Oligonucleotide Labels

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A Photoreactive Ruthenium(II) Complex Tethered to a Guanine-Containing Oligonucleotide: A Biomolecular Tool that Behaves as a "Seppuku Molecule"**

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The design of specific DNA or RNA damaging agents may be achieved by anchoring a reactive species to an oligonucleotide (ODN) probe which, by duplex or triplex formation, should direct the irreversible chemical modification towards the targeted sequence.[1] This approach is certainly interesting in the context of gene silencing, particularly for the development of anticancer agents.[2] However, the achievement of a high level of selectivity remains a challenge because many side reactions such as interaction with proteins can occur in biological systems.[3]

In this context, the tethering of a transition metal complex to an ODN strand and subsequent activation by light is an attractive strategy. Indeed, the reactivity of the metallic compound towards the genetic material can be 1) triggered by light to act on specific tissues and 2) directed towards a DNA or RNA target sequence to cause damage.[4] We have prepared oligonucleotides labeled with ruthenium(II) complexes (Ru-ODNs) that are able to photo-cross-link with their complementary strand under visible irradiation (Figure 1 a).[5] The different parameters that govern the photo-cross-linking efficiency have been extensively studied,[5,6] these conjugates could be promising as anticancer agents based on gene silencing. Indeed, this sequence-specific photo-cross-linking process has been shown to inhibit in vitro DNA polymerase with a high efficiency.[6] The prerequisites for this photochemical reaction are 1) the presence of at least two π-deficient polyazaaromatic ligands such as 1,4,5,8-tetraaza-

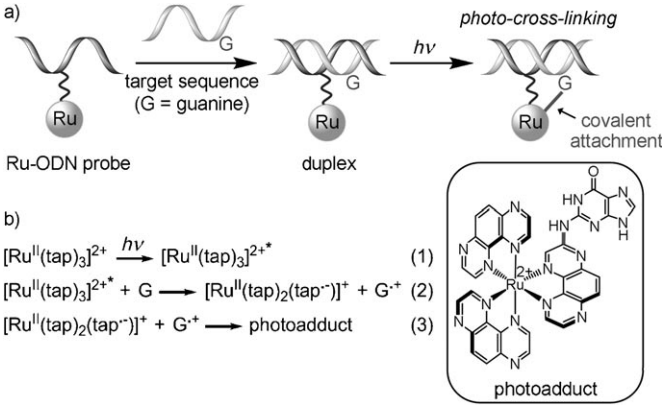

Figure 1. a) Photo-cross-linking processes that occur between an ODN probe labeled with a suitable Ru^{II} complex and the complementary G-containing ODN strand and b) formation and structure of the photoadduct produced upon illumination of $[Ru(tap)_3]^{2+}$ in the presence of a guanine residue in GMP (guanosine-5′-monophosphate) or DNA after acid hydrolysis.

phenanthrene (tap) in the tethered metallic complex and 2) the presence of at least one guanine base (G) in the target sequence, in the vicinity of the tethered complex after hybridization. The mechanism involves a photoinduced electron transfer (PET) from the G base towards the oxidizing excited Ru^{II} compound with a recombination of the produced radicals, which covalently links one of the tap ligands to the G unit [Figure 1 b, Eqs. (1)–(3)]. The structure of the photoadduct (Figure 1 b) has been determined by ESI mass spectrometry and NMR spectroscopy.[7]

This strategy was thought to be restricted to target sequences without cytosine in order to avoid the presence of a guanine in the Ru-ODN probe, which would be unfavorable for the photo-cross-linking. In spite of these limitations, we decided to investigate the effect of the presence of a G base in the Ru-ODN probe sequence on the photoreactivity of such systems. Herein, we report the unique photochemical behavior of a new generation of photoreactive G-containing Ru-ODN probes that can self-inhibit in the absence of their specific target strands. We have named these molecules "seppuku molecules" because their photochemical behavior reminds us of the suicide of someone who has not accomplished his attributed duty in antic Japanese society.

