Probing the interaction between DNA and cell transfection polymers with luminescent Ru^{II}complexes†

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The luminescence properties of Ru^{II} complexes [Ru(TAP)$_2$(phen)]Cl$_2$ (**1**), [Ru(TAP)$_2$(TPAC)]Cl$_2$ (**2**) and [Ru(phen)$_2$(TPAC)]Cl$_2$ (**3**) (TAP = 1,4,5,8-tetraazaphenanthrene and TPAC = tetrapyrido-acridine) were used for probing the interactions of cell transfection (co)polymers, such as poly[2-dimethylaminoethyl-methacrylate] (PDMAEMA) and poly[2-dimethylaminoethyl-methacrylate]-b-poly[(ethylene glycol-α-methylether-ω-methacrylate] (P(DMAEMA-b-MAPEG)), with different polynucleotides (poly[(dA–dT)]$_2$ and poly[(dG–dC)]$_2$) and nucleic acids (calf thymus DNA and pBR322 plasmid DNA). It turned out that **1** was not a useful probe because its affinity constant for DNA was too weak. Moreover, **3** was also excluded because DNA–Ru complex aggregates complicated the interpretation of the data. **2** was the only candidate that could be used as a luminescent probe of "DNA–polymer" self-assemblies that would penetrate cell membranes. Different (co)polymer/polynucleotide ratios were tested with complex **2** by luminescence measurements. The results showed that **2** is an interesting probe, which is very sensitive to changes in the polynucleotide double helix structure that are induced by interactions with the synthetic (co)polymer. Moreover, complex **2** has a mode of action of emission that is different from that of the classically used ethidium bromide, *i.e.* an increase in emission when certain "polymer/DNA" ratios are reached during the titration of DNA with the polymer.

Introduction

Research on non-viral synthetic transfecting agents for the efficient delivery of genetic material has increased tremendously during the last decade.[1] These specially designed vectorizing agents are either positively charged proteins, like protamines, liposomes or lipid derivatives,[2] or synthetic polymers[3] or dendrimers.[4] Different methods have been developed for studying the interactions between transfected DNA and these vectorizing agents. For example, the aggregates or particles formed between interacting partners can be examined by gel electrophoretic mobility, dynamic linear scattering (DLS) or atomic force microscopy (AFM), *i.e.* techniques that provide information on the sizes and shapes of these particles. The classical "fluorescence quenching exclusion assay", first used for the interaction between DNA and small molecules,[5] has also been applied to DNA transfecting agents.[1c,2,3c] This emission spectroscopy is based on the competition of interaction with DNA between a fluorescent organic dye (the most widely used is ethidium bromide (ETB)) and the species under investigation. ETB has a fluorescence intensity and lifetime

that increase when it intercalates into the DNA double helix, due to a decrease of proton exchange with the DNA duplex compared to water.[6] In the presence of another interacting species, ETB is expelled from DNA into water and regains its original fluorescence. This spectroscopic method allows quantitative determinations as long as no particles are formed in the medium; in such a case, light scattering perturbs quantitative measurements. On the other hand, during the last decade, it has been shown that Ru^{II} complexes can behave as interesting DNA photoprobes.[7] In this case, the luminescence of the Ru complexes generally increases by interacting with DNA, due to protection of their metal-to-ligand charge transfer excited triplet state (^{3}MLCT) by the double helix. In our laboratory, we have more particularly examined photo-oxidizing Ru^{II} complexes whose luminescence is, in contrast, quenched by interacting with DNA, due to an electron transfer process from guanine bases.[8] In the context of our research on synthetic polymeric vectorizing agents, we have shown[9] that this kind of Ru complex, when tethered to a cell-penetrating polymer, is able to photo-crosslink guanine-containing oligo-nucleotides with the synthetic polymer *via* a photo-electron transfer process. In the present work, we wondered whether this same type of complex, not attached, but free in solution, could be used like ETB for studying the interaction of synthetic transfecting polymers with DNA. These Ru complexes could indeed offer some advantages over ETB. Being very sensitive to the DNA microenvironment and good emitters from the ^{3}MLCT state, and therefore having a much

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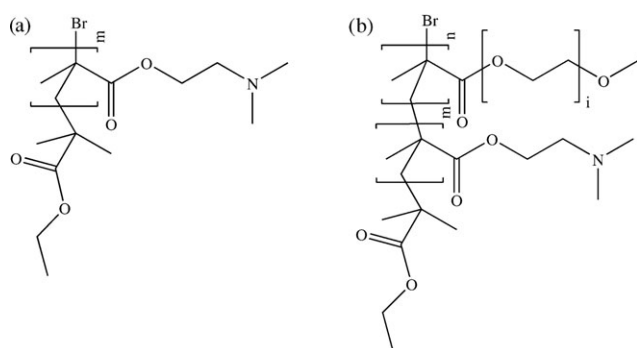
Fig. 1 (a) PDMAEMA (trunk homopolymer); (b) P(DMAEMA-b-MAPEG) (palm tree co-polymer).

longer excited state lifetime than that of the ETB singlet excited state, they could probe the DNA dynamics and local structures of the double helix over a longer timescale. Therefore, they might be more sensitive to DNA structural changes induced by interactions with a synthetic polymer. Moreover, their mode of action as a sensor could be different depending on the ligands of the Ru complex. Thus, with oxidizing ligands and due to a photo-induced electron transfer as mentioned above, the emission could also be quenched instead of being enhanced by DNA.

As promising results had been obtained previously by our co-workers with positively charged transfecting polymers,[10] examined by DLS and AFM, and tested by cell transfection experiments, we decided to choose for this study two of these polymers at our disposal: the poly[2-dimethylaminoethyl-methacrylate] homopolymer [P(DMAEMA); Fig. 1(a)], which will be called the "trunk" polymer, and poly[2-dimethyl-aminoethyl-methacrylate]-b-poly[(ethylene glycol-α-methyl-ether-ω-methacrylate] [P(DMAEMA-b-MAPEG); Fig. 1(b)], which will be called the "palm tree" co-polymer. Binary associations of the trunk or palm tree polymer with the pCMVb-plasmid (Fig. 2) are able to cross the cellular membrane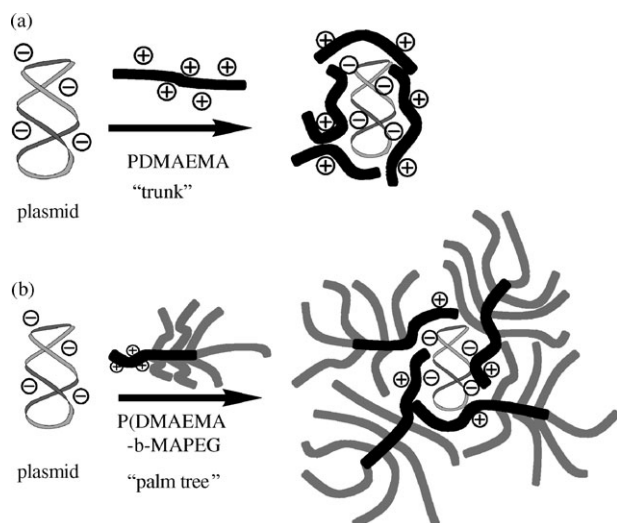s of Cos-7 cells in the absence of serum. Ternary associations containing both polymers (trunk + palm tree) and pCMVb-plasmid (Fig. 3) have also been shown to transfect these cells, even in the presence of serum.

