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ARTICLE *in* DALTON TRANSACTIONS · APRIL 2005

Impact Factor: 4.2 · DOI: 10.1039/b415898a · Source: PubMed

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Detection of secondary structures in 17-mer Ru(II)-labeled single-stranded oligonucleotides from luminescence lifetime studies†

D. García-Fresnadillo,[a] O. Lentzen,[b] I. Ortmans,[b] E. Defrancq[c] and A. Kirsch-De Mesmaeker*[b]

[a] *Department of Organic Chemistry, Faculty of Chemistry, Universidad Complutense de Madrid, Avenida Complutense s/n, E-28040 Madrid, Spain. E-mail: dgfresna@quim.ucm.es; Fax: +34 91394 4103; Tel: +34 91394 4220*

[b] *Université Libre de Bruxelles, Organic Chemistry and Photochemistry, CP 160/08, 50 Avenue F. D. Roosevelt, B-1050 Brussels, Belgium. E-mail: akirsch@ulb.ac.be; Fax: +32 2 650 30 18; Tel: +32 2 650 30 17*

[c] *LEDSS, UMR CNRS 5616, ICMG FR2607, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France. E-mail: Eric.Defrancq@ujf-grenoble.fr; Fax: +33(0)4 76 51 49 46; Tel: +33(0)4 76 51 44 33*

Received 14th October 2004, Accepted 13th January 2005
First published as an Advance Article on the web 1st February 2005

The emission properties of a non intercalating complex, $[Ru(TAP)_2(dip)]^{2+}$ (TAP = 1,4,5,8-tetraazaphenanthrene; dip = 4,7-diphenyl-1,10-phenanthroline), tethered to 17-mer single-stranded oligodeoxyribonucleotides (ODNs) either in the middle or at the 5′-end of the sequence, are determined. The results highlight the fact that the luminescence of this metallic compound is sufficiently sensitive to its microenvironment to probe self-structuration of these short single-stranded ODNs. It is shown that the weighted averaged emission lifetimes (τ_M) along with the quenching rate constants of luminescence by oxygen reflect particularly well different structures adopted by the different ODNs sequences. The determination of these parameters thus offers an elegant way to examine possible structurations of synthetic single-stranded ODNs that play important roles in biological applications.

Introduction

The self-structuration of single-stranded (ss) poly(deoxy)ribonucleotides play important roles in various biological functions of DNA/RNA biomolecules. For instance, RNA stem-loops are specifically recognized by proteins, or some U-turn structures belong to recognition units of antisense/target pairs.[1,2] Self-structuration of ss synthetic oligodeoxyribonucleotides (ODNs) can also have dramatic influences in the frame of the antigene or antisense strategies, since they compete with the formation of the triple or double helix in the recognition process. Because of this self-structuration the synthetic ss ODN is thus less efficient for its hybridisation with the target sequence.[3,4] In the same way, the structuration of ss ODNs has to be considered when molecular tools in DNA diagnostics, or in nanobiotechnology are developed.[5]

These examples illustrate the important consequences of possible self-pairing in synthetic ODNs and the need to know whether structurations such as hairpin formations exist for the synthesized ODNs. This is not always an easy task especially when one deals with short synthetic ODNs.

We have recently investigated the photophysical and photochemical behaviour of a series of 17-mer duplex ODNs where one of the strands (the probe-sequence) is derivatised with a photoreactive non intercalating Ru(II) complex, $[Ru(TAP)_2(dip)]^{2+}$ (TAP = 1,4,5,8-tetraazaphenanthrene; dip = 4,7-diphenyl-1,10-phenanthroline), tethered either in the middle or at the end of the sequence (Fig. 1).[6–12] These systems represent models for studying the photocrosslinking, which is triggered by the Ru(II) complex, between the probe-strand and target-strand. [13] Such Ru-ODN systems could be developed in the future either in the frame of the antisense strategy or as molecular tools in DNA diagnostics with however the problem related to a possible structuration as mentioned above. On the other hand, it has been