The Ru^{II} complexes contain a phenanthroline ligand derivatized by a linker, namely phen″ (phen″ = *N*-(2-(1,10-phenanthrolin-5-ylamino)-2-oxoethyl)-2-(aminooxy)aceta-

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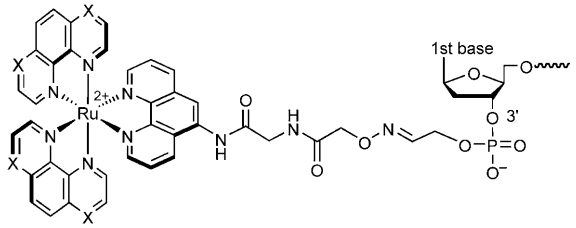
mide), which allows their tethering at the 3′ end of the modified ODNs. The two polyazaaromatic Ru^{II} complexes, $[Ru(tap)_2phen'']^{2+}$ (Ru(T)) and $[Ru(phen)_2phen'']^{2+}$ (Ru(P)), were anchored on a 14-mer ODN strand with or without a guanine base ($ODN_{(G)}$ and $ODN_{(T)}$, respectively), to afford the conjugates Ru(T)-$ODN_{(G)}$, Ru(P)-$ODN_{(G)}$, and Ru(T)-$ODN_{(T)}$ (Figure 2).[8,9] The other polyazaaromatic ligands are



- The three Ru-ODN sequences (3′→5′):

Ru(T)-$ODN_{(G)}$: TAC CAC TC**G** TTC CC
Ru(P)-$ODN_{(G)}$: TAC CAC TC**G** TTC CC
Ru(T)-$ODN_{(T)}$: TAC CAC TCT TTC CC

- $ODN_{(G)}$ complementary sequence (5′→3′):

Targ1: AT**G** **GTG** AGC AA**G** **GG**

- Noncomplementary target ODN sequences (5′→3′):

Targ2: TAA ATT TAA **GG**A AAA AA
Targ3: **GG**C T**G**A **GAG** **GG**T AA
Targ4: AT**G** **GTT** ATC AA**G** **GG**

Figure 2. Ruthenium complexes chemically tethered to the 3′ end of the probe ODN strands and the target sequences used in this study.

either two 1,10-phenanthroline (phen) or two tap ligands, whose difference in π-deficient character allows the tuning of the oxidizing power of the resulting metal complex. Hence, a photoreaction could be expected with Ru(T)-$ODN_{(G)}$. The two other conjugates, Ru(P)-$ODN_{(G)}$ and Ru(T)-$ODN_{(T)}$, were used as controls because, in these cases, the two prerequisites (that is, a photoreactive complex and a guanine unit, respectively) for the above-mentioned photoadduct formation are not fulfilled.

Luminescence lifetime measurements of the tethered Ru^{II} complexes were first carried out with single-strand ODN in buffered aqueous solution (Table 1). The weighted average lifetime (τ_m) for Ru(T)-$ODN_{(G)}$ is shorter than the lifetime of the corresponding free Ru^{II} complex (τ_0; Table 1, entry 3), with a short component of 89 ns. In contrast, neither τ_{tm} nor the discrete lifetime components are shorter than the corresponding τ_0 values for Ru(P)-$ODN_{(G)}$ and Ru(T)-$ODN_{(T)}$ (Table 1, entries 1,2).[10] These data show clearly that no PET, which is responsible for the luminescence quenching [Figure 1b, Eq. (2)], occurs for Ru(P)-$ODN_{(G)}$ and Ru(T)-$ODN_{(T)}$ but occurs only for Ru(T)-$ODN_{(G)}$. The possible production of photoadducts from Ru(T)-$ODN_{(G)}$ was monitored by

Table 1: Luminescence lifetimes of the Ru-ODNs conjugates.[a]

Entry	Conjugate	τ_1[b][ns] (C_1)[c]	τ_2[ns] (C_2)	τ_3[ns] (C_3)	τ_m[d][ns]	τ_0[e][ns]
1	Ru(P)-$ODN_{(G)}$	1224 (17)	555 (83)	–	669	540
2	Ru(T)-$ODN_{(T)}$[f]	705 (44)	407 (56)	–	538	411
3	Ru(T)-$ODN_{(G)}$	708 (10)	301 (62)	89 (28)	282	725