Concerning the Ru complexes, the three compounds, $[Ru(TAP)_2(phen)]Cl_2$ (1), $[Ru(TAP)_2(TPAC)]Cl_2$ (2) and $[Ru(phen)_2(TPAC)]Cl_2$ (3) (TAP = 1,4,5,8-tetraazaphen-anthrene and TPAC = tetrapyrido-acridine), shown in Fig. 4, were selected. Complex 1 produces a photo-electron transfer process with the guanine bases and displays a moderate affinity for nucleic acids. The emission of complex 2 is also quenched by DNA, but this compound exhibits a high affinity for DNA due to its intercalation between the stacked base pairs, thanks to the extended TPAC ligand. Complex 3 also interacts via intercalation, but displays no photo-reactivity with DNA, and should therefore behave like ETB. Complexes 1–3 were thus tested in quantitative investigations of the interaction between (co)polymer transfecting agents and polynucleotides.

Experimental

Materials

Ru^{II} complexes 1–3 (Fig. 4) were prepared according to literature procedures.[11,12] The cationic trunk homopolymer, with M_n = 27 500 (determined by gel permeation chromatography (GPC)) and PDI = 1.39, and the palm tree co-polymer, with M_n = 27 800 and M_w/M_n = 1.4 (determined by size exclusion chromatography (SEC) and relative to PMMA standards, relative compositions expressed in mol% and determined by ^1H NMR: P(DMAEMA) = 82.7% and MAPEG = 17.3%, based on a P(DMAEMA) trunk: M_n = 13 600 and M_w/M_n = 1.25, and short PEG branches, each with M_w = 475), were synthesized according to published methods; their pK_a values were ca. 7.[10,13,14] All chemicals and reagents were obtained from Fluka, Aldrich or Merck, and were used without further purification. Calf thymus DNA (CT-DNA), dialysed several times against a buffer solution (~8 kbp), was purchased from Sigma, and the pBR322 plasmids (4361 bp), [poly(dA–dT)]$_2$ and [poly(dG–dC)]$_2$ were purchased from Amersham Biosciences.

Instruments

For the characterization of the Ru complexes, ^1H NMR (300 MHz) spectra were obtained on a Bruker Avance 300 instrument, absorption spectra were recorded on a Perkin-Elmer Lambda UV-VIS spectrophotometer, and emission spectra were recorded with a Shimadzu RF-5001 PC spectrometer equipped with a Hamamatsu R-928 photomultiplier tube and a 250 W xenon lamp as the excitation source. The spectra were corrected for instrument response.

Luminescence titrations

Luminescence titrations were carried out as follows. A certain volume of solution of the metallic complex (1×10^{-5} M or 5×10^{-6} M) in buffer (155 mM NaCl, 20 mM HEPES, pH 7.4), with the highest number of DNA equivalents (equivalents in phosphate or base) in the same buffer, was introduced into a spectroscopic cell. The solution obtained was stirred, and after
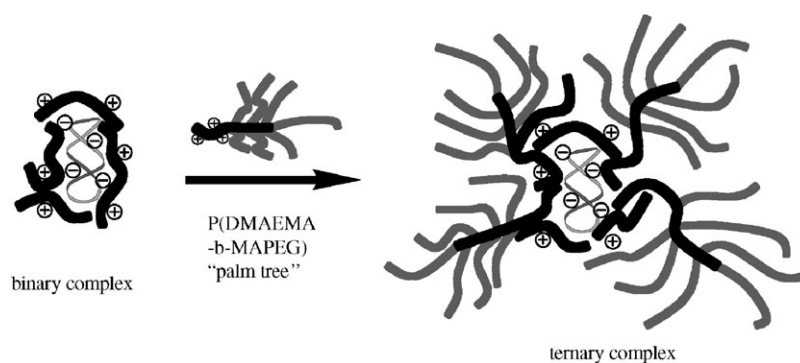

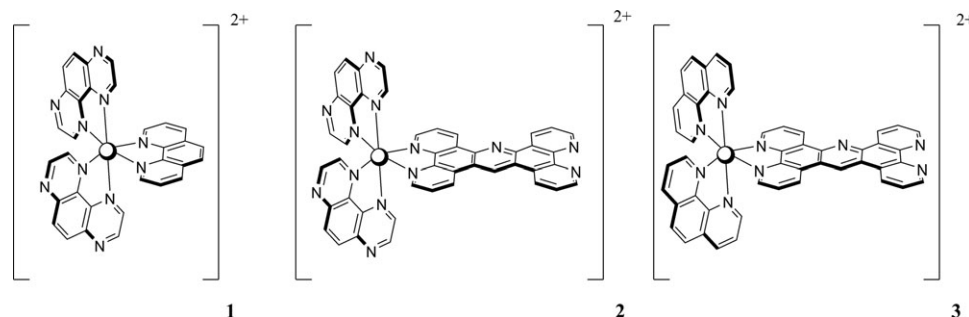Fig. 2 A hypothetical structure of the association between a plasmid and (a) the trunk polymer, and (b) the palm tree co-polymer.

Fig. 3 A hypothetical structure of the association between a plasmid and the trunk + palm tree polymers.

Fig. 4 The structures of [Ru(TAP)$_2$(phen)]Cl$_2$ (**1**), [Ru(TAP)$_2$(TPAC)]Cl$_2$ (**2**) and [Ru(phen)$_2$(TPAC)]Cl$_2$ (**3**).

10 min the absorption and emission spectra were recorded. A stock solution, which contained the same concentration of metallic complex in the same buffer, was also prepared. After the measurement, a certain volume of the DNA/Ru^{II} solution was removed from the cell and the same volume of stock solution of the Ru^{II} complex was added. In this way, the Ru^{II} complex concentration was kept constant for each sample, while the DNA concentration was diluted from sample to sample. This method avoided prolonged illumination of the same Ru complexes, which would induce photo-electron transfer with the guanine bases, leading to photo-adduct formation.[8b] The highest and lowest concentrations of DNA were 1×10^{-3} M and 5×10^{-6} M, respectively, expressed in equivalents of nucleotide bases.

When the titration was carried out with the trunk or palm tree (co)polymer, the solution in the cell consisted of two components, the Ru^{II} complex and the polynucleotide, both kept at a constant concentration. This mixture was titrated with varying concentrations of polymer. Thus, for the first measured sample, the cell contained metal complex (1×10^{-5} or 5×10^{-6} M), buffer solution (155 mM NaCl, 20 mM HEPES, pH 7.4), polynucleotide (CT-DNA, [poly(dA–dT)]$_2$ or [poly(dG–dC)]$_2$ at a concentration such that it corresponded to the luminescence plateau of the complex in the presence of the polynucleotide) and the highest concentration of polymer equivalents. This sample was then progressively diluted with a stock solution, which always contained the same concentration of metal complex, buffer, NaCl and polynucleotide. The same procedure was adopted for the titration of the ternary association "metal complex + polynucleotide + trunk polymer" with the palm tree co-polymer. In this case,

the palm tree co-polymer was diluted from sample to sample by a stock solution of the ternary association.

