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# Tuning Sensitivity in Paramagnetic NMR Detection of Ligand–DNA Interactions

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The derivatization of DNA with paramagnetic tags has been used both to get information about the properties of DNA itself, and to characterize its interaction with proteins.[1–3] More recently the insertion of a spin label or a paramagnetic metal ion in proteins has been exploited as a means to increase sensitivity in drug-screening experiments.[4–7] A widely used ligand screening technique is based on the recording of $^1$H NMR spectra by using a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence, on a mixture of compounds in a library of chemicals.[8,9] By varying the length of the CPMG delay the intensity of each signal varies according to its transverse relaxation rate ($R_2$). In the presence of micromolar quantities of the target protein, the intensity of signals of compounds which interact in fast exchange with the macromolecule, is strongly affected. The larger the macromolecular adduct, the higher the sensitivity of the method. For a target with a given molecular weight, the sensitivity of the method can be significantly increased by placing a paramagnetic center in the target, which will cause a stronger effect on $R_2$ values of NMR-active nuclei belonging to the ligand.[10]

Nucleic acids functional properties are nowadays attractive targets in drug discovery to treat several severe pathologies such as cancer and infectious and immunological diseases.[11–14] Therefore, there is an increasing interest in developing strategies to speed-up the screening of libraries of chemicals to target nucleic acids. To this end, we were prompted to test an NMR approach based on $R_2$-edited experiments to detect ligand–DNA interactions. Short synthetic fragments of nucleic acids are easily available, permitting the generation of models for in vitro screening; nevertheless their low molecular size proportionally decreases the sensitivity of the NMR strategy based on CPMG experiments. The insertion of a paramagnetic metal ion in the target synthetic oligonucleotides permits these limitations to be overcome, increasing the sensitivity of the method, and making possible the identification of weak binders as a basis for a further iterative optimization process which can transform them from hit compounds to lead compounds. The possibility of using different metal ions is a further advantage as it permits modulation of the paramagnetic contribution in the experiment, to affect binding sites located at different distances from the paramagnetic probe.

Herein, we demonstrate the feasibility of this strategy for a case in which the target macromolecule is a short synthetic DNA fragment. In particular, we choose as the model system a double strand 12-mer DNA containing a GC site as target, and norfloxacin known to interact preferentially with such a site, as ligand (Figure 1).[15]

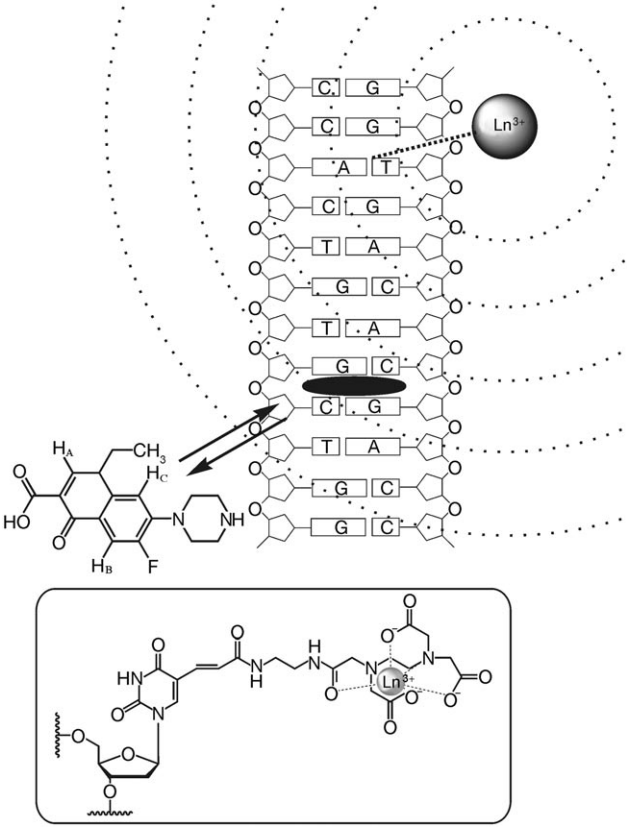


**Figure 1.** Representation of the model system consisting of a double strand 12-mer DNA containing a GC site as target and norfloxacin, known to interact preferentially with the ligand binding site.

Norfloxacin is an antibiotic drug which belongs to the quinolone family.[16] Quinolone, synthetic fluorinated analogues of nalidixic acid like norfloxacin, are antibacterial and anticancer drugs,[17] acting as inhibitors of type II and type IV topoisomerase.[18,19] The interaction of DNA with quinolones has been extensively studied[20,21] and in particular the interaction with norfloxacin has been characterized by NMR.[22] The drug proved to partially intercalate at GC sites and to exhibit nonspecific groove binding. The complex resulted in fast exchange on the NMR timescale,[22] thus making possible the application of the $R_2$-edited experiments.

