Luminescent conjugates between dinuclear rhenium(I) complexes and peptide nucleic acids (PNA) for cell imaging and DNA targeting†

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New luminescent dinuclear rhenium(I) tricarbonyl complex–PNA conjugates have been synthesized through a reliable solid-phase synthetic methodology. Their photophysical properties have been measured. The most luminescent Re–PNA conjugate 7 showed interesting two-photon absorption (TPA) properties, that were exploited for imaging experiments, to demonstrate its easy uptake into living cells.

Peptide nucleic acid (PNA) is a DNA mimic,[1] in which *N*-(2-aminoethyl)glycine units form a pseudo-peptide chain bearing the four nucleobases. Because of its neutral chain, PNA exhibits stronger and more selective binding affinity for complementary nucleic acid (DNA and RNA) strands than natural nucleic acids. PNA also shows higher mismatch selectivity and noticeable chemical and enzymatic stability compared to natural oligonucleotides.[2]

The above advantages have addressed many research efforts to the use of PNA for DNA detection, by providing PNA of appropriate analytical probes (electrochemical,[3,4] fluorescent,[5] radioactive,[6] *etc.*). Recently some of us have used magnetic PNA as biosensor for the rapid and reliable assessment of DNA hybridization by T_2 relaxation measurements.[7] In any case luminescence detection is one of the most widely used techniques to identify the DNA/PNA hybridization event.[5]

Some of us are currently developing a novel family of dinuclear tricarbonyl rhenium(I) derivatives, of general formula $[Re_2(\mu\text{-}X)(\mu\text{-}Y)(\mu\text{-}1,2\text{-diazine})(CO)_6]$, which exhibit intense emission in the range 550–620 nm, originating from triplet metal-to-ligand-charge transfer (^{3}MLCT) excited states.[8–10] Thanks to their photophysical properties, this new family of luminescent complexes appears a promising tool for PNA labelling, in view of biomedical applications. In addition, these complexes show the characteristic intense IR absorption bands due to the stretching of the CO ligands, about 2000 cm^{-1}, that allows the use of organometalcarbonyl compounds as bioprobes.[11]

A number of metal complexes have been conjugated to PNA oligomers with the aim of imparting to them new biochemical and spectroscopic properties.[3,4,12,13] Some examples of mononuclear rhenium complex–PNA conjugates have been reported in the literature[14–16] but, as far as we know, no luminescent and no dinuclear rhenium complexes have been used in PNA labelling.

In the present work we focused our attention on two dinuclear rhenium(I) complexes, namely **1** and **2** of Fig. 1, containing a carboxyl group on the diazine ligand, for the conjugation to PNA oligomers through an amide bond with the terminus NH_2 group. A reliable solid-phase synthetic methodology has been established, which provided a luminescent rhenium–PNA decamer conjugate, whose easy uptake into living cells has been demonstrated by imaging through two-photon excitation.

The new complexes **1** and **2**, which are air stable orange and yellow solids, were prepared in 60% and 90% yields, respectively, by refluxing $[Re(CO)_5Cl]$ with 0.5 equivalents of the corresponding diazine for 2 h in toluene solution.[9,10] The pyridazine-4-carboxylic acid ligand is commercially available, while the 4-(pyridazin-4-yl)butanoic acid ligand was prepared by a [4+2] Diels–Alder cycloaddition reaction between 1,2,4,5-tetrazine and 1-hexynoic acid.[17] In order to find the best conditions for binding these complexes to a PNA oligomer, the model reactions between the thymine monomer **3** and complex **1** or the free pyridazine-4-carboxylic acid ligand were studied (see ESI for details), showing that the best route to obtain the conjugate **4** consists in binding the free diazine ligand to the PNA monomer **3** to give the modified monomer **5**, which was then reacted with $[Re(CO)_5Cl]$ (Scheme 1).

Therefore this procedure was used for preparing the rhenium PNA decamer conjugates **6** and **7** (Scheme 2). The PNA homo-thymine decamer was prepared by an automated solid phase synthesis,[2] using standard Boc-procedure conditions (see ESI). Then the corresponding free diazine ligands were manually coupled to the terminal NH_2 group of

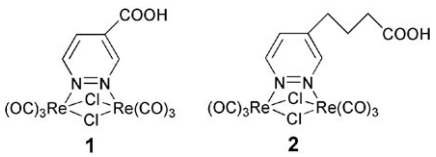

Fig. 1 Structure of complexes **1** and **2**.