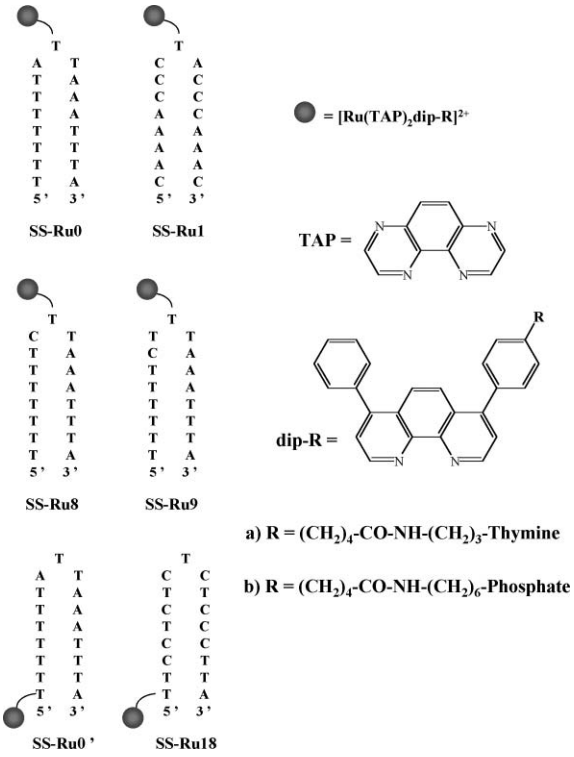

Fig. 1 The different oligonucleotides derivatised by the $[Ru(TAP)_2(dip)]^{2+}$ complex. The complex is attached either in the middle of the sequence at position 5 of a thymine (see (a)) or at the end of the strand at the 5′ terminal phosphate group (see (b)). A = adenine, T = thymine, C = cytosine (for the chemical attachment, see ref. 6–12, the numbering of the oligonucleotides has remained the same as in these references).

shown in the literature[14] that with an ODN labeled with a Ru-dppz complex, which is considered as a double-stranded DNA light-switch,[15–17] the luminescence of this complex is switched on by attachment to the ss ODN, although no intercalation

† D. García-Fresnadillo and O. Lentzen. Both authors equally contributed to this work.

DOI: 10.1039/b415898a

is predicted *a priori*. In this case, no particular self ss ODN structuration was considered but the simple attachment induces some protection of the complex from water, maybe by some π interaction with the bases.

In the present work, considering the importance which could be played by self-structuration of a ss ODN, we have selected, among the previously prepared $[Ru(TAP)_2(dip)]^{2+}$ derivatised ODNs, a few sequences. The goal is to investigate the possible effects of the ss ODN microenvironment on the luminescence of the attached complex and to evaluate whether the emission characteristics can be used for the detection of ss secondary structures.

Since self-structured ss ODNs (such as hairpin structures) may involve the formation of a few Watson–Crick base-pairs, some 17-mer sequences, which would allow formation of such base-pairs, have been selected along with some sequences that do not contain complementary bases (Fig. 1). Thus, the various conjugates with at least three possible base pairings are **SS-Ru0**, **SS-Ru8** and **SS-Ru9** (complex anchored in the middle) and **SS-Ru0′** (complex anchored at the 5′-extremity). The luminescence behaviour of these sequences is compared (i) to that of conjugates containing one or none self-complementary bases (**SS-Ru1** and **SS-Ru18**) and (ii) to that of the duplex sequences **DS-Ru0** and **DS-Ru0′** (DS = double strand), which contain thus the **SS-Ru0** or the **SS-Ru0′** hybridized to the corresponding complementary strand. The characteristics of luminescence of these double stranded ODNs can be taken as references for an excited complex which is protected from water by a double helix.

Experimental

The synthesis and purification of $[Ru(TAP)_2(dip)]^{2+}$, the preparation of the oligonucleotides, and the coupling procedures have been reported earlier.[7] The different $[Ru(TAP)_2(dip)]^{2+}$-labeled oligonucleotides were characterized by ElectroSpray Mass Spectrometry (ES-MS). **SS-Ru0**: calcd. mass 6077.5, found 6076.2; **SS-Ru0′**: calcd. mass 6213.5, found 6210.7; **SS-Ru1**: calcd. mass 5984.4, found 5982.7; **SS-Ru8**: calcd. mass 6053.5, found 6051.0; **SS-Ru9**: calcd. mass 6053.5, found 6051.8; **SS-Ru18**: calcd. mass 6057.3, found 6057.6.