All of the absorption spectra were measured in the 200–800 nm range. For the emission spectra, the excitation wavelengths for **1**, **2** and **3** were 450, 450 and 415 nm, respectively, and the luminescence intensity was integrated over the whole emission spectrum. The absorption data were used to correct the luminescence titrations in order to report emission intensities for the same percentage of absorbed light. The emission intensity (I) of the samples that contained the above mentioned mixtures, in which the Ru^{II} concentration was always kept constant was recorded as a function of the concentration (in equivalents) of the component, which was variable. One equivalent of polymer is defined as one equivalent of monomer unit containing an amine function (protonated or not). In order to be able to compare the plots, the measured emission intensity (I) was divided by the emission intensity corresponding to that of the solution for zero equivalents of titrating agent (I_0). Thus, if the solution contained only the Ru^{II} complex and was titrated with DNA, I_0 was the emission intensity of the Ru^{II} complex alone; if the solution contained the Ru^{II} complex plus DNA, both at constant concentration, and was titrated with the polymer, I_0 corresponded to the emission intensity of the constant Ru^{II} complex plus DNA. For all the titrations, I/I_0 was plotted on the ordinate. On the abscissa, the number of equivalents of the titrating component was plotted with respect to the Ru^{II} complex, which was chosen as the reference point. If the titrated solution contained more components than the Ru^{II} complex, then the ratio of these components was kept constant during the whole titration, as outlined above.

Results and discussion

Choice of systems: Ru complexes, polynucleotides and polymers

As outlined in the introduction, when polyazaaromatic Ru^{II} complexes are excited, they emit in the visible region, and if they interact with polynucleotides, their luminescence intensity may increase or decrease, depending on the nature of the polyazaaromatic ligands and the guanine content of the polynucleotide. Thus, for complexes **1** and **2**, which contain two TAP π-accepting ligands,[12,15,16] their luminescence is quenched by the guanine bases of the polynucleotides. As explained above, this inhibition is caused by an electron transfer from the guanine base to the excited complex. Therefore, emission quenching is measured for **1** and **2** with CT-DNA and [poly(dG–dC)]$_2$. With the addition of increasing amounts of polynucleotide at constant Ru^{II} complex concentration, I decreases until a plateau value is reached, which indicates that all of the metallic complex is interacting with the polynucleotide. In contrast, the same complexes exhibit an increase in luminescence intensity with increasing amounts of polynucleotide if the latter does not contain any guanine base, for example with [poly(dA–dT)]$_2$. On the other hand, when the Ru^{II} complex contains two phen ligands instead of two TAP ligands (in the case of complex **3**; Fig. 4), I increases with increasing amounts of polynucleotide, independently of the guanine content. This is caused by protection of the excited state by the double helix (i) from the aqueous solution and/or (ii) from oxygen quenching. On the basis of these different luminescence behaviors, we tested complexes **1–3** by recording the titration curves corresponding to their luminescence as a function of increasing amounts of polynucleotide (measured in number of equivalents of phosphate or base). The behaviors of the three complexes could be different because **1** interacts with DNA with a weak affinity constant by adsorption inside the DNA grooves,[17] whereas **2** and **3** intercalate into the DNA stacks with a high affinity constant, due to the presence of the planar extended TPAC ligand. Therefore, in these cases, the interaction with polynucleotides is not only driven by Coulombic interactions, but also by entropic factors. The characteristic behaviors of complexes **1–3** should be perturbed by the presence of the positively charged synthetic polymer, which interacts with the negatively charged polynucleotide. These effects should depend in some way on the interaction geometry and affinity constant of the complex for DNA. For instance, the influence of the polymer at the level of the "DNA–Ru complex" interaction could not be purely electrostatic for **2** and **3**. Thus, complex **1** was chosen as a photoreactive groove binder, and complexes **2** and **3** were selected for their intercalation ability, the former being photo-reactive, whereas the latter cannot photo-oxidize guanine bases.

The chosen trunk homopolymer and palm tree co-polymer have pK_a values around 7 and, at the pH needed in these experiments (pH 7.4), they both dissolve well. For this study, we considered one polymer equivalent as being one equivalent of monomer unit with an amine function (protonated or not). Thus, for the palm tree co-polymer, as the palms have no amine function, we considered only the number of equivalents of monomer units in the trunk, which contains the amine functions.

The first step in the study consisted of determining the number of equivalents of nucleotide base (or phosphate) needed for each Ru complex (as a reference point), so that the whole amount of luminescent complex interacts with the polynucleotide. The number of phosphate equivalents was found at the plateau obtained by luminescence titration of the complex with increasing amounts of DNA (in number of bases or phosphates). In the second step, this pre-formed polynucleotide–Ru^{II} binary association (at the plateau) was titrated with either the trunk polymer or the palm tree co-polymer until another plateau of emission was reached. Because of the polymer–polynucleotide association, and probably because of partial or total neutralization of the charges, the Ru complex should, at the end of the titration (thus at the plateau), be completely ejected from the polynucleotide; therefore, its luminescence should be restored and correspond to that of the free Ru complex in the buffer solution. This restoration of emission in the titration curve should correspond either to an increase of luminescence, if the Ru excited species was quenched by the guanine content of the polynucleotide, or to a decrease of luminescence, if the emission of the Ru excited species was exalted by the polynucleotide. In other words, the plateau of the titration curve for a constant polynucleotide/Ru ratio with increasing amounts of polymer should indicate the largest amount of polymer for which the complexes are released in solution.

Tests with complexes 1, 2 and 3 as photoprobes

[Ru(TAP)$_2$(phen)]Cl$_2$ (1). As outlined above, the first step in this test consists of determining the number of equivalents of nucleotide base needed to reach a plateau value in the emission intensity of the Ru complex, by successive additions of polynucleotide. The changes in the emission of **1** (at a constant concentration of 1×10^{-5} M), caused by its interaction with different polynucleotides (CT-DNA, [poly(dA–dT)]$_2$ and [poly(dG–dC)]$_2$), were examined at pH 7.4 (20 mM HEPES) in the absence, as well as in the presence, of NaCl (155 mM). As expected with CT-DNA in the absence of NaCl, I/I_0 decreased until a plateau was reached at a nucleotide : Ru ratio of 40 : 1 (Fig. 5). However, in the presence of 155 mM NaCl, which represents the physiological concentration used for the transfection experiments,[8] the luminescence of **1** was not quenched (Fig. 5). This indicates that **1** does not interact with CT-DNA under these conditions. The same conclusion was applicable to the other polynucleotides because the affinity constant of this complex remained in the region of 10^3 M^{-1},[17] independent of the type of polynucleotide, and this value is too weak for the complex to compete in DNA interactions at such high NaCl concentrations. Consequently, complex **1** could not be used in these investigations in the presence of 155 mM NaCl.

