments. Moreover, as their activity does not seem to be correlated with the presence of oxygen, like other organic dyes in photodynamic therapy (PDT) [86–88], they could act differently than in classical PDT.

4.2. Photo-crosslinking between two single-stranded oligonucleotides

The use of synthetic oligonucleotides (ODNs) for the inhibition of gene expression (in this case, it is a specific gene targeting) represents an attractive therapeutic approach. The antigen (triple helix formation between an ODN and a targeted double stranded DNA) [89,90] or antisense (double helix formation between an ODN and mRNA) [91,92] strategies are well known and provide means to inhibit specific genes. A major problem related to this inhibition process arises from: (i) the poor stability of the hybridization of the ODN with the target and (ii) the presence of enzymes that recognize the hybridization with the synthetic ODN and destroy the system. Many efforts have been focussed on the different possible ways for increasing the stability of the system "synthetic ODN-targeted sequence" [93,94].

4.2.1. Working principles

In connection with these strategies, the efficient photoreactivity of the TAP complexes towards DNA, combined with a selectivity of their photoreaction with the guanine units of a targeted gene, would offer a solution to this stability problem. Indeed, if a specific synthetic ODN is derivatized with a highly photo-oxidising and photoreactive Ru(II) compound (probe sequence) and then hybridised to a specific gene (target complementary sequence), irradiation of the attached complex should lead to the formation of a covalent bond between the Ru-probe sequence and a guanine moiety (if present) of the target sequence via photo-induced adduct formation. This should result into a photo-crosslinking between the two strands (see schematic representation in Fig. 9). Thus, the stability of the hybridization of the Ru-ODN with the targeted sequence should be increased. Moreover, such photo-crosslinking would perhaps be more resistant to the enzymes.

4.2.2. Experiments

4.2.2.1. Preparation of the system. In order to implement experimentally the system of Fig. 9, the probe Ru(II)-labelled sequence is prepared by attaching chemically a Ru(II) complex containing two TAP ligands to a 17-mer oligonucleotide. This ODN derivatization has been performed with the $[Ru(TAP)_2(dip)]^{2+}$ (dip = 4,7-diphenylphenanthroline) complex [95]. The dip ligand offers the possibility to be easily derivatized by a carboxylic group (Fig. 10A). The subsequent amide bond formation between this carboxylic acid function and a thymine modified nucleobase (Fig. 10B) allows the chemical anchoring of the oxidising Ru(II) complex to a modified thymine in the middle of the probe ODN sequence.

The probe sequence may not contain any guanine residues to avoid photoreaction in the probe sequence itself. In contrast, the target strands, solely, should contain guanine units. On the other hand, the anchoring of the complex in the middle of the probe sequence offers several advantages. It allows one to target guanine bases of the complementary strand in both directions, i.e. towards either the 3′-end or the 5′-end. In addition, the microenvironment of the nucleobases in the middle of the derivatized duplex should be closer to that of normal double-stranded structures, because the attached complex induces only a small perturbation [95].

Thus, according to the "working principles" outlined above, hybridization of the probe Ru(II) derivatized sequence with the G-containing target sequence, depending on the position of the

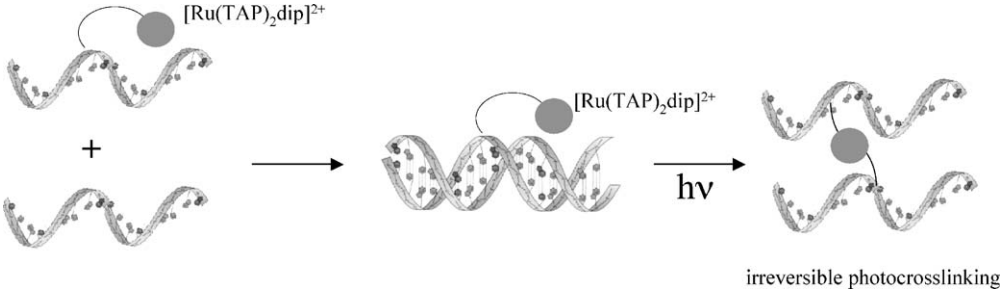

Fig. 9. Schematic representation of the photo-crosslinking process for the complex $[Ru(TAP)_2(dip)]^{2+}$ anchored onto an oligodesoxynucleotide (ODN).