The conjugate solutions (10 μM, 600 μL) were prepared by diluting the appropriate volume of stock solution (in water) in aqueous buffer (50 mM NaCl, 10 mM Tris, pH 7). The samples were left to equilibrate for 1 h and stored in the dark at −20 °C.

All measurements (denaturation curves, emission) were carried out in a 600 μL quartz cell (1.0 × 0.2 cm) UV Select (Warrington, UK) and each experiment was performed in triplicate with at least two different solutions of each conjugate, in order to evaluate the reproducibility of the experiments. The results were averaged.

The denaturation curves of single-stranded oligonucleotides were recorded on a Perkin-Elmer Lambda 40 UV/VIS Spectrophotometer (Norwalk CT, USA) equipped with a thermostated cell-holder under a nitrogen atmosphere. The temperature was controlled with a Peltier Temperature Programmer PTP-1, DBS Strumenti Scientifici (Padova, Italy). The temperature of the solutions was increased from 5 to 75 °C at a rate of 0.5 °C min^{-1} and the changes in absorption were monitored at 260 nm. The denaturation curves were analyzed with UV TempLab software package. They were also recorded with a Cary 3-Bio Spectrometer equipped with a multiple cell-holder thermostated by a Cary Temperature Controller (Mulgrave, Australia) and interfaced to a Pentium 4 PC equipped with the Cary WinUV software package containing the Scan and Thermal applications for instrument control, data acquisition and thermal analysis of nucleic acids. In this case, the temperature of the cell was decreased from 75 to 1 °C at a rate of 1 °C min^{-1} under nitrogen atmosphere. The denaturation temperatures of the oligonucleotides were calculated from the first derivative of the recorded curves.

The emission spectra were recorded at room temperature (23 ± 2 °C) on a Shimadzu RF-5001PC spectrofluorimeter (Duisburg, Germany) equipped with a Hamamatsu R928 red-sensitive photomultiplier tube. Excitation wavelengths were 379 and 422 nm and the spectra were recorded from 500–760 nm and 500–800 nm, respectively, and corrected for the photomultiplier response. The measurements of the quenching of luminescence by oxygen were carried out with a Perkin-Elmer LS-5 spectrofluorometer (Überlingen, Germany) equipped with a Hamamatsu R928 red-sensitive photomultiplier.

The emission lifetimes were measured by using the single-photon counting (SPC) technique with an Edinburgh Instruments FL900 spectrometer (Edinburgh, UK) equipped with a hypobaric nitrogen discharge lamp and a Hamamatsu R928 red-sensitive photomultiplier tube. The excitation wavelength was 379 nm and the scattered light was removed with a 420 nm cut-on filter, Coherent-Ealing 26–4267 (Auburn CA, USA). The emission monochromator was positioned at the maximum luminescence wavelength of each sample (650 nm). 10^4 counts were collected in the peak channel. The temperature of the cell holder was thermostated at 25.0 ± 2.0 °C with a Haake NB22 temperature controller (Berlin, Germany). The emission profiles were analysed using the original Edinburgh Instruments software. The decays were fitted from the peak channel to the baseline of the experimental decay. An increasing number of exponentials was used until the fit was statistically acceptable as judged by the χ^2 test (value near 1), the appearance of the weighted residuals plot, the value of the Durbin–Watson parameter, the % of weighted residuals < 3 standard deviations, and the autocorrelation plot.

The measurements with Ar and O_2 purged solutions were carried out with Ar 99.999% (Praxair, Madrid) and O_2 99.95% (Praxair, Madrid). The solutions were purged for 30 min. The parameters from the analysis of the luminescence decays in Tables 1 and 2 (τ_1,%C_1, τ_2,%C_2) result from the average of 3–4 different experiments. Therefore an estimated error can be associated for each value.

Results and discussion

Absorption and emission

As reported for the corresponding duplexes,[8] the absorption spectrum of the ss conjugates corresponds to the superposition of the absorption of the $[Ru(TAP)_2(dip)]^{2+}$ complex and that of the oligonucleotide bases.