[Ru(phen)$_2$(TPAC)]Cl$_2$ (3). The changes in the emission of **3** (at a constant concentration of 5×10^{-6} M) in the presence of CT-DNA, [poly(dA–dT)]$_2$ and [poly(dG–dC)]$_2$ were also examined under physiological conditions. As expected (see above), the interaction of **3** with these three polynucleotides, was accompanied by an emission increase (Fig. 6). However, the value of the polynucleotide/Ru ratio (expressed
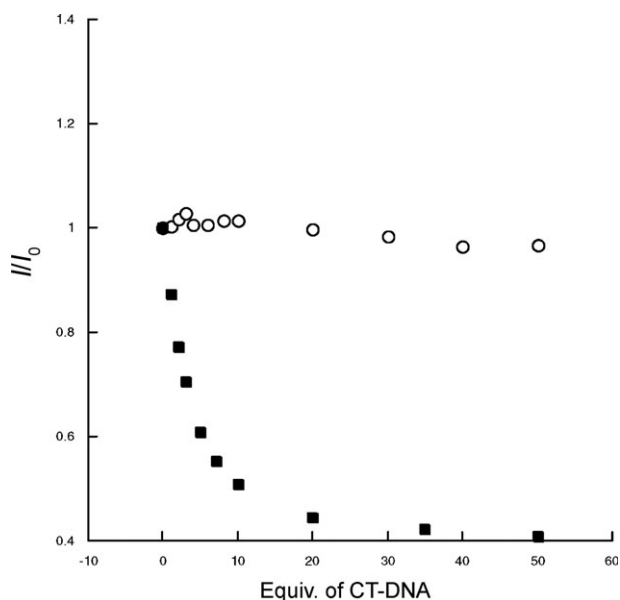
Fig. 5 The relative emission intensity (I/I_0) of **1** (at a concentration of 1×10^{-5} M) as a function of the number of equivalents of CT-DNA at pH 7.4, in the absence of NaCl (■) and in the presence of 155 mM NaCl (○).

in equivalents of polynucleotide) at which a plateau is reached in the titration curve is not quite clear. Indeed, I/I_0 seemed to increase in two steps: from 0 to ~10 equiv., and from 10–20 to ~50–60 equiv. The origin of this enhancement in two phases is not clear, though it might be related to the participation of two equilibrium constants between the complex and the polynucleotide. The degree of protection of **3** *vs.* the aqueous phase
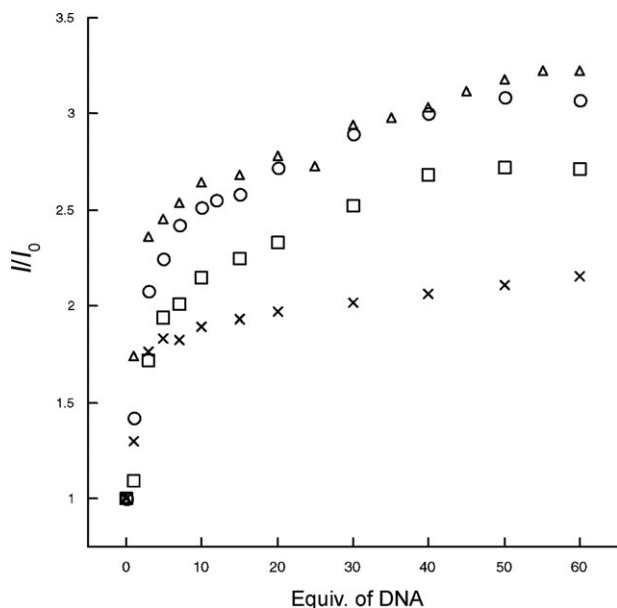

Fig. 6 The relative emission intensity (I/I_0) of **3** (at a concentration of 5×10^{-6} M) as a function of the number of polynucleotide equivalents (pH 7.4, 20 mM HEPES, 155 mM NaCl) of CT-DNA (□), [poly(dA–dT)]$_2$ (○) and [poly(dG–dC)]$_2$ (△); these three curves correspond to a waiting time of 10 min between the measurements, whereas for [poly(dG–dC)]$_2$ (×), the waiting time was 1 h.

would be different according to these two equilibrium interactions. Thus, in addition to the intercalation of the complex, polynucleotide aggregates incorporating complex **3** (which is more hydrophobic than complex **2**) might be present. Only in the presence of [poly(dG–dC)]$_2$ as a polynucleotide did this second phase enhancement seem to be absent with increasing amounts of polynucleotide, and the waiting time between each polynucleotide addition extended from 10 min (see experimental) to 1 h (Fig. 6). The same behaviors as those described above were observed using a 1×10^{-5} M concentration of metal complex **3**, and a lower NaCl concentration (80 mM) did not change the shape of the titration curve. The formation of DNA–Ru complex aggregates complicates the interpretation of the data, making complex **3** unsuitable as a photoprobe.

In spite of these problems, we tried nevertheless to titrate [poly(dA–dT)]$_2$–Ru with the trunk polymer (ESI, Fig. S1†) and palm tree co-polymer (ESI, Fig. S2†), with a [poly(dA–dT)]$_2$: Ru ratio of 50 : 1. Three main problems occurred with this: (i) with the addition of only a few equivalents of polymer, I/I_0 had already started to drop; (ii) the points were somewhat scattered in the region that looks like a plateau (at around 20 equiv. of polynucleotide), and (iii) the initial emission intensity of the complex in the absence of polynucleotide was not completely recovered after the addition of 20 equiv. of polymer. These observations could be attributed to the problem of aggregate formation, as described above, and to some light scattering from small particles. The same experiment could not be performed with CT-DNA. Indeed, when one or two equivalents of trunk or palm tree (co)polymer were added to the CT-DNA (CT-DNA : Ru = 50 : 1), precipitation occurred.

In conclusion, for the reasons outlined above, the Ru complexes **1** and **3** could not be easily used for probing the "polynucleotide–polymer" interactions.