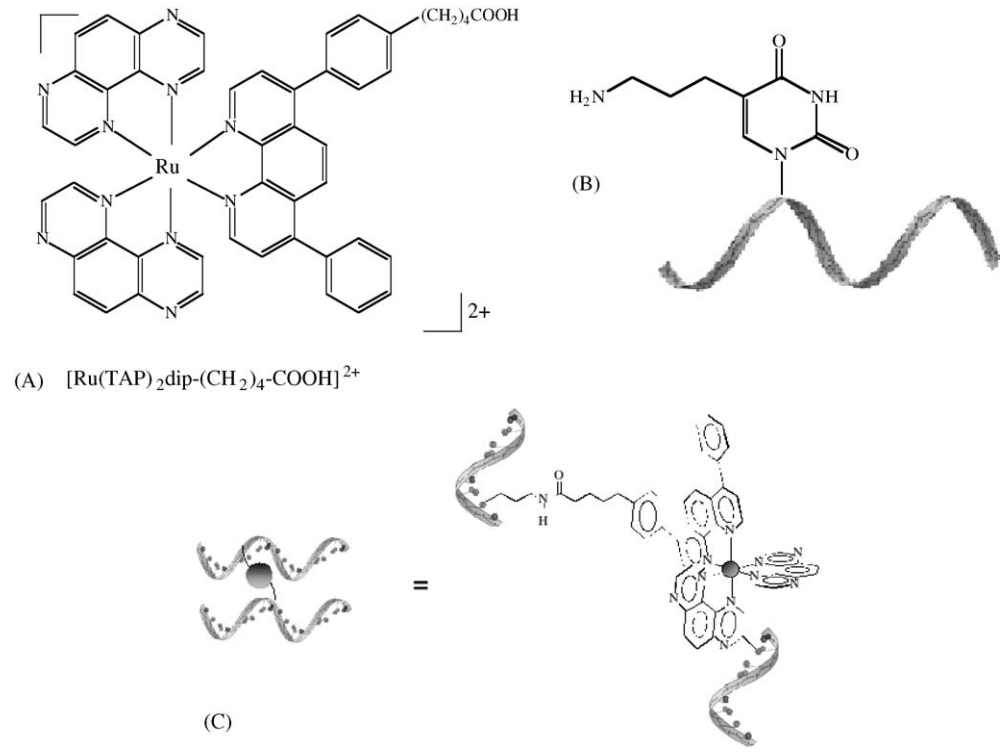

Fig. 10. (A) $[Ru(TAP)_2(dip)]^{2+}$ with the dip ligand (dip = 4,7-diphenylphenanthroline) derivatized by an alkyl chain with a carboxylic acid function; (B) the 17-mer probe oligonucleotide, containing a thymine modified nucleobase; (C) most likely structure for the photo-crosslinked system.

G base in the target sequence, may bring the oxidising metallic moiety in close contact with the guanine unit of the complementary sequence. In such a case, under irradiation of the complex, a photo-induced electron transfer, followed by an adduct formation should produce a photo-crosslinking (see Fig. 10C). Actually, Fig. 10C corresponds to the most likely structure on the basis of previous results obtained with GMP as discussed above.

In order to check precisely the different parameters that could control: (i) the electron transfer process (Table 2, vide supra) and (ii) the subsequent adduct formation or the photo-crosslinking, different duplex sequences were prepared (Fig. 11A, vide supra). In these sequences, the relative position of the guanine residues versus the anchoring site is different.