The emission maximum of the free complex in water is 658 nm and is shifted bathochromically by 10–16 nm in the double stranded oligonucleotide depending on the anchoring position of the complex (middle or end of the sequence) and on the oligonucleotide sequence.[8] These shifts have been attributed to the protection of the tethered luminophore from water by the hydrophobic grooves of the double helix.[8] Interestingly, the emission maximum for $[Ru(TAP)_2(dip)]^{2+}$ anchored on ss oligonucleotides is shifted bathochromically by ~4–10 nm as compared to the free complex. This indicates that there is also some protection of the complex by the ss oligonucleotides, however weaker than by the duplex oligonucleotides.

Time-resolved emission of air-equilibrated solutions

The excited state lifetime of the free complex in water or tris buffer medium is 0.55 μs.[8] The time-resolved luminescence decay profiles of the conjugates (**SS-Ru0**, **SS-Ru0′**, **SS-Ru1**, **SS-Ru8**, **SS-Ru9** and **SS-Ru18**) and the reference duplexes (**DS-Ru0**, **DS-Ru0′**) in air-equilibrated buffered solutions are bi-exponential functions. The corresponding values of the lifetimes components, pre-exponential factors, and weighted averaged lifetimes (τ_M) are collected in Table 1.

Table 1 Luminescence lifetimes of the conjugates in air-equilibrated solutions[a]

	τ_1/μs	$\%C_1$	τ_2/μs	$\%C_2$	τ_M/μs
DS-Ru0	0.63 ± 0.06	29 ± 3	1.16 ± 0.10	71 ± 7	1.00 ± 0.04
SS-Ru0	0.61 ± 0.08	39 ± 11	1.12 ± 0.09	61 ± 11	0.92 ± 0.03
SS-Ru8	0.56 ± 0.05	34 ± 4	1.06 ± 0.02	66 ± 4	0.89 ± 0.04
SS-Ru9	0.66 ± 0.04	46 ± 8	1.16 ± 0.05	54 ± 8	0.93 ± 0.01
SS-Ru1	0.38 ± 0.10	13 ± 2	0.70 ± 0.01	87 ± 2	0.66 ± 0.02
DS-Ru0′	0.55 ± 0.05	31 ± 3	1.00 ± 0.10	69 ± 7	0.86 ± 0.02
SS-Ru0′	0.54 ± 0.07	23 ± 10	1.02 ± 0.02	77 ± 10	0.91 ± 0.04
SS-Ru18	0.26 ± 0.04	30 ± 2	0.76 ± 0.01	70 ± 2	0.61 ± 0.01
SS-Ru0′ + 7 M urea	0.38 ± 0.04	10 ± 2	0.82 ± 0.02	90 ± 2	0.78 ± 0.01
SS-Ru18 + 7 M urea	0.30 ± 0.04	15 ± 2	0.74 ± 0.03	85 ± 2	0.68 ± 0.03

[a] The decay profiles were fitted to a sum of exponential functions. $I(t) = \sum(B_i \exp(-t/\tau_i))$; $(i = 1, 2)$ where B_i is the corresponding preexponential factor and τ_i the discrete lifetime component. The normalized preexponential factor $\%C_i$ were calculated as $\%C_i = B_i/\sum B_i$; $(i = 1, 2)$ and the weighted averaged lifetime τ_M, as $\tau_M = (\sum B_i\tau_i)/(\sum B_i)$. The luminescence lifetime of the free complex in water or in tris buffer is 0.55 μs, and 0.63 μs in the presence of 7 M urea.

Table 2 Luminescence lifetimes of Ar-purged solutions and quenching constants of the $[Ru(TAP)_2(dip)]^{2+}$-labeled single stranded oligonucleotides[a]