[a] Measured in tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl) buffer (10 mM, pH 7) and NaCl (150 mM). [b] The decay profiles were fitted according to $I(t)=\Sigma(B_i\exp(-t/\tau_i))$, $(i=1, 2, 3)$. B_i are the corresponding pre-exponential factors and τ_i the discrete lifetime components. [c] Normalized pre-exponential factor: $\%Ci=B_i/\Sigma B_i$, $(i=1, 2, 3)$. [d] Pre-exponential weighted average lifetime: $\tau_m=\Sigma(B_i\tau_i)/(\Sigma B_i)$. [e] Lifetime of the free complex. [f] Measured in oxygen-saturated solution.

UV/Vis absorption spectroscopy. Upon visible irradiation of Ru(T)-$ODN_{(G)}$, an hyperchromic effect around 400 nm was observed, whereas such a change was not detected with Ru(P)-$ODN_{(G)}$ and Ru(T)-$ODN_{(T)}$ (see the Supporting Information). This hyperchromic effect is characteristic of the formation of a photoadduct that arises from the presence of a G base in the sequence.[11]

The formation of photoadducts was further investigated by polyacrylamide gel electrophoresis (PAGE) experiments. Thus, Ru(T)-$ODN_{(G)}$ was 5′-end ^{32}P-labeled, then loaded onto a gel after increasing irradiation times (Figure 3, lanes 1–6). The starting Ru-ODN (lane 1) was found to be transformed as a function of illumination time into a new product, which was observed on the gel as a faster migrating band (lanes 2–6). This result suggests the formation of a less sterically hindered ODN such as a cyclic ODN (cycRu(T)-$ODN_{(G)}$), which arises from an intramolecular photoreaction of Ru(T)-$ODN_{(G)}$ (Figure 4a). Despite the distance (eight base units) between the two reactive intermediates (that is, the attached reduced

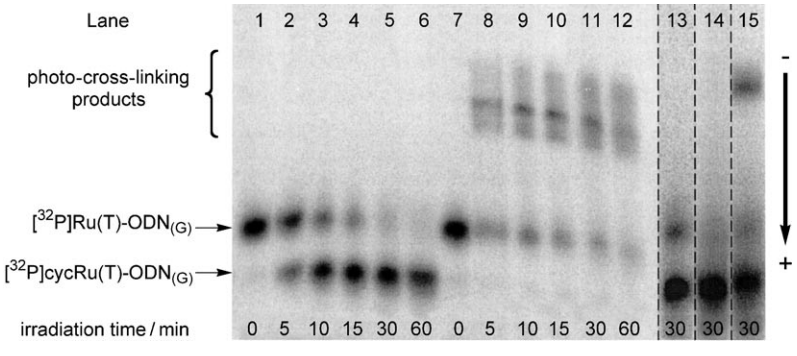


Figure 3. PAGE monitoring of the photochemical behavior of Ru(T)-$ODN_{(G)}$ in the absence (lanes 1–6) and in the presence (lanes 7–12) of the complementary target strand (Targ1, 1.1 equiv). The Ru-ODN strand was 5′-end ^{32}P-labeled, the gels were performed in denaturing conditions (urea, 7 M), and the samples in aqueous buffer solution (4 μM in Tris-HCl 10 mM, NaCl 150 mM, pH 7) were irradiated for up to 60 minutes with a laser source ($\lambda=442$ nm). Lanes 13, 14, and 15 correspond to Ru(T)-$ODN_{(G)}$ that was irradiated for 30 minutes in the presence of noncomplementary sequences (1.1 equiv) Targ2 at 22 °C with NaCl (150 mM) and Targ3 and Targ4 at 33 °C with NaCl (50 mM, which is closer to cellular conditions), respectively.