Sequence 0 which does not contain any guanine unit in the target strand has been used as reference.

4.2.2.2. Luminescence quenching by electron transfer. In comparison to the duplex sequence 0, the presence of guanine residues in the Ru(II)-labelled duplexes of Table 2 induces, upon irradiation, luminescence quenching due to the electron transfer. This process is however not possible if the G units are too far away from the attachment site of the complex (case of sequences 4 and 5, see Fig. 11A, vide supra).

Except for this distance factor, the results of Table 2 show clearly that the percentage of quenching in the different duplexes can be correlated to the ionisation potential (IP) of the guanine residues, the lower the ionisation potential, the higher the quenching. It is indeed well known that the IP value is related to the presence and number of other neighbouring guanine units. A triplet GGG has a lower ionisation potential than a doublet GG, which has in turn a lower oxidation potential than an isolated G [96–98].

The question that can be raised is whether some static quenching would participate in the overall quenching process. Therefore, the luminescence lifetimes (the ratios τ/τ_0 where τ_0 and τ are the luminescence lifetimes in the absence and presence of

Table 2
Percentage of quenching for $[Ru(TAP)_2(dip)]^{2+}$ anchored to different oligonucleotide duplexes [99,100]

Sequence	% Quenching[a]	Calc. IP (eV)[b]
5′-TTT TTT **CCT*** TAA ATT TA-3′ 3′-AAA AAA **GG**A ATT TAA AT-5′	59 ± 2	6.32
5′- TTT TTT TAT* **CC**A ATT TA-3′ 3′-AAA AAA ATA **GG**T TAA AT-5′	49 ± 2	6.42
5′- TTT TTT T**CT*** TAA ATT TA-3′ 3′-AAA AAA A**G**A ATT TAA AT-5′	38 ± 2	6.55
5′- TTT TTT **CTT*** TAA ATT TA-3′ 3′-AAA AAA **G**AA ATT TAA AT-5′	30 ± 3	6.55
5′- TTT TTT TAT* **C**TA ATT TA-3′ 3′-AAA AAA ATA **G**AT TAA AT-5′	31 ± 2	6.60
5′- TTT TTT TAT* **TC**A ATT TA-3′ 3′-AAA AAA ATA A**G**T TAA AT-5′	23 ± 3	6.65

[a] Percentage quenching (Q) of luminescence of the thymine anchored $[Ru(TAP)_2(dip)]^{2+}$ (T^*), where $Q = 1 - I/I_0 = 1 - \tau_a/\tau_{a0}$; τ_a is the pre-exponential (α) weighted average lifetime $[\tau_a = (\Sigma\alpha_i\tau_i)/\Sigma\alpha_i]$ and τ_{a0} is the corresponding value for the reference sequence 0.

[b] Calculated ionisation potential based on 6-mer model systems for the guanine residues present in the different sequences.