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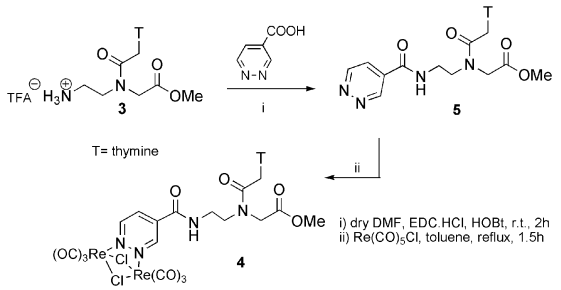
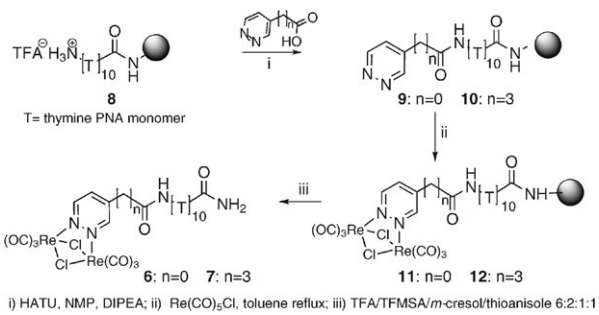


Scheme 1 Synthesis of the Re–PNA monomer conjugate **4**.

the resin-supported PNA **8**, by forming the amide bond with HATU as the condensing agent and DIPEA, in NMP as the solvent. The resin supported PNA decamers **9** and **10**, bearing terminal diazine ligands, were then reacted with an excess of $[Re(CO)_5Cl]$ in refluxing toluene. The results of this solid phase complexation were excellent and the supported Re–PNA decamer conjugates **11** and **12** could be easily purified by washing the resin with toluene.

Thanks to the stability of these Re complexes (which was checked by treating **1** with a TFA/TFMSA solution from which it was recovered unchanged), the final cleavage from the resin was carried out in the standard acidic conditions.[2] Therefore, **11** and **12** were treated with TFA/TMSA/*m*-cresol/thioanisole 6 : 2 : 1 : 1 for 1 h and the corresponding rhenium–PNA conjugates **6** and **7** were recovered, as orange and pale yellow solids respectively, by precipitation with diethyl ether. Both compounds were purified by means of semipreparative RP-HPLC and their structures confirmed with MALDI, LC-MS, and UV-vis spectroscopy (see ESI).

The photophysical properties of complexes **1** and **2** and of their PNA conjugates **4**, **6** and **7** are reported in Table 1. Complexes **1** and **2** show properties typical of excited states arising from $d\pi$(Re)–π^*(diazine) charge transfer:[9,10] (*i*) blue shift of the absorption and red shift of the emission by increasing solvent polarity, (*ii*) modulation of the emission on varying the diazine substituents (*i.e.* bathochromic shift and quenching of the emission by introducing the electron-withdrawing carboxyl group). Then complex **1** is completely non-emitting, whilst **2**, owing to the presence of the alkyl spacer between pyridazine and the carboxyl group, gives intense broad emission (quantum yields 12%), centred at 586 nm in toluene solution, with the long lifetime typical of triplet emission (1600 ns in deareated solution).



Scheme 2 Solid phase synthesis of the Re–PNA conjugates **6**, **7**.

Table 1 Photophysical properties of complexes **1**, **2**, **4**, **6** and **7** at 295 K

		λ_{abs}/nm ($\varepsilon/10^3$)	λ_{em}/nm	$\Phi_{em} \times 100$	τ/ns
1	[a]	409 (6.5)	—	—	—
	[b]	355 (7.1)	—	—	—
2	[a]	370 (8.6)	586	12(1)	1600
	[b]	335 (7.5)	610	1.7(1)	535
	[c]	335 (7.5)	610	1.0(1)	320
4	[a]	390 (6.9)	660	0.4(1)	20
	[b]	364 (8.1)	—	—	—
6	[b]	360 (n.d.)	—	—	—
7	[b]	333 (7.9)	610	1.7(2)	546
	[c]	333 (7.9)	610	1.1(2)	316

[a] Deaerated toluene solution. [b] Deaerated and [c] aerated CH_3CN–H_2O 1 : 1 solution. In parentheses the uncertainty on the last digit of the photoluminescence quantum yields (Φ_{em}). For the lifetimes uncertainties see ESI.

Interestingly, complex **1** becomes weakly emitting when bound to PNA monomer (conjugate **4**, emission: λ_{max} 660 nm, Φ_{em} 0.4% in deaerated toluene), probably because, owing to the presence of the amide group, in this complex the diazine is less electron-poor than in **1**. Such poor luminescence is completely lost in aerated CH_3CN–H_2O solution, both for **4** and for **6** (which is insoluble in toluene).

In contrast, the binding with PNA does not change the emission properties of complex **2**, since the carboxyl group is well spaced from the diazine ring. So, both **2** and its conjugate **7** show almost identical absorption and emission features in aerated CH_3CN–H_2O solution, as well as identical PLQY and similar lifetimes (λ_{max} absorption 333–335 nm, λ_{max} emission 610 nm, Φ_{em} 1.0–1.1%, τ 316–320 ns, Table 1). Both lifetimes and quantum yields increase in deaerated solution, in agreement with a substantial triplet character of the excited state.