[Ru(TAP)$_2$(TPAC)]Cl$_2$ (2). For **2** (at concentrations of 1×10^{-5} M and 5×10^{-6} M; pH 7.4, 20 mM HEPES, 155 mM NaCl), I/I_0 decreased with increasing amounts of CT-DNA and [poly(dG–dC)]$_2$. Again, as mentioned above, this quenching was expected, since the complex contains two TAP ligands and its excited state is thus capable of abstracting an electron from a guanine base of these polynucleotides. Plateau values were reached at polynucleotide : Ru ratios of ~5 : 1 for CT-DNA and [poly(dG–dC)]$_2$ (Fig. 7). In the presence of [poly(dA–dT)]$_2$, the emission intensity of **2** increased, since in that case, no guanine bases were present and consequently the excited state of the complex was protected by the double helix without any photo-reaction. A plateau value was reached at a polynucleotide : Ru ratio of ~15 : 1 (Fig. 7). Because a plateau can clearly be obtained for complex **2** with the three polynucleotides without complications as described for complex **3** (the plateau value remained the same with time intervals of 10 min or 1 h between each measurement), we could proceed to the second step of our analysis, *i.e.* the titration of each "polynucleotide loaded with Ru complex **2**" system with the polymer. Complex **2** should be especially interesting for probing the "polynucleotide–polymer" interaction since its mode of action depends on the
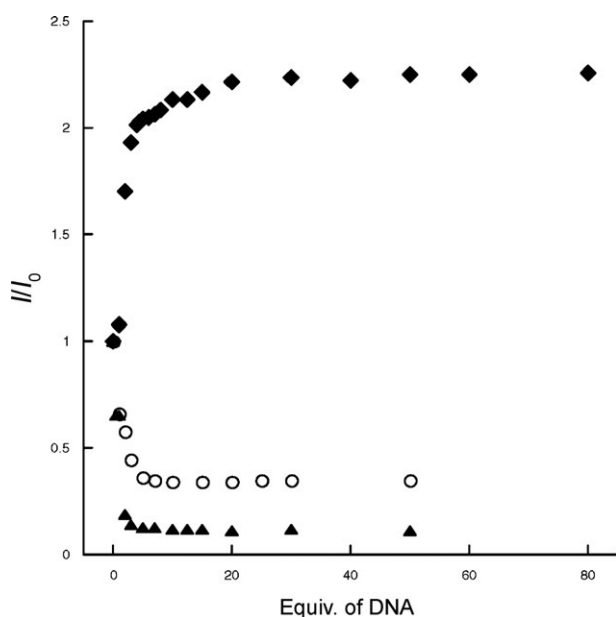
Fig. 7 The relative emission intensity (I/I_0) of complex **2** (at a concentration of 1×10^{-5} M) as a function of the number of polynucleotide equivalents (pH 7.4, 20 mM HEPES, 155 mM NaCl) of CT-DNA (○), [poly(dA–dT)]$_2$ (◆) and [poly(dG–dC)]$_2$ (▲).

Fig. 8 The relative emission intensity (I/I_0) of CT-DNA–**2** = 10 : 1 (▲), [poly(dA–dT)]$_2$–**2** = 20 : 1 (●) and [poly(dG–dC)]$_2$–**2** = 10 : 1 (■) as a function of the number of equivalents of trunk polymer. Concentration of Ru complex = 5×10^{-6} M.

polynucleotide, *i.e.* an increase (as with ETB) or a decrease of luminescence depending on the guanine content of the polynucleotide.

A question that can be raised concerning complex **2** is why it does not lead to the same types of problems as those described for complex **3**, taking into account the fact that both complexes intercalate into the polynucleotides. This difference should be attributable to differences in their hydrophobicity/ hydrophilicity properties. Indeed, due to its two TAP ligands, complex **2** is more hydrophilic than complex **3** with its two phen ligands. Consequently, complex **2** should be less inclined than complex **3** to lead to polynucleotide aggregates.

Titrations of "polynucleotide–2" systems with the trunk polymer

For titrations with the trunk polymer, several trials were performed in order to determine the best number of equivalents of nucleotide at which the effect of adding increasing amounts of trunk polymer could be conveniently observed. As the ratios CT-DNA : **2** = 5 : 1, [poly(dG–dC)]$_2$: **2** = 5 : 1 and [poly(dA–dT)]$_2$: **2** = 15 : 1 represent the ratios to reach the beginning of the plateau (Fig. 7), higher ratios (more poly- nucleotide equivalents) were first chosen so that the whole amount of Ru complex could be safely assumed to be inter- acting with the polynucleotide. However, for ratios of CT-DNA : **2** = 15 : 1, [poly(dG–dC)]$_2$: **2** = 15 : 1 and [poly(dA–dT)]$_2$: **2** = 25 : 1, the solution did not remain clear upon addition of the trunk polymer. Actually, the best titra- tion results with the trunk polymer were obtained with an intermediate amount of polynucleotide, *i.e.* for the ratios of DNA : **2** = 10 : 1, [poly(dG–dC)]$_2$: **2** = 10 : 1 and [poly(dA–dT)]$_2$: **2** = 20 : 1, in which the concentration of the Ru complex was 5×10^{-6} M. For these ratios, as can be seen
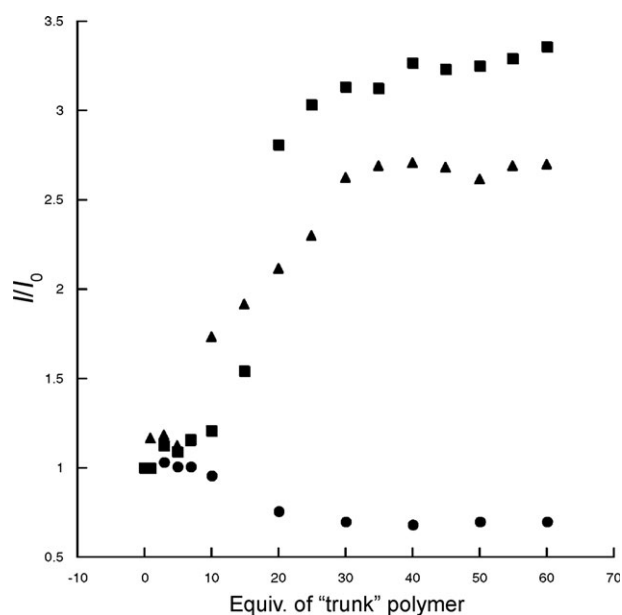
in Fig. 7, a plateau value for I/I_0 is clearly reached. Fig. 8 shows the titration curves as a function of increasing equiva- lents of trunk polymer. After the addition of a certain number of equivalents of trunk polymer, Ru complex **2**, as explained previously, should be expelled from the polynucleotide, and the luminescence intensity should be restored in the case of CT-DNA and [poly(dG–dC)]$_2$, whereas it should decrease in the case of [poly(dA–dT)]$_2$. These behaviors can indeed be observed in the curves in Fig. 8. However, with [poly(dG–dC)]$_2$, 90% of the luminescence was quenched at the beginning of the experiment (see Fig. 7); thus, a factor of 3.5 for the emission restoration in the presence of polymer is too low. Three possibilities can be considered to explain this weak emission. (i) Complex **2** could also interact with the polymer and give rise to an emission quenching, but this possibility has to be excluded because the polymer does not induce any change in the Ru emission (data not shown). (ii) Another possibility would be that even outside the [poly(dG–dC)]$_2$ double helix, the emission of complex **2** is dynamically quenched by this polynucleotide. This possibility also has to be discarded because the dynamic quenching calculated from the Stern–Volmer relation would lead to only 7% quenching (with quenching rate constant $k_q < 2 \times 10^9$ M^{-1} s^{-1}, luminescence lifetime τ = 760 ns and an equivalent guanine concentration of 5×10^{-5} M).[16] (iii) The remaining possibility is the presence of light scattering for the measurements at the plateau value. This is a recurrent problem (see later), which cannot be avoided if particles are formed (see introduction). Once light scattering is present, the quantitative measurement of light intensity compared to the initial I_0 of the Ru complex is, of course, no longer possible. Therefore, we will consider the appearance of the plateau value as being due to the occurrence of a compact assembly between the poly- nucleotide and the polymer, assuming that the complex may or

may not have been completely expelled from these particles. A non-complete recovery of the initial properties of a probe has also been observed in the literature.[3c]

The addition of the trunk polymer to the [poly(dA–dT)]$_2$–**2** or [poly(dG–dC)]$_2$–**2** associations does not change the luminescence until ∼10 equiv. of trunk polymer is reached, and for CT-DNA until ∼5–10 equiv. of trunk polymer is reached (see Fig. 8 and the second column of Table 1). This means that for these amounts of polymer, the trunk polymer does not distort the structure of the polynucleotide double helix too much, because the Ru complex remains inside the double strands.