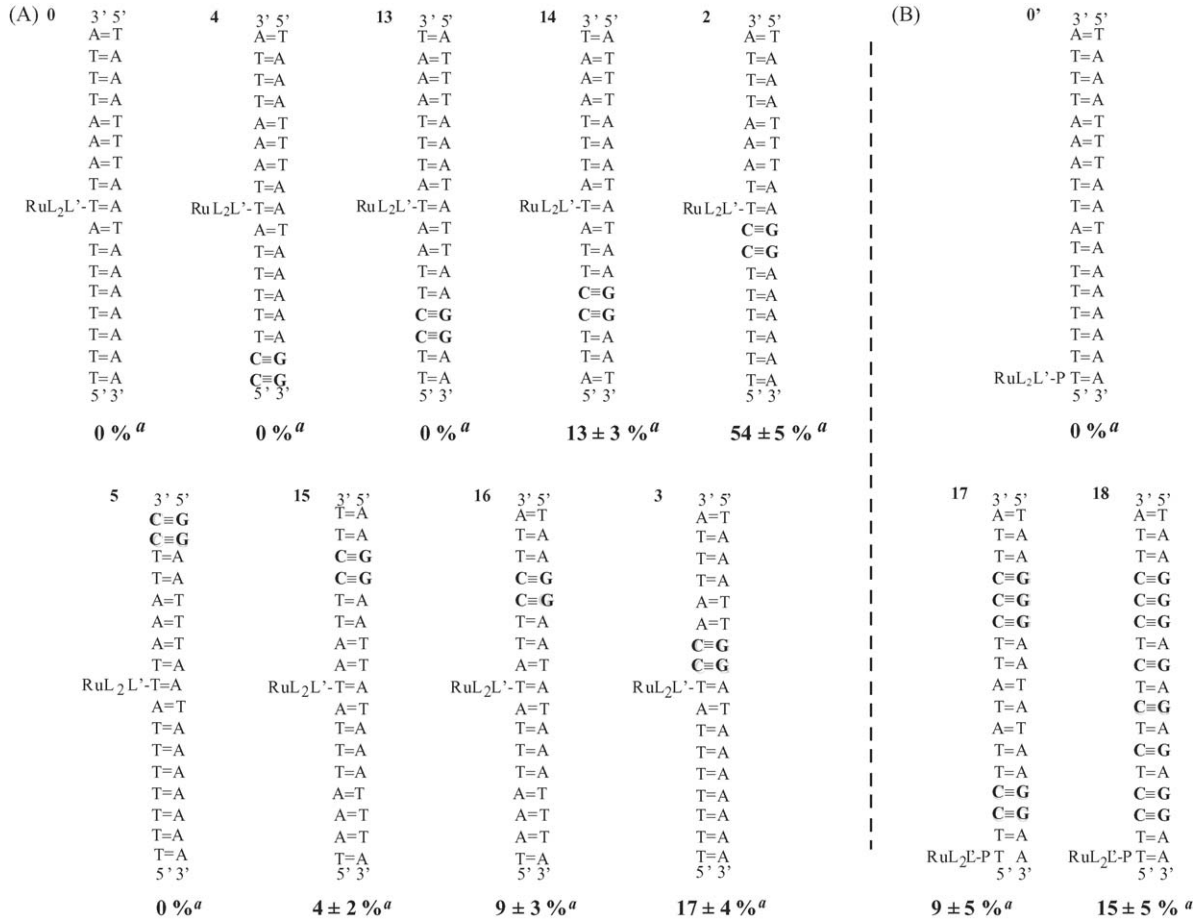

Fig. 11. The different Ru(II)-labelled duplex sequences. RuL_2L' = anchored $[Ru(TAP)_2(dip)]^{2+}$. (A) For the study of photo-crosslinking; (B) for the study of competition between photo-crosslinking and hole transfer. ([a]Percentage of ODN adduct formation. The intensity of the spot corresponding to the ODN adduct was compared to the total radioactivity observed in the experiment [99,104].)

the guanine units in the target strand, respectively) have been measured and compared to the emission intensities (the ratios I/I_0 where I_0 and I are the emission intensities in the absence and presence of the guanine units in the target strand, respectively). However, as the lifetime values depend very much on the microenvironment around the excited complex, two, three or even four different luminescence lifetime components were detected. Therefore, instead of considering each emission component, a weighted average lifetime has been used. This method was successfully applied for the determination of quenching in microheterogeneous systems such as polymers [101,102]. The comparison of the values of the ratios τ/τ_0 (in weighted average emission lifetime) and I/I_0 show that they are equal within the experimental errors [103]. Therefore, the quenching can be regarded as a pure dynamic process, which probably stems from the non-intercalative mode of interaction of the complex with the duplex sequence.