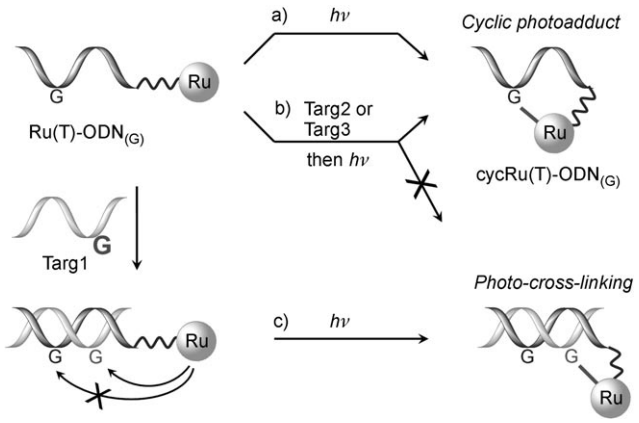


Figure 4. Schematic representation of the photochemical behavior of Ru(T)-ODN$_{(G)}$ in the absence (a) and in the presence (b, c) of G-containing ODN target strands, which illustrates a "seppuku process".

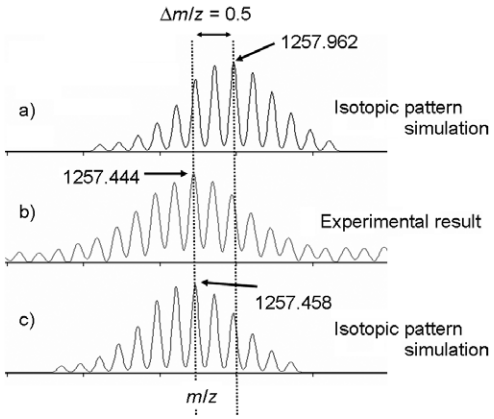


Figure 5. Mass spectrometric analysis of Ru(T)-ODN$_{(G)}$ after irradiation: nano-ESI mass spectrum (positive ion mode) of the crude reaction mixture. Expanded peaks for the isotopic distribution patterns at m/z 1257 (quadruply charged species): a) isotopic pattern simulation for Ru(T)-ODN$_{(G)}$, b) mass spectrum obtained after irradiation of Ru(T)-ODN$_{(G)}$, and c) isotopic pattern simulation for cycRu(T)-ODN$_{(G)}$.

complex $[Ru(tap)(tap^{\cdot-})(phen'')]^{+}$ and oxidized guanine unit $G^{\cdot+}$), the yield of formation of this new photoadduct reached 80% after an irradiation time of 30 minutes (see the Supporting Information). Moreover, no other band was observable, which indicates a very selective and highly efficient reaction process. In contrast, the faster band was not observed upon irradiation of Ru(P)-ODN$_{(G)}$ and Ru(T)-ODN$_{(T)}$ under the same conditions (see the Supporting Information).

Other experiments were conducted with Ru(T)-ODN$_{(G)}$ under denaturing conditions (urea, 7 M) during the illumination. The luminescence lifetimes (see the Supporting Information) indicated, even under these conditions, the presence of a luminescence quenching and the corresponding PAGE experiments confirmed the formation of cycRu(T)-ODN$_{(G)}$ (data not shown). Consequently, a self-organization of the single-strand ODN is not required for this intramolecular photoreaction to occur.

The cyclic photoproduct cycRu(T)-ODN$_{(G)}$ was further characterized by nano-ESI mass spectrometry. A mass spectral analysis was performed on the crude mixture obtained after irradiation of Ru(T)-ODN$_{(G)}$ under the same conditions as those described for the PAGE experiments (see the Supporting Information). Figure 5 shows the comparison between the experimental result (Figure 5b) and two isotopic pattern simulations (Figure 5a,c) that correspond to the calculated positions of quadruply charged cations derived from Ru(T)-ODN$_{(G)}$ and cycRu(T)-ODN$_{(G)}$. The $\Delta m/z$ value of 0.5 between these two simulated data arises from the production of a covalent bond in the cyclic species and the inherent loss of two hydrogen atoms (Figure 1b). Hence, a very good agreement with the mass spectrum simulated for the quadruply charged species of cycRu(T)-ODN$_{(G)}$ is observed after illumination of Ru(T)-ODN$_{(G)}$ (Figure 5b,c), which clearly confirms the hypothesis of formation of an intramolecular photoadduct (Figure 4a).