	τ_1/μs	$\%C_1$	τ_2/μs	$\%C_2$	τ_M/μs	$k_{q1}/M^{-1}\,s^{-1}$	$k_{q2}/M^{-1}\,s^{-1}$
DS-Ru0	0.63 ± 0.06	25 ± 3	1.20 ± 0.11	75 ± 7	1.06 ± 0.04	3.0×10^8	2.3×10^8
SS-Ru0	0.61 ± 0.02	26 ± 2	1.11 ± 0.01	74 ± 2	0.98 ± 0.01	4.7×10^8	3.0×10^8
SS-Ru8	0.55 ± 0.17	30 ± 11	1.13 ± 0.04	70 ± 11	0.95 ± 0.01	4.6×10^8	3.0×10^8
SS-Ru9	0.79 ± 0.07	54 ± 14	1.32 ± 0.09	46 ± 14	1.03 ± 0.01	3.8×10^8	4.3×10^8
SS-Ru1	—	—	0.78 ± 0.01	100 ± 1	0.78 ± 0.01	6.3×10^8	8.5×10^8
DS-Ru0′	0.51 ± 0.05	16 ± 2	1.03 ± 0.10	84 ± 8	0.94 ± 0.02	5.8×10^8	4.0×10^8
SS-Ru0′	0.61 ± 0.03	21 ± 2	1.12 ± 0.01	79 ± 2	1.01 ± 0.01	5.5×10^8	4.1×10^8
SS-Ru18	0.32 ± 0.01	30 ± 1	0.90 ± 0.01	70 ± 1	0.72 ± 0.01	5.5×10^8	10.0×10^8

[a] The luminescence lifetime of the free complex in Ar-saturated water is 0.70 μs and the corresponding bimolecular quenching constant is $1.3 \times 10^{+9}$ $M^{-1}\,s^{-1}$. k_{q1} = bimolecular quenching constant by oxygen obtained from steady-state emission measurements. k_{q2} = bimolecular quenching constant by oxygen obtained from time-resolved emission measurements.

The weighted averaged lifetime (τ_M) is a good parameter for characterizing complicated luminescence decays in microheterogeneous media.[18] For the **SS-Ru0**, **SS-Ru0′**, **SS-Ru8** and **SS-Ru9** conjugates, the τ_M values range from 0.89 μs to 0.93 μs and are close to the values found for the reference duplexes (**DS-Ru0** and **DS-Ru0′**, 1.00 and 0.86 μs, respectively). This suggests that the excited state of the attached Ru(II) complex in these ss oligonucleotides is protected from its deactivation by water or oxygen and this protection is quasi as efficient in the single strands as in the duplexes **DS-Ru0** or **DS-Ru0′**. This would indicate that some secondary structures would be adopted by **SS-Ru0**, **SS-Ru0′**, **SS-Ru8** and **SS-Ru9**.

In contrast, for the conjugates **SS-Ru1** and **SS-Ru18**, the τ_M values are 0.66 and 0.61 μs, respectively, thus slightly longer than the lifetime of the luminophore in water or tris buffer medium (0.55 μs) but much shorter than the values determined for the other ss sequences or duplexes. Consequently, this would indicate a lack of secondary structure in these ss ODNs.

As developed below, a closer examination of the values of the lifetime components in Table 1 strengthens these conclusions and provides complementary information. The bi-exponential profile of the emission decays of the conjugates **SS-Ru0**, **SS-Ru0′**, **SS-Ru8** and **SS-Ru9** is similar to that of the duplexes **DS-Ru0** and **DS-Ru0′**. In these two categories of ODNs (SS and DS) the emitting species occupy thus at least two different sites that would correspond to two different degrees of protection from water. Actually one can imagine two situations for the self structuration of the ss oligonucleotides, which would result in lifetime values similar to those in the double strands. (i) The ss conjugates could all be present in the form of hairpin structures with some pairing of self-complementary bases. In such a case, the situation would be quite similar to that of a double stranded helix. Thus the excited species with the longer lifetime (between 1.00 and 1.16 μs) would be protected by the stacking of hydrophobic base pairs, exactly like in a duplex.[8] In this situation there would be only one form present (hairpin structure). (ii) The ss conjugates could be present in two forms *i.e.* an unstructured ss oligonucleotide (or a random-coil form), in equilibrium with a structured form like a hairpin structure. In this second case, the two forms should be in slow equilibrium as compared to the kinetic rate constants controlling the emission lifetimes. Indeed this condition has to be fulfilled because a biexponential decay is observed. If the two forms were in fast equilibrium with a relaxation time constant for the equilibrium shorter than the lifetime of the excited species, the decays should correspond to single exponentials (the excited species should feel an averaged environmental influence), which is not the case. Consequently if there is an equilibrium, this latter has to be slow, which would explain that denaturation-like curves can be measured for these ODNs (see below). Since the conjugates **SS-Ru0**, **SS-Ru0′**, **SS-Ru8** and **SS-Ru9** had been chosen because they contain self-complementary bases susceptible to form Watson–Crick hydrogen bonds, these sequences are indeed susceptible to adopt a hairpin-like secondary structure responsible for the longer emission lifetime components.