4.2.2.3. Photo-crosslinking (adduct formation). The photo-crosslinking can be detected by polyacrylamide gel electrophoresis experiments (PAGE) in denaturing conditions (7 M urea). For this purpose, the target strand has to be ^{32}P-labelled at the 5′-end for its radioactive detection. When a duplex is deposited onto a gel, after migration in denaturing conditions, the two strands are separated and one spot corresponding to the ^{32}P-labelled target strand is observed. However, if after illumination, the two strands are photo-crosslinked, they migrate together even in denaturing conditions. A spot of much lower mobility is observed and its intensity depends on the percentage of photo-crosslinking, which can thus be measured quantitatively in this way.

As the photo-induced electron transfer process leads to adduct formation, the percentage of quenching should also be correlated to the percentage of photo-adduct or photo-crosslinking. This has been verified by PAGE experiments in denaturing conditions. For the illuminated duplexes that contain guanine units closer to the attached complex than in sequences 4 and 5 (Fig. 11A), the gel analyses reveal indeed the presence of a retarded band (except for sequence 13, see further), which migrates like a duplex (i.e. a 34-mer) and originates thus from a photo-crosslinking. In contrast, for the reference sequence 0—which does not contain any guanine units—or for sequences 4 and 5—for which no emission quenching is observed—there is no indication of the presence of such a retarded band. Actually, for the other G-containing duplexes the percentage of photo-crosslinking varies from 4 to 54% (Fig. 11A) [99,104] depending

on the ionisation potential of the guanine (singlet G or doublet GG) and the distance of the guanine moiety from the attached complex. Although the percentage of photo-crosslinking can be more or less related to these factors, the data show that it depends also (for a same ionisation potential and distance of the G unit) on the direction of the G position from the attachment site, i.e. towards the 5′- or 3′-end direction of the complementary sequence (compare for example sequence 2 with sequence 3).

Moreover, interestingly, the distance that can be reached by the complex (i.e. largest number of base pairs) when the linker stretches inside the DNA groove depends on the direction of the stretching. When it stretches towards the 5′ direction of the target strand (sequence 15, still 4% photo-crosslinking), the distance is larger than towards the 3′ direction (sequence 13, no more photo-crosslinking). This longer stretching towards the 5′ side is qualitatively confirmed by computer modelling [103]. This maximum distance for which a photo-crosslinking is obtained is shorter than for observing a luminescence quenching. This can reasonably be explained since the photoreaction and subsequent formation of a covalent bond between the TAP ligand and the guanine unit should require many more constraints than emission quenching. Indeed, after the electron transfer, a close contact between the reduced metal complexes and the guanine radical cation is necessary for the bond formation. In contrast, the electron transfer is expected to be less dependent on these geometrical factors.

4.2.2.4. Complex anchored to the 5′-terminal phosphate. Although most of this work is focused on the study of complexes chemically tethered in the middle of the duplexes, other studies have also examined the behaviour of complexes anchored to the 5′-terminal phosphate (Fig. 11B). The goal in that case was to test the possible competition between a long-range hole migration in the duplex ODN (revealed, after a piperidine treatment, by cleavage at the level of the G where the hole has been trapped) [81,105] and the formation of a photo-adduct. Therefore, the duplex sequences 0′, 17, and 18 have been examined (Fig. 11 B). In the sequence 17, the two G residues of the target sequence are close to the attached complex of the probe sequence and are separated by 7 A–T or T–A base pairs from a GGG triplet. In this sequence 17, the hole injected into the GG doublet by the excited complex should not migrate towards the GGG triplet because of the presence of seven A–T (T–A) base pairs that do not allow the migration of the hole [106]. Sequence 17 under illumination should thus produce only photo-crosslinking and no GGG triplet photo-oxidation. This has indeed been observed experimentally [104]. In contrast, in sequence 18, the two G units close to the attached complex are separated from the GGG triplet by C–G base pairs alternating with T–A base pairs. The photo-injected hole into the GG doublet close to the complex can in that case either migrate and be trapped by the GGG triplet or produce an adduct by reaction with the reduced complex. Experimentally however, sequence 18 does not produce clear and well detectable cleavage at the level of the GGG triplet. This result shows that the migration of the injected hole cannot compete with the formation of photo-adduct. Therefore, the photo-adduct formation turns out to be a rather efficient process.