In order to test whether Ru(T)-ODN$_{(G)}$ could still behave as specific photoreagent of a target ODN strand for gene silencing, we examined its photochemistry as a probe in the presence of different G-containing ODN targets (Figure 2), among which only Targ1 corresponds to the complementary sequence. Thus, when Ru(T)-ODN$_{(G)}$ was hybridized with Targ1, then illuminated and afterwards analyzed by PAGE experiments under denaturing conditions, only retarded bands were observed (Figure 3, lanes 7–12). Following our previous work,[5,6] these bands were assigned to the formation of photo-cross-linked strands, which arise from an intermolecular reaction between one of the tap ligands of the probe sequence and a guanine base of the complementary sequence, in the vicinity of the complex after hybridization (Figure 4c).[12] The yield of this interstrand reaction reaches 80% after an illumination time of 30 minutes (the same yield as for the non-G-containing Ru(T)-ODN$_{(T)}$ with its complementary sequence). Interestingly, no trace of the adduct cycRu(T)-ODN$_{(G)}$ was observed, which shows that the intramolecular process does not take place in the presence of the G-containing complementary strand. Thus, Ru(T)-ODN$_{(G)}$ does not self-inhibit upon illumination in the presence of its specific target and should therefore be suitable for gene silencing applications. This highly selective process, which favors inter- over intramolecular reactions, can be attributed to the fact that, upon duplex formation, the Ru-ODN strands remain in a more rigid helical conformation in which the G units of the probe cannot be reached by the Ru^{II} complex.[9]

Finally, in order to test the specificity of the photoreaction (that is, inter- over intramolecular selectivity), we used G-containing ODNs as nonspecific potential targets, which differ in their degree of complementarity with the probe sequence (Figure 2). Figure 3, lane 13 shows that no photo-cross-linking was detected after irradiation of Ru(T)-ODN$_{(G)}$ with Targ2. Very interestingly, the gel reveals the exclusive formation of cycRu(T)-ODN$_{(G)}$ (80% yield, Figure 4b), that is, the photoreactive probe Ru(T)-ODN$_{(G)}$ self-inhibits in the presence of an "incorrect" target.

We tested also nonspecific targets at 33 °C in NaCl (50 mM), which is closer to cellular conditions. With the scrambled sequence Targ3, which contains the same number of G bases as the nonscrambled target, again no photo-cross-

linking was detected (lane 14). With the sequence Targ4, which contains just two mismatches, only weak photo-cross-linking (20%) was observed and most of the Ru(T)-$ODN_{(G)}$ (60%) self-inhibited (lane 15). This unique "seppuku process" is of particular interest for in vivo applications since it is expected to reduce or avoid the appearance of undesired secondary photoeffects with proteins for example.[13]

In conclusion, the combination of a photoreactive Ru^{II} complex with a G-containing ODN probe has led to unique photochemical behavior with an interesting potential application not only in the context of gene silencing but also for in vitro experiments to detect mismatches. This work could constitute the basis of a new type of "intelligent" biomolecular tools, namely "seppuku molecules", which use their photoreactivity against themselves if they do not find their specific target.

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[9] The position of the guanine base in the sequence of Ru(T)-$ODN_{(G)}$ was chosen such as not being directly accessible by the metal complex without a folding of the strand.

[10] In these cases, the presence of long-lived components, that is, longer than τ_0, indicates a protection of the excited state from the aqueous phase, which suggests that the Ru-ODN adopts a secondary structure, see: D. García-Fresnadillo, O. Lentzen, I. Ortmans, E. Defrancq, A. Kirsch-De Mesmaeker, *Dalton Trans.* **2005**, 852 – 856.

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