As seen from Fig. 8, it is not easy to determine the upper limit of equivalents of trunk polymer for which the emission remains equal to ∼1. Therefore, we preferred to determine the number of equivalents of trunk polymer at which a plateau was reached (maximum of emission intensity; see Fig. 8 and column 3 of Table 1), thus, as mentioned above, when a tight polynucleotide–trunk polymer association is formed. In column 5 of Table 1, the number of equivalents (expressed in monomer units) of trunk polymer is given per one equivalent of polynucleotide (expressed in terms of phosphate groups), at which the Ru complex emission remains constant. From column 5 of Table 1, the following conclusions can be drawn. (i) Even in the case of [poly(dA–dT)]$_2$, for which only 1 equiv. of trunk polymer is needed to reach an emission plateau, this result does not correspond to a charge neutralization of the polynucleotide by the polymer. Indeed, there are still negative charges in this polynucleotide–trunk polymer association, since the polymer at pH 7.4 is very close to its pK_a (at ca. 7); thus, not all of the polymer equivalents are protonated. Therefore, this means that well before charge neutralization, the Ru complex is released into the medium, not only because of a decrease in the negative charge of the polynucleotide, but also because of a change of structure or a distortion of the double helix, introduced by the trunk polymer. (ii) If we compare the three polynucleotides, column 5 of Table 1 indicates that for [poly(dG–dC)]$_2$ and CT-DNA, more equivalents of polymer (2–3 and ∼3 equiv., respectively) are needed to reach the plateau value to thus distort these polynucleotides. [Poly(dA–dT)]$_2$, which is indeed slightly more flexible (composed of base pairs with only two hydrogen bonds) than the two other polynucleotides, is therefore the more easily deformable by the polymer, whereas CT-DNA is less so. One could speculate, for example, that the synthetic polymer could change the degree of winding of the double helix and/or increase the distance between the bases.

Titrations of "polynucleotide–2" systems with the palm tree co-polymer

In order to compare the effect of adding the trunk polymer with that of adding the palm tree co-polymer, we also titrated the "polynucleotide–**2**" association with the palm tree co-polymer (see Fig. 9 and Table 2). Column 4 of Table 2 (equivalents of palm tree co-polymer per one equivalent of polynucleotide) indicates, as for the trunk polymer, that [poly(dA–dT)]$_2$ is the polynucleotide for which the double helix or the stacking of bases is the most easily destroyed by the palm tree co-polymer, whereas the structure of CT-DNA is the least easily modified. On the other hand, comparing Table 2 with Table 1 indicates that [poly(dG–dC)]$_2$ and CT-DNA can accommodate more palm tree co-polymer than trunk polymer before reaching a constant Ru complex emission (compare column 5 of Table 1 with column 4 of Table 2). This suggests that the density of charges of the polymer plays a role in polynucleotide deformation. When the density is weaker, due to the "palms" of polyethylene glycol that in some way dilute the charges of the trunk polymer, the distortion at the level of the polynucleotide is weaker. In other words, the "palms" of polyethylene glycol might provide steric hindrance to further interaction of the trunk of the palm tree with the polynucleotide. This would allow the double helix to retain its structure intact with more positive charges than with the trunk polymer.

Titrations of "polynucleotide–2–trunk polymer" systems with the palm tree co-polymer

This case was also examined because it has been shown that this type of "polynucleotide–polymer mixture" association can give the best results for transfection.[10a] Thus, we titrated with the palm tree co-polymer, the "polynucleotide–Ru complex–trunk polymer" association, in which the Ru complex's luminescence intensity remained constant with the addition of trunk polymer, thus with a number of trunk polymer equivalents less than or equal to that given in column 2 of Table 1. The following associations were used per equivalent of Ru complex (see column 1 and 2 of Table 1): poly(dA–dT)]$_2$: trunk = 20 : 5, [poly(dG–dC)]$_2$: trunk = 10 : 5 and CT-DNA : trunk = 10 : 5. The results of the titrations are shown in Fig. 10 and summarized in Table 3.

A curve with CT-DNA could not be obtained because of the appearance of turbidity or even precipitation with the addition of the palm tree co-polymer to the system. The results in Table 3 have been treated in the following way. We have calculated the sum of the number of equivalents of palm tree co-polymer obtained at the plateau of luminescence and the

Table 1 Titration of the "Ru–polynucleotide" association with the trunk polymer (see also Fig. 8), using Ru complex **2**

Equiv. of polynucleotide per 1 Ru	Max. equiv. of trunk polymer for constant emission	Equiv. of trunk polymer at the plateau	Equiv. of trunk polymer/equiv. of polynucleotide at the plateau	Equiv. of trunk polymer per 1 equiv. polynucleotide at the plateau
[Poly(dA–dT)]$_2$ = 20	10	25–30	25/20 to 30/20	∼1
[Poly(dG–dC)]$_2$ = 10	10	20–30	20/10 to 30/10	∼2–3
CT-DNA = 10	5	30	30/10	∼3
pBR322 = 10	≈5		20/10	∼2
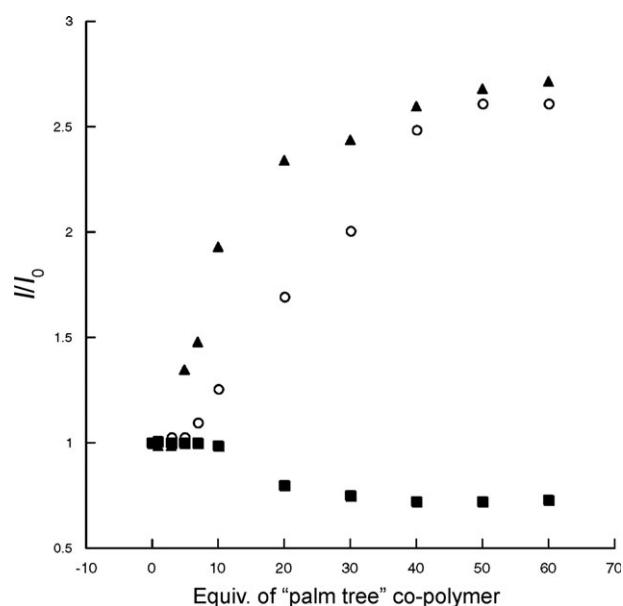
Fig. 9 The relative emission intensity (I/I_0) of CT-DNA–**2** = 10 : 1 (○), [poly(dA–dT)]$_2$–**2** = 20 : 1 (■) and [poly(dG–dC)]$_2$–**2** = 10 : 1 (▲) as a function of the number of equivalents of palm tree co-polymer. Concentration of Ru complex = 5×10^{-6} M.

number of equivalents of the trunk polymer already present in the associations (*i.e.* column 2 + column 3). In column 4, we give the ratio of the total amount of the two polymers to the amount of polynucleotide (expressed in equivalents per equivalent of Ru), and column 5 gives the value of this ratio, and thus the number of equivalents of the two polymers per one equivalent of polynucleotide. This ratio indicates again that [poly(dA–dT)]$_2$ (ratio = 1.5 : 1) is more sensitive than [poly(dG–dC)]$_2$ (ratio = 2.5 : 1) to the addition of the two polymers. Moreover, a comparison of the last columns of Table 1, Table 2 and Table 3 indicates that (i) there is almost no difference between the effect of adding the trunk polymer and adding the mixture of trunk + palm tree co-polymer (Table 1 and Table 3), and (ii) the palm tree co-polymer alone distorts the polynucleotide by less (Table 2), at least when [poly(dG–dC)]$_2$ and CT-DNA are used.