4.2.3. In vitro applications

The photo-adduct formation between a target sequence and a probe Ru(II)-labelled sequence could be exploited to perturb the normal functions of enzymes involved in gene expressions. Important effects of inhibition of the enzymatic functions are actually interesting in view of developing new therapeutic drugs for gene-silencing (vide supra).

In this connection, it has been shown that the function of two DNA-polymerases (Klenow fragment and polymerase β) is inhibited with 100% efficiency at the level of the photo-crosslinking between the probe Ru-labelled strand and a guanine unit of a longer oligonucleotide template [104]. This is an interesting effect since the DNA-polymerases are responsible for DNA replication during cell division. This inhibition was demonstrated in the following way. A 17-mer Ru(II)-labelled oligonucleotide was hybridised to its complementary sequence located on the 5′ side of a 40-mer matrix (template for the enzyme). After illumination, the elongation by the DNA-polymerase of a 13-mer DNA primer hybridised to the 3′-end of the same matrix, stopped at a position corresponding to the formation of the photo-adduct with an efficiency of 100% (Fig. 12).

Other experiments have also been performed with the exonuclease III (an enzyme which digests an oligonucleotide from the 3′-end). They clearly demonstrate that in that case too, this enzyme is blocked with 100% efficiency at the level of the photo-crosslinking. These results suggest then, that oligonucleotides derivatized with photoreactive Ru-TAP complexes might be promising molecular tools activated under illumination, useful in experiments of specific gene-silencing.

4.2.4. Perspectives

On the basis of the results obtained with the photo-oxidising complexes and amino acids such as trytophan as described above, other possible applications for these complexes can be considered. Indeed, as photo-electron transfer and photo-adduct formation also take place between the Ru-TAP complexes and tryptophan, photo-crosslinking could be conceivable with a target containing a tryptophan moiety. In order to test this possibility, preliminary experiments have been carried out with a

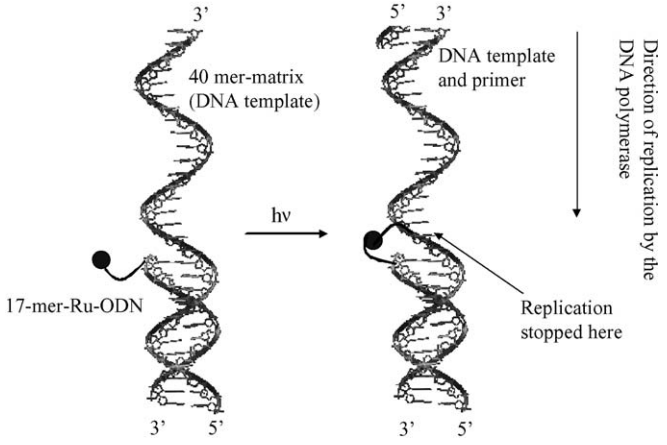

Fig. 12. Schematic representation of the inhibition of the elongation of a DNA primer by a DNA-polymerase due to photo-crosslinking.

photoreactive $[Ru(TAP)_2(phen)]^{2+}$ species attached to the 5′-phosphate end of a probe ODN sequence in the presence of a target strand. In this case, the target strand does not contain any guanine unit but is chemically anchored to its 3′-end to a tripeptide, Lys-Trp-Lys (Lys = lysine). Gel electrophoresis analyses after hybridization and illumination, show that photo-crosslinking of the two strands is observed and should be due in this case to the presence of the tryptophan unit in the tripeptide. These preliminary results could thus enlarge the field of applications of photo-crosslinking to polypeptides and proteins.