In addition, the ability of the new Re–PNA conjugate **7** to recognize and bind the complementary DNA strand was evaluated by measuring the Tm (see ESI).

Compound **7** possesses therefore a series of very favourable properties for biological applications. It not only maintains a good emission in the presence of oxygen and water, but also exhibits a large Stokes shift (277 nm) and long lifetime of the excited state, that eliminate self-quenching problems and allow easy distinction from the cell background auto-fluorescence, which is normally very short lived (less than 10 ns).[18]

Moreover, both complexes **2** and **7** can be excited by two photon absorption using near IR laser light, with a maximum effect around 750 nm (see ESI). This red shifted excitation profile (already established for mononuclear Re complexes, but rarely exploited)[19] is advantageous over one-photon absorption, since it allows the achievement of deeper tissue penetration with less damage to the cell tissues.[20]

Recently, many papers have appeared dealing with the use of phosphorescent metal complexes as targeting molecules in cell imaging.[18] Moreover, a few studies reported enhanced cellular uptake of PNA oligomers containing *N*-terminally attached Zn^{2+} or Co^{2+} complexes.[21,22] We have therefore investigated the ability of the conjugate **7** to permeate cell membranes, by measuring the cellular uptake from HEK-293 cells in tissue plated cells. A small aliquot (50 μL) of a

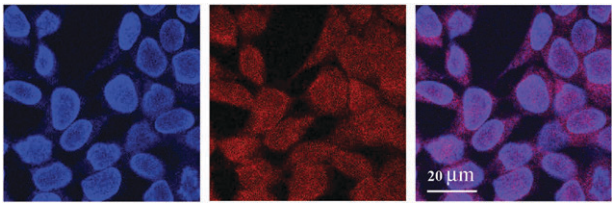


Fig. 2 Images of HEK-293 cells stained with the Re–PNA conjugate **7**, recorded about 10 min after the addition of the complex, through a 485/30 (left) and a 600/40 (center) band pass filter and their superposition (final **7** concentration 3 μM; field of view: 78 × 78 μm^2). Analogous images through the 485 and 535 filters are shown in Fig. S7 of ESI.

0.12 mM DMSO solution of **7** was added to 2 mL of phosphate buffer solution (PBS) in different wells of the plate, giving a 3 μM concentration of **7** in the wells. The samples were imaged using two photon excitation at 770 nm and the luminescence of the complex was detected in a wide spectral range, through 485/30, 535/50 and 600/40 band pass filters. Measurements performed under the same laser power in the absence of **7** showed that cells autofluorescence was negligible (see ESI). After incubation for 10 min at 37 °C, intense luminescence was observed, indicating that **7** penetrates the cell membrane, staining both the cytoplasm and the nucleus (see Fig. 2, whose image plane was chosen through a *z*-scan of the cells in a 40 μm range). The emission of **7** inside the nucleus appears to be blue shifted with respect to that in the cytoplasm (enhanced visibility of the nucleus in the blue filtered image of Fig. 2). Both the reduced mobility and the more hydrophobic character of the nuclear environment, with respect to the cytoplasm, could be responsible for this hypsochromic shift of the ^{3}MLCT emission. Interestingly, in the same conditions the homothymine PNA decamer labeled with fluorescein appears to not permeate small vesicles inside the cytoplasm region of cells (see ESI), whilst the neutral complex **2** alone shows roughly the same behaviour as **7** (see ESI).

Wide field transmitted light images confirmed that the cells were alive during all the imaging experiments, ruling out the hypothesis that cellular uptake was promoted by the onset of cell apoptosis, due to singlet oxygen formation (triplet emitters being potential sensitizers of singlet oxygen).

These preliminary results indicate that **7** is viable as a fluorophore for cell imaging, although more detailed experiments are needed in order to establish the kinetics and mechanism of the process, the influence of the kind of cells used and the lowest limits of complex and DMSO concentration. In fact, a role of DMSO in enhancing membrane permeability can be anticipated (in line with recent studies on cellular delivery of proteins, drugs and organometallic complexes),[23] since no staining of the cells was detected using an aqueous solution of **7**. At the very low concentrations used in the present study (2.5% v/v), DMSO does not appear to significantly affect cells morphology and viability (see ESI). Moreover, viability studies have indicated that neither **7** nor **2** shows any specific toxicity (see ESI).

In conclusion, in this paper we have shown that the conjugation of PNA with dinuclear rhenium(I) complexes provides a new bioorganometallic system with many favourable properties: it is photostable and non cytotoxic, it very quickly permeates living cells, in the presence of small amounts of DMSO and it stains with different colours nucleus and cytoplasm (suggesting a possible use for highlighting environments with different lipophilicity or rigidity). So far, no PNA with such photophysical property has been reported and we think that this study can open new perspectives for biological PNA applications.

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