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The DNA sequence chosen for the study is 5′-CCA CTG TGC TGG-3′/5′-CCA GCA CAG T*GG-3′, where T* represents a thymidine analogue nucleoside to which EDTA is attached through a linker [5-(*N*-ethylenediamminetriacetate, monoacetylamminoethyl-3-acrylamido)-2′-deoxyuridine]. The automatic synthesis of the oligonucleotides was carried out according to the phosphoramidite chemistry standard procedures by the use, in particular, of the EDTA-C2-dT-CE phosphoramidite from Glen Research Corp. As previously reported[1,2] in the (T*) EDTA-functionalized DNA, the complex is placed in the major groove, without any significant alteration of the double helix structure. Furthermore, in the model investigated herein, the modified nucleoside is four bases from the norfloxacin binding site and close to the terminal part of the strand, so reducing any risk of structural modification of the binding site and any possible alteration of its affinity for the ligand.

For most of lanthanides, but not for $Gd^{III}$, the increase of $R_2$ of neighboring nuclei is mainly due to Curie relaxation, which depends on the type of paramagnetic metal ion, the distance of the nuclei from such probe, and on the applied magnetic field. In the present work, $Ce^{III}$ and $Dy^{III}$ have been chosen as metal ions on the basis of their sizably different paramagnetic properties. Although the binding constants of lanthanides for this EDTA derivative have not been determined, $K_D$ values lower than $10^{-10}$ M are reported in the case of the weaker bisamide-EDTA chelators.[23] This large stability ensures selective metal binding and prevents any nonspecific interaction with DNA.

Oligonucleotides bind lanthanides themselves with a good affinity ($K_D \sim 10^{-4}$–$10^{-3}$ M)[24] but the nonspecific binding cannot be exploited for ligand screening. In fact DNA condensation, competition phenomena, alteration of the binding site, and dissociation at nanomolar concentration make this approach not feasible.

On the contrary the affinity of this EDTA monoamide for the lanthanides and for other metals such as $Mn^{II}$ provides the possibility of fine tuning the paramagnetic contribution. In this system the paramagnetic center is constituted by a metal complex covalently bound to the DNA oligonucleotide by a seven atom linker and it is not immobilized.

The EDTA linked to the oligonucleotide was titrated up to 95% with the above mentioned metal ions to generate the paramagnetic species and with $Ca^{II}$, to generate the diamagnetic no-effect reference analogue, with the titration being followed by monitoring the EDTA peaks in the $^1$H NMR spectrum of the derivatives. Each chelated-metal DNA-complex was then extensively washed by a centricon device with NaCl 100 mM water solution at pH 7.0, to remove any possible trace of unbound paramagnetic metal ion. The samples were then lyophilized and redissolved in $D_2O$ to give stock solutions 360 μM of $Ca^{II}$-EDTA-DNA, $Ce^{III}$-EDTA-DNA, and $Dy^{III}$-EDTA-DNA, respectively. Aliquots of paramagnetic- and diamagnetic-DNA samples were used to titrate $D_2O$ solutions of norfloxacin 300 μM, in 5 mM phosphate buffer containing NaCl 100 mM at pH 7.0.

The NMR experiments were performed on a DRX Bruker 700 MHz spectrometer equipped with a TXI probe at 298 K. Concentrations of 0.7, 1.4, 2.8, 5.6, 11.2, and 22.4 μM of metal-EDTA-DNA have been examined. A series of $^1$H NMR experiments with increasing duration of the CPMG delay were performed for all the sample concentrations. The signals by far more affected by the extension of the CPMG delay are those relative to the norfloxacin aromatic protons. At concentrations of 5.6 μM and 1.4 μM for $Ce^{III}$ and $Dy^{III}$ derivatives respectively, the signal intensity of the two aromatic protons $H_A$ and $H_B$ protons was completely eliminated, and that of $H_C$ strongly reduced.

The signals of the norfloxacin aromatic protons in the presence and in the absence of the functionalized oligonucleotides (0.7 μM) at 150 ms of CPMG spin-lock time are shown in Figure 2.

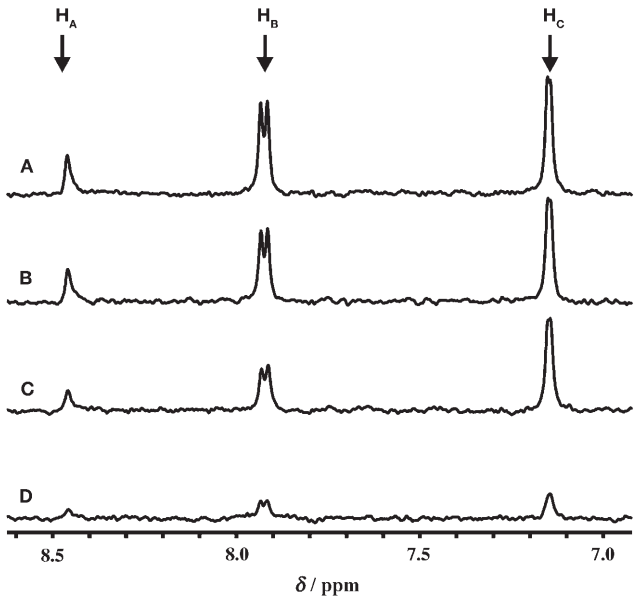


**Figure 2.** Section of relaxation-edited $^1$H NMR experiments of norfloxacin at 150 ms of CPMG spin-lock time. Reference spectrum of a) norfloxacin (300 μM) alone, and spectra recorded in the presence of b) $Ca^{II}$-EDTA-DNA (0.7 μM), c) $Ce^{III}$-EDTA-DNA (0.7 μM), and d) $Dy^{III}$-EDTA-DNA (0.7 μM).