[Ru(TAP)$_2$(TPAC)]Cl$_2$ as a photoprobe for the interaction between plasmid pBR322 and the polymers

For the transfection experiments,[10a] none of the poly-nucleotides studied in this work were used. Therefore, we have also performed luminescence measurements of complex **2** with plasmid DNA, which was the material tested for the biological assays. The addition of plasmid pBR322 to Ru complex **2** led
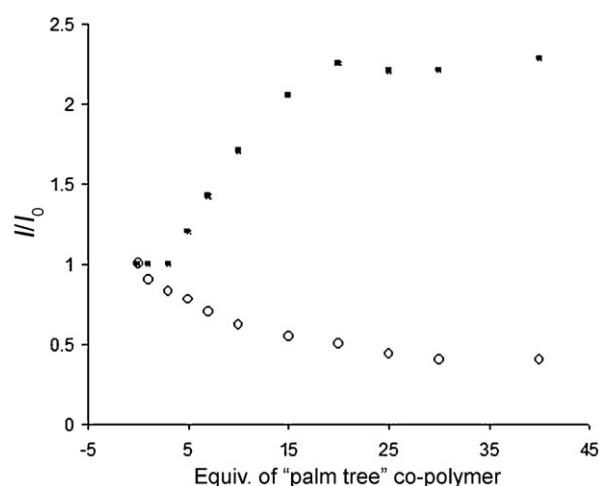

Fig. 10 The relative emission intensity (I/I_0) of the **2**–[poly(dG–dC)]$_2$–trunk polymer association = 1 : 10 : 5 (■) and of the **2**–[poly(dA–dT)]$_2$–trunk association = 1 : 20 : 5 (○) as a function of the number of equivalents of palm tree co-polymer.
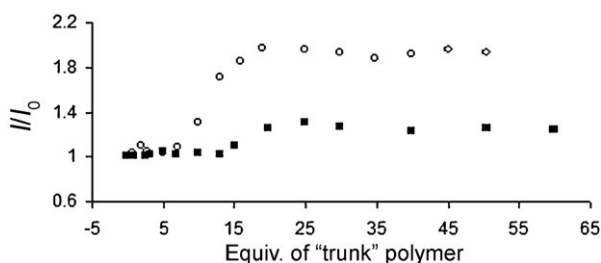
to its luminescence quenching, because the plasmid contains guanine bases. Under the same physiological conditions as before, a plateau in the emission of **2** was reached at a polynucleotide : Ru ratio of 5 : 1. Thus, for titrations with the polymer, higher pBR322 : Ru ratios were used, *i.e.* with 10 and 20 equiv. of pBR322 (expressed in equivalents of phosphate). With 10 equiv. of pBR322 (Fig. 11 and column 2 of Table 1), 5 equiv. of trunk polymer could be added without changing the emission intensity, *i.e.* the same amount as with CT-DNA. Column 5 of Table 1 indicates that per equivalent of pBR322 plasmid, the plateau of luminescence is reached at ~2 equiv. of trunk polymer, compared to ~3 equiv. for CT-DNA. It seems that the plasmid is slightly more sensitive than CT-DNA to deformation by the trunk polymer. At higher concentra-tions of pBR322 plasmid (pBR322 : Ru = 20 : 1), Fig. 11 shows that the plateau value is abnormally small. This means that the problem of light scattering causing too weak an emission restoration, as discussed before for the first titrations with the polymers presented in Fig. 8, has become very important in this case. This is not surprising, because a plasmid has a supercoiled circular form, and therefore it has a greater propensity to form aggregates or particles in the presence of a compacting agent, such as cationic polymers. Thus, when too high concentrations of plasmids are used, the titrations are no longer possible because of the formation of particles. As mentioned in the introduction, spectroscopic methods, such as emission intensity measurements, are no longer applicable in such cases.

Table 2 Titration of the "Ru–polynucleotide" association with the palm tree co-polymer (see also Fig. 9), using Ru complex **2**

Equiv. of polynucleotide per 1 Ru	Equiv. of palm tree co-polymer at the plateau	Equiv. of palm tree co-polymer/equiv. of polynucleotide at the plateau	Equiv. of palm tree co-polymer per 1 equiv. of polynucleotide at the plateau
[Poly(dA–dT)]$_2$ = 20	25	25/20	~1
[Poly(dG–dC)]$_2$ = 10	40–50	40/10–50/10	~4–5
CT-DNA = 10	40–50	50/10	~5

Table 3 Titration of the "Ru–polynucleotide–trunk polymer" association with the palm tree co-polymer (see also Fig. 10), using Ru complex **2**

Equiv. of polynucleotide/ equiv. of trunk polymer per 1 Ru	Equiv. of palm tree co-polymer at the plateau	Equiv. of "palm tree + trunk" polymers at the plateau	Equiv. of the sum of the polymers/equiv. of polynucleotide at the plateau	Equiv. of the sum of the polymers per 1 equiv. of polynucleotide at the plateau
$[Poly(dA–dT)]_2 = 20/5$	25–30	30–35	30/20–35/20	~1.5
$[Poly(dG–dC)]_2 = 10/5$	20	25	25/10	~2.5
CT-DNA $= 10/5$	precipitation	—	—	—

Fig. 11 The relative emission intensity (I/I_0) for the pBR322–**2** (10 : 1) (○) and pBR322–**2** (20 : 1) (■) associations as a function of the number of equivalents of trunk polymer. Concentration of Ru complex $= 5 \times 10^{-6}$ M.