Interestingly, the **SS-Ru1** and **SS-Ru18** conjugates, which had been chosen for their non-capability to form base-pairs, exhibit different lifetime characteristics. For these conjugates, the long-lived component reaches only 0.70 and 0.76 μs, which indicates little protection from the aqueous phase. On the other hand, the short-lived component is shorter than the lifetime of the excited Ru(II) complex in water. This unexpected result, which obviously cannot be attributed to a luminescence quenching, could be attributed to an equilibrium, in the same time scale as the deactivation of the excited state, between conformations adopted by the labeled ss oligonucleotides and which would be close to random-coil forms. Thus for **SS-Ru1** and **SS-Ru18**, the rate constants associated to the equilibrium between the different forms would be in the same order of magnitude as the decay constants of the excited state of the anchored complex. Actually, in such a case, two pseudo-lifetimes are measured and their inverses (*i.e.* the rate constants) correspond to a complicated combination of

the kinetic constants associated to the equilibrium and the excited state deactivation. A fully resolved kinetic system, which corresponds to this case, is given in ref. 19. In such a situation, the lifetimes of the biexponential decay correspond thus to combinations of the excited states lifetimes of the species and the lifetimes related to the equilibrium between these species.[19]

Time-resolved emission in the presence of urea

To test the possible structuration of ss oligonucleotides, time-resolved emission measurements were carried out in denaturing conditions (7 M urea). The conjugates **SS-Ru0′** and **SS-Ru18** have been chosen for this study because they represent the two different situations considered for the emission lifetimes.

The results are presented in Table 1 and should be compared with the emission lifetime of the free complex with 7 M urea *i.e.* 0.63 μs. The weighted averaged lifetime τ_M for the conjugate **SS-Ru0′** is shortened by the presence of urea. This result agrees with the denaturation of a secondary structure of the oligonucleotide by urea since the complex should be less protected in the presence of a denaturing agent. Moreover, the shorter component of the emission lifetime for this conjugate decreases from 0.54 μs (without urea) to 0.38 μs in the presence of urea, which is much shorter than the lifetime of the free complex (0.63 μs with urea). This would indicate that the disappearance of the secondary structure due to the denaturing agent induces the appearance of equilibriums between different conformations with an associated relaxation time in the same order of magnitude as the excited state lifetime so that, as explained above, the discrete emission lifetime components do not correspond anymore to real excited state lifetimes.

For the conjugate **SS-Ru18**, the weighted averaged lifetime τ_M with urea is 0.68 μs, slightly longer than without urea (0.61 μs). This suggests that even without urea, there is no structuration of the oligonucleotide. The fact that two lifetime components are again detected even with urea indicates the existence of an equilibrium between different conformations.

The slightly longer τ_M values in presence of urea for both **SS-Ru0′** (0.78 μs) and **SS-Ru18** (0.68 μs) than the luminescence lifetime of the free complex with urea (0.63 μs)[20] could be indicative of some remaining π stacking between the bases and the ligands of the complex, even with urea.

Quenching by oxygen

To demonstrate the higher degree of protection experienced by the Ru(II) complex when it is tethered to single-stranded oligonucleotides with secondary structure as compared to unstructured ODNs, oxygen quenching experiments were carried out. The emission lifetimes for argon-purged solutions and the bimolecular quenching constants by oxygen calculated from steady state and time-resolved emission measurements under air and argon are gathered in Table 2.

An increment of about 6% in the τ_M values is measured for the oxygen-free solutions of **DS-Ru0**, **SS-Ru0**, **SS-Ru8** and **SS-Ru9** (Ru(II) complex tethered to the middle of the strand and therefore less exposed to the aqueous environment), while this increment reaches 10% for **DS-Ru0′** and **SS-Ru0′** (Ru(II) complex tethered to the 5′ extremity and therefore more exposed to the aqueous environment). In contrast to these 6–10% increments, the increment of lifetime reaches 18% for **SS-Ru1** and **SS-Ru18**. These results show that the sequences, which cannot form a secondary structure (**SS-Ru1** and **SS-Ru18**), do not protect the excited Ru(II) complex efficiently from quenching by oxygen and therefore show the highest increments of their weighed averaged emission lifetime (τ_M) from an air to an argon saturated solution. In contrast, the luminophore in **SS-Ru0**, **SS-Ru8** and **SS-Ru9** and **SS-Ru0′** has a good degree of protection from quenching by oxygen (thus weak increment of the τ_M values from air to argon saturated solutions), which is similar to that experienced by the excited Ru(II) complex attached to the double strands.