The comparison of the spectra acquired under the same experimental conditions nicely prove that the increased sensitivity of the NMR methodology is due to the presence of the paramagnetic probe. The spectrum recorded with the solution containing nanomolar concentration of $Ca^{II}$-EDTA-DNA, shows a small reduction in the signal intensity of the aromatic protons because of the interactions occurring. With the same concentration of $Ce^{III}$-EDTA-DNA, the effect is larger than in the presence of the diamagnetic analogue and becomes dramatic in the spectrum recorded in the presence of the same amount of $Dy^{III}$-EDTA-DNA.

A careful analysis of the data reveals a gain in sensitivity by a factor of three for $Ce^{III}$ and of six for $Dy^{III}$ with respect to the diamagnetic adduct, proving that the mobility of the paramagnetic probe related to linker extension and flexibility is not a limiting factor for screening applications.

  

To evaluate the effect on the proton-relaxation behavior produced by possible nonspecific interactions of the ligand with the paramagnetic complex present in solution, all the experiments were repeated in the presence of the simple $Ce^{III}$-EDTA and $Dy^{III}$-EDTA complexes, at the same concentration as the (T*) EDTA-functionalized DNA (0.7, 1.4, 2.8, 5.6, 11.2, and 22.4 μM). In both cases a signal intensity reduction in the order of 19% of that produced by an equivalent concentration of the corresponding paramagnetic DNA-adduct was seen. Possible false positives in ligand screening related to nonspecific interactions with the paramagnetic complex present in the lanthanide-EDTA-DNA can be easily excluded by performing the reference spectrum in the presence of an equivalent concentration of the corresponding lanthanide-EDTA complex.

Besides the increased ligand screening sensitivity, the comparison of the results with the theoretical relaxation effects expected for $Ce^{III}$ and $Dy^{III}$ have provided qualitative information on the specificity of norfloxacin binding to the DNA oligonucleotide. According to a model generated in silico exploring all the conformations allowed by the linker, the minimum distance between the chelated metal ion and the GC site was estimated to be around 11 Å. Even in this more favorable condition, at this distance the contribution of $Ce^{III}$ to $R_2$ is negligible as calculated and shown in Figure 3. Therefore the effect detected for $Ce^{III}$-EDTA-DNA should be related to binding at sites at least 5 Å closer to the paramagnetic center along the DNA strand with respect to the GC base pair. This is in agreement with previous investigations[15,22] where, besides the preference for the GC site, nonspecific groove binding was reported for norfloxacin. On the contrary, for $Dy^{III}$ the paramagnetic enhancement is large enough at 11 Å and effective up to around 16 Å. Therefore for $Dy^{III}$-EDTA-DNA, a relevant contribution to the increased sensitivity monitored by the CPMG experiment is expected to come from the ligand interacting at the GC site. The presence of nonspecific binding makes the structural characterization of the adduct not feasible even using the approach recently developed to exploit flexible paramagnetic probes.[1]

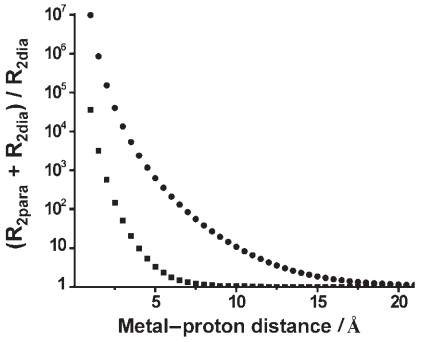


**Figure 3.** Theoretical $R_2$ enhancements at 700 Mhz and at 298 K calculated for a ligand proton at various distances from a $Ce^{III}$ ion (■) and $Dy^{III}$ ion (●) covalently linked to the oligonucleotide. $R_{2dia}$ values around 30 $s^{-1}$ were estimated for a ligand proton bound to a diamagnetic DNA oligonucleotide of the size of the present.

In summary, we proved the applicability of the described method for ligand screening relative to DNA. The increased sensitivity compared to the traditional $R_2$-based screening technique has to be considered a relevant empowering feature because it allows consideration of low-molecular weight oligonucleotides as target molecules, making the screening process cheaper and easier to exploit. The capability of detecting weak interactions with poorly soluble small binders and the possibility to get information on ligand specificity by playing with the different paramagnetic properties of the metal ions and with the position of the probe, make this approach feasible and attractive when the use of other techniques, such as SPR, is problematic or less informative. Also relevant is the possibility of inserting different lanthanides that permit modulation of sensitivity and detection of interactions, even those far from the probe along the DNA strand.

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