4.3. Ru(II)-labelled single-stranded oligonucleotides as probes of secondary structures

The luminescence of Ru(II) complexes (oxidising and non-oxidising compounds) are extremely sensitive to their microenvironment [107–109]. Therefore, Ru(II) complexes tethered to oligonucleotides may be used interestingly for the detection of self-structuration of single-stranded (ss) oligonucleotides. The adoption of secondary structures by single-stranded oligo- or poly(deoxy)ribonucleotides plays important roles in various biological functions of DNA/RNA biomolecules. For example, certain proteins are known to interact with specific RNA loop-structures [110,111], which are thus crucial for the recognition process. Moreover, the preparation of molecular beacons for DNA diagnostics, are based on the self-structuration of a single-stranded oligonucleotide [112,113]. However, the detection of a secondary structure has always been a difficult task and a real challenge especially in the case of short oligonucleotides. There is therefore a constant need for new methods to probe and detect self-structuration in the oligo- or polynucleotides.

Ru(II)-labelled single-stranded oligonucleotides are good candidates to explore the presence of such secondary structures adopted by the single strand because the tethered metallic moiety exhibits different luminescence behaviour depending on the microenvironment produced by the possible self-structuration.

For this purpose, $[Ru(TAP)_2(dip)]^{2+}$ derivatized single-stranded (ss) oligonucleotides have been selected from the previous list of sequences because they might form some kind of hair-pin structure by a self-pairing of a few bases (Fig. 13).

Table 3
Weighted average luminescence lifetimes (τ_a) in air-equilibrated aqueous solutions for some sequences

	Sequence	τ_a (μs)[a]
0	5′-TTTTTTTA**T***TAAATTTA-3′	0.92 ± 0.03
8	5′-TTTTTTTC**T***TAAATTTA-3′	0.89 ± 0.04
9	5′-TTTTTTCT**T***TAAATTTA-3′	0.93 ± 0.01
1	5′-CAAAACCC**T***ACCCAAAC-3′	0.66 ± 0.02
0′	5′-**T***TTTTTTATTAAATTTA-3′	0.91 ± 0.04
18	5′-**T***TCCTCTCTCTCCCTTA-3′	0.61 ± 0.01
DS 0	5′-TTTTTT TA**T***TAAATTTA-3′ 3′-AAAAAAATA ATTTAAAT-5′	1.00 ± 0.04
DS 0′	5′-**T***TTTTTTATTAAATTTA-3′ 3′-A AAAAAATAATTTAAAT-5′	0.86 ± 0.02

T^* = attachment site of the complex.
[a] Pre-exponential (α) weighted average lifetime $\tau_a = (\Sigma\alpha_i\tau_i)/\Sigma\alpha_i$.

In order to investigate the possible self-structuration of these ss sequences, the luminescence lifetimes of the anchored Ru(II) complexes were measured and compared to those of the Ru(II)-labelled ss sequences that do not have any self-complementary bases in their sequence [114]. In all the cases, the sequences do not contain any guanine residues. Consequently, no photo-induced electron transfer (and thus luminescence quenching) and subsequent adduct formation are expected.

The luminescence lifetimes of the anchored complexes should reflect some contribution of the effect of the microenvironment to the deactivation processes of the excited state. As expected, the time-resolved luminescence decay profiles for all the sequences were biexponential functions. Therefore, as explained in Section 4.2.2.2, weighted average emission lifetimes (τ_a, see definition in the table) have been used. The values are listed in Table 3 for the selected sequences. The τ_a values for sequences 0 and 0′ hybridised to their complementary strand (duplexes DS) are also given for comparison and used as references for an excited complex, which is protected from water by a double helix.