Melting temperatures

Since the denaturation of secondary structures in nucleic acids is usually accompanied by an increase of the absorption at 260 nm, it should be possible to test the base-pairing of the ss Ru-derivatised oligonucleotides by the measurement of the melting temperatures (T_m).

Fig. 2 shows the curves obtained for the different conjugates; the curves are normalized by the corresponding initial absorption.

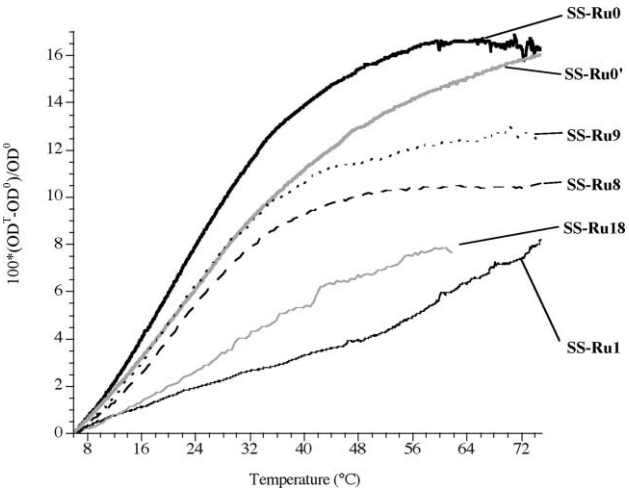

Fig. 2 Denaturation curves measured for the different single-stranded conjugates. The curves were normalized by the absorption at 1 °C (OD^T = optical density at temperature T; OD^0 = optical density at 1 °C).

In the case of conjugates **SS-Ru0**, **SS-Ru0′**, **SS-Ru8** and **SS-Ru9**, the curves are characteristic of a denaturation of a secondary structure. The corresponding hyperchromicity is of course weaker than in the case of the duplex oligonucleotides. The denaturation temperature is about 20 °C for these ss ODNs (Table 3) as compared to 40 °C for the **DS-Ru0**. This low temperature indicates the possibility of equilibrium between the structured form (hairpin) and a random-coil form of the conjugates at room temperature.

In contrast, the increase of absorption with temperature is quasi linear in the case of conjugates **SS-Ru1** and **SS-Ru18** and does not allow the determination of a denaturation temperature. This confirms that these conjugates do not form stable structures at room temperature and that the two emission lifetimes for these two conjugates originate from fast equilibrium between random-coil forms in the time-scale of the emission lifetime.

Conclusions

The results show that the determination of characteristics such as the emission lifetimes of ss Ru-labeled oligonucleotides, in the absence and in the presence of urea or oxygen offers an elegant way to investigate the possible secondary structurations and dynamics of ss oligonucleotides. As compared to denaturation

Table 3 Melting temperatures (T_m) of the conjugates

Conjugate	T_m/°C
SS-Ru0	20 ± 1
SS-Ru8	21 ± 3
SS-Ru9	21 ± 3
SS-Ru1	—[a]
SS-Ru0′	27 ± 2
SS-Ru18	—[a]

[a] Not measurable.

curves, more detailed information on the structuration can be obtained, for example even a simple π stacking of the bases in the ss **ODN** can be detected. These data could be of significant value in studies where such Ru-derivatised oligonucleotides would be applied in the frame of the antigene or antisense strategies or as diagnostic tools in DNA studies.

Acknowledgements

D. G.-F. is grateful to Dr E. Jiménez Hernández for helpful discussions, and to Complutense University of Madrid (Spain) and the European T.M.R. program (ERBFMRXCT980226) for a postdoctoral grant. A.K.D. and O.L. thank the ARC (Action de Recherche Concertée, 2002–2007) for financial support. E.D. and A.K.D are also grateful to the Laboratoire Européen Associé (FNRS-Belgium and CNRS-France) for their support.

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