Conclusions

We have learnt from this study that the selection of a Ru complex that behaves as a good photoprobe for the interaction between a polymeric cationic transfecting agent and genetic material is not straightforward. The metal-based probe must have a high affinity for the polynucleotide, even in the presence of high concentrations of salts. However, metal complexes like $[Ru(phen)_2(TPAC)]Cl_2$ (**3**) might have a disadvantage, due to their higher hydrophobicity, as compared to $[Ru(TAP)_2TPAC]^{2+}$ (**2**), giving rise to complicated associations with the polynucleotides (not only intercalation) (Fig. 6). This is not the case with complex **2**, which turned out to be the best candidate selected among the three investigated possibilities. However, as explained in the introduction, one has to keep in mind that a spectroscopic method, such as the measurement of the emission intensity, is useful, but only for solutions. If particles start forming, the measured luminescence intensity is disturbed by light scattering. Other methods must then be applied to characterize these aggregates. In this work, we chose the plateau value reached after the addition of polymer as corresponding to the upper limit of the number of polymer equivalents that interact with the polynucleotides without causing too much distortion of the double helix structure. As shown and discussed in this work (Table 1, Table 2 and Table 3), the number of equivalents depends on the polynucleotide and the polymer. The smallest value determined for the amount of polymer was 1 equiv., and corresponded to $[poly(dA–dT)]_2$. Taking the pK_a value of the polymer into account, this means that only about half of this equivalent is positively charged. Consequently, the double helix, at least for $[poly(dA–dT)]_2$, is already distorted well before neutralization of the negative charges of the oligonucleotide by the positive charges of the polymer. Below half an equivalent of polymer, the emission intensity remains constant (compare for $[poly(dA–dT)]_2$ columns 1 and 2 in Table 1).

In conclusion, our titration experiments with complex **2** can be considered as a rapid method for the determination of the maximum number of equivalents of polymer that can be used without introducing too many changes into the DNA double helix or plasmid structure. This highlights the fact that **2** is a very good sensor, sensitive to its microenvironment, and which, in contrast to ETB, indicates the presence of a "DNA-transfecting polymer" association by light emission instead of an extinction.

In spite of the problem of light scattering, turbidity, or even precipitation, which we met upon addition of polymer, and which was expected since it is well known that aggregates or particles can be formed, we nevertheless succeeded in performing quantitative measurements. As shown in this work, the polynucleotide double helix structure might not have conserved its geometrical integrity, which might have consequences for transfected cells. Therefore, we think that a method such as the one described in this work could be useful to determine whether genetic material has retained its complete original geometry with a selected ratio of polymer.

Acknowledgements

References

1 (a) D. Luo and W. M. Saltzman, *Nat. Biotechnol.*, 2000, **18**, 33–37; (b) M. E. Davis, *Curr. Opin. Biotechnol.*, 2002, **13**, 128–131; (c) D. J. Gary, N. Puri and Y.-Y. Won, *J. Controlled Release*, 2007, **121**, 64–73.

2 (a) A. D. Miller, *Angew. Chem., Int. Ed.*, 1998, **37**, 1768–1785; (b) C. Prata, Y. Zhao, P. Barthélémy, Y. Li, D. Luo, T. J. McIntosh, S. J. Lee and M. W. Grinstaff, *J. Am. Chem. Soc.*, 2004, **126**, 12196–12197; (c) H. Gershon, R. Ghirlando, S. B. Guttman and A. Minsky, *Biochemistry*, 1993, **32**, 7143–7151; (d) M. Singh, A. Hawtrey and M. Ariatti, *Int. J. Pharm.*, 2006, **321**, 124–137.

3 (a) J. H. Jeong, S. W. Kim and T. G. Park, *Prog. Polym. Sci.*, 2007, **32**, 1239–1274; (b) D. M. Lynn, D. G. Anderson, D. Putman and R. Langer, *J. Am. Chem. Soc.*, 2001, **123**, 8155–8156; (c) J. S. Choi, D. K. Joo, C. H. Kim and J. S. Park, *J. Am. Chem. Soc.*, 2000, **122**, 474–480.

4 (a) J. F. Kukowska-Latallo, A. U. Bielinska, J. Johnson, R. Spinder, D. A. Tomalia and J. R. Baker, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 4897–4902; (b) D. Luo, K. Haverstick, N. Belcheva, E. Han and W. M. Saltzman, *Macromolecules*, 2002, **35**, 3456–3462.

5 H. W. Zimmerman, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 115–130.

6 S. J. Atherton and P. C. Beaumont, *J. Phys. Chem.*, 1987, **91**, 3993–3997.

7 (a) B. Norden, P. Lincoln, B. Akerman and E. Tuite, *Met. Ions Biol. Syst.*, 1996, **33**, 177–252; (b) C. Moucheron, A. Kirsch-De Mesmaeker and J. Kelly, in *Less Common Metals in Proteins and Nucleic Acid Probes (Structure and Bonding)*, ed. M. J. Clarke, Springer-Verlag, Berlin, 1998, vol. 92, pp. 163–216; (c) K. E. Erkkila, D. T. Odom and J. K. Barton, *Chem. Rev.*, 1999, **99**, 2777–2795; (d) C. Metcalfe and J. A. Thomas, *Chem. Soc. Rev.*, 2003, **32**, 215–224; (e) F. Pierard and A. Kirsch-De Mesmaeker, *Inorg. Chem. Commun.*, 2006, **9**, 111–126.

8 (a) C. Moucheron, A. Kirsch-De Mesmaeker and J. M. Kelly, *J. Photochem. Photobiol., B*, 1997, **40**, 91–106; (b) B. Elias and A. Kirsch-De Mesmaeker, *Coord. Chem. Rev.*, 2006, **250**, 1627–1641.

9 S. Deroo, V. Toncheva, E. Defrancq, C. Moucheron, E. Schacht and A. Kirsch-De Mesmaeker, *Biomacromolecules*, 2007, **8**, 3503–3510.

10 (a) S. Pirotton, C. Muller, N. Pantoustier, F. Botteman, S. Collinet, C. Grandfils, G. Dandrifosse, P. Degée, P. Dubois and M. Raes, *Pharm. Res.*, 2004, **21**, 1471–1479; (b) C. Volcke, S. Pirotton, C. Grandfils, C. Humbert, P. A. Thiry, I. Ydens, P. Dubois and M. Raes, *J. Biotechnol.*, 2006, **125**, 11–21.

11 J.-P. Lecomte, A. Kirsch-De Mesmaeker, M. Demeunynck and J. Lhomme, *J. Chem. Soc., Faraday Trans.*, 1993, **89**, 3261–3269.

12 B. Elias, L. Herman, C. Moucheron and A. Kirsch-De Mesmaeker, *Inorg. Chem.*, 2007, **46**, 4973–4988.

13 N. Pantoustier, S. Moins, M. Wautier, P. Degée and P. Dubois, *Chem. Commun.*, 2003, 340–341.

14 S. Liu, J. V. M. Weaver, Y. Tang, N. C. Billingham and S. P. Armes, *Macromolecules*, 2002, **35**, 6121–6131.

15 I. Ortmans, B. Elias, J. M. Kelly, C. Moucheron and A. Kirsch-De Mesmaeker, *Dalton Trans.*, 2004, 668–676.

16 (a) L. Herman, B. Elias, F. Pierard, C. Moucheron and A. Kirsch-De Mesmaeker, *J. Phys. Chem. A*, 2007, **111**, 9756–9763; (b) L. Herman, *PhD Thesis*, Université Libre de Bruxelles, 2008.

17 A. Del Guerzo and A. Kirsch-De Mesmaeker, *Inorg. Chem.*, 2002, **41**, 938–945.

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