From these measurements, it turned out that all the τ_a values are close to the values obtained for the reference duplex

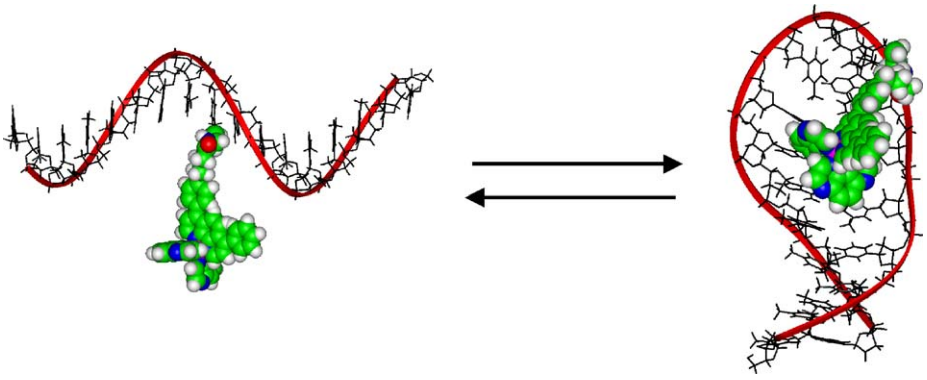

Fig. 13. Change of the microenvironment induced by self-pairing of the single-stranded oligonucleotide.

sequences 0 and 0′, except for sequences 1 and 18. Thus, this similarity between the τ_a values indicates the presence of some secondary structures adopted by the single-stranded oligonucleotides, probably due to the self-pairing of the base(s). Furthermore, only sequences 1 and 18 exhibit a τ_a lifetime that is shorter than the values obtained for the other ss sequences. This indicates that 1 and 18 are not self-structured (1 has indeed no possibility of self-pairing).

Further investigation based on careful analyses of each component of the luminescence decays, in the presence and absence of oxygen, and in a denaturing medium (7 M urea), in addition to measurements of the melting temperatures, clearly confirm the above conclusions. Moreover, the results show also that, even in the absence of base pairing due to the presence of urea, there is some protection of the excited complex probably by π stacking interaction with the nucleo-bases.

In conclusion, this detailed study of luminescence lifetimes has shown that the detection of the presence of secondary structuration is possible and that an investigation of the dynamics of folding/unfolding of single-stranded oligonucleotides can also be performed.

5. General conclusions

The interesting consequences of the excited state properties of a series of polyazaaromatic Ru(II) complexes based on the TAP and HAT ligands have been highlighted. It is explained how one can take advantage of these properties in the presence of biomolecules. Thus, under illumination, these oxidising metal complexes undergo a reductive photo-induced electron transfer by bio-reducing agents such as guanine residues or tryptophan moieties. This charge transfer process with plasmid DNA induces single-strand breaks with transformation of the ccc into the oc form as demonstrated by gel electrophoresis experiments and Atomic Force Microscopy. Moreover the elementary charge transfer process is responsible for the formation of adducts. Interestingly these adducts produced on a DNA template inhibit the RNA synthesis by RNA-polymerase. Furthermore, adducts formation has been used to photo-crosslink two single-stranded oligonucleotides. For that purpose, an oxidising complex is tethered to a probe single-stranded sequence. After irradiation in the presence of the target complementary sequence, which contains a guanine unit, the two strands are photo-crosslinked. This type of photo-crosslinking inhibits with 100% efficiency two types of enzymes: DNA-polymerase, responsible for DNA replication, and exonuclease III, capable of digesting the DNA from the 3′-end.

In conclusion, the synthetic Ru-labelled oligonucleotides could be applied for the specific detection and isolation of a targeted gene (if it contains a G unit) for rapid diagnostics. In the frame of the antigen or antisense strategy, the Ru derivatized oligonucleotides could also find future applications for the treatment of cancers by gene-silencing with specific gene targeting more particularly in dermatology. On the other hand, in the absence of photo-induced electron transfer, the use of a Ru(II)-labelled single-stranded oligonucleotide allows the detection of its possible self-structuration by measurements of the emission lifetime.

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