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Combined neutron and X-ray diffraction studies of DNA in crystals and solutions

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Recent developments in instrumentation and facilities for sample preparation have resulted in sharply increased interest in the application of neutron diffraction. Of particular interest are combined approaches in which neutron methods are used in parallel with X-ray techniques. Two distinct examples are given. The first is a single-crystal study of an A-DNA structure formed by the oligonucleotide d(AGGGGCCCCT)$_2$, showing evidence of unusual base protonation that is not visible by X-ray crystallography. The second is a solution scattering study of the interaction of a bisacridine derivative with the human telomeric sequence d(AGGGTTAGGGTTAGGGTTAGGG) and illustrates the differing effects of NaCl and KCl on this interaction.

1. Background

DNA is a highly flexible molecule that is known to adopt markedly different conformations depending on the base sequence and the prevailing conditions. Despite the huge amount of work that has been carried out since the first fibre-diffraction studies of double-stranded DNA, fundamental questions remain about the nature of DNA polymorphism, its role in biological processes and its potential exploitation in the development of targeted therapies. The molecule can adopt single-, double-, triple- and quadruple-stranded variants and within each of these categories is capable of major structural changes between well defined molecular conformations. This is well illustrated by the structural polymorphism observed for double-stranded DNA, for which five major structural variants have been observed (Fuller *et al.*, 2004) and where structural transitions between these have been followed using synchrotron X-ray sources (Forsyth *et al.*, 1986; Mahendrasingam *et al.*, 1986). While the biological significance of this conformational polymorphism is not clear, there has been considerable speculation about the role of different double-stranded forms and it seems inconceivable that this capability would not be exploited in biological function. For example, the fact that cytosine methylation, which has known consequences for regulatory processes, has such a profound effect on DNA structure and transitions is unlikely to be without biological significance. For triplex and quadruplex DNA structures there are again good reasons to believe that the structures are important in biology and many such systems are the subject of intense investigation as potential targets for drug/ligand interactions.

As in other areas of structural and molecular biology, a fundamental concern is always the degree to which the techniques used perturb the information sought. In the case of nucleic acids, a considerable amount of information has been

provided by X-ray fibre diffraction. While such studies provide data from the continuous long polymer that are relatively unaffected by end effects and crystal-packing forces, the resolution is typically limited to 2–3 Å and traditional crystallographic approaches for structure determination can rarely be used. A further difficulty is that it is often difficult to identify structured water reliably. This problem has been addressed through the development and use of neutron fibre diffraction (Forsyth *et al.*, 1989; Langan *et al.*, 1992; Shotton *et al.*, 1997), which has allowed structured water around various DNA polynucleotide conformations to be determined. Single-crystal studies of short oligonucleotide duplexes allow high-resolution studies to be carried out and such studies were directly responsible for the discovery of left-handed DNA (Wang *et al.*, 1979) and its subsequent identification in fibres (Arnott *et al.*, 1980). Solution scattering studies further broaden the scope of nucleic acid work by providing low-resolution information in free solvent as well as the option of parametric studies (*e.g.* temperature, pH, ligand concentration *etc*.). In all of the approaches summarized above, the application of neutron methods alongside X-ray approaches offers strongly complementary information. For crystallography and fibre diffraction, major gains may occur in terms of identifying hydration and protonation states. In solution studies, numerous options that exploit contrast variation exist.

This paper illustrates these points by describing two separate studies using completely different approaches. The first is a preliminary single-crystal neutron diffraction study of the oligonucleotide d(AGGGGCCCCT)$_2$ in the A conformation. Both neutron and X-ray crystallographic data were recorded at room temperature from the same sample and refinement was carried out against a joint target function. The second study is a solution scattering study of the quadruplex-forming human telomeric sequence d(AGGGTTAGGGTTAGGGTTAGGG). Here, the interest was in obtaining low-resolution information on the conformational changes that occur as a function of ionic strength and the degree to which this is altered by the presence of a bisacridine derivative (I) with several aromatic rings in the linker (Fig. 1). (I) was originally synthesized as a putative bisintercalator and was subsequently shown to have low activity in a human cytosine methyltransferase inhibition screen (Denny *et al.*, unpublished work).

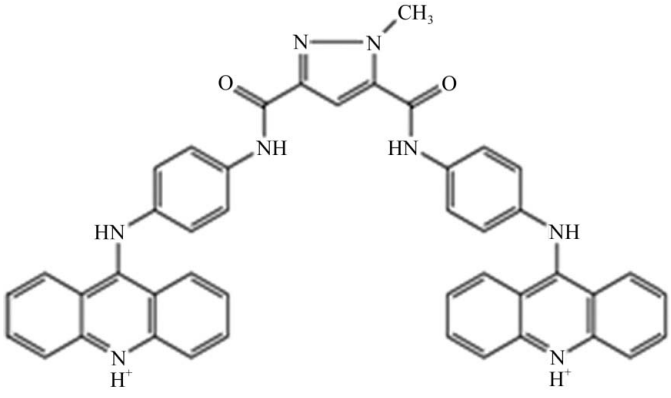

Figure 1
The ligand (I) used in the solution scattering studies of the quadruplex d(AGGGTTAGGGTTAGGGTTAGGG) (Denny *et al.*, unpublished work).

2. Methods

2.1. Sample preparation

2.1.1. Preparation of A-DNA crystals from the oligonucleotide d(AGGGGCCCCT)$_2$. The oligonucleotide d(AGGGGCCCCT) was purchased from MWG Biotech and further purified by anion-exchange chromatography using a Pharmacia MonoQ HR 10/10 column, dialysis and a final desalting step using Bio-Rad Econo-Pac 10DG columns. The oligonucleotides were flash-frozen and lyophilized prior to solubilization in water to obtain the final stock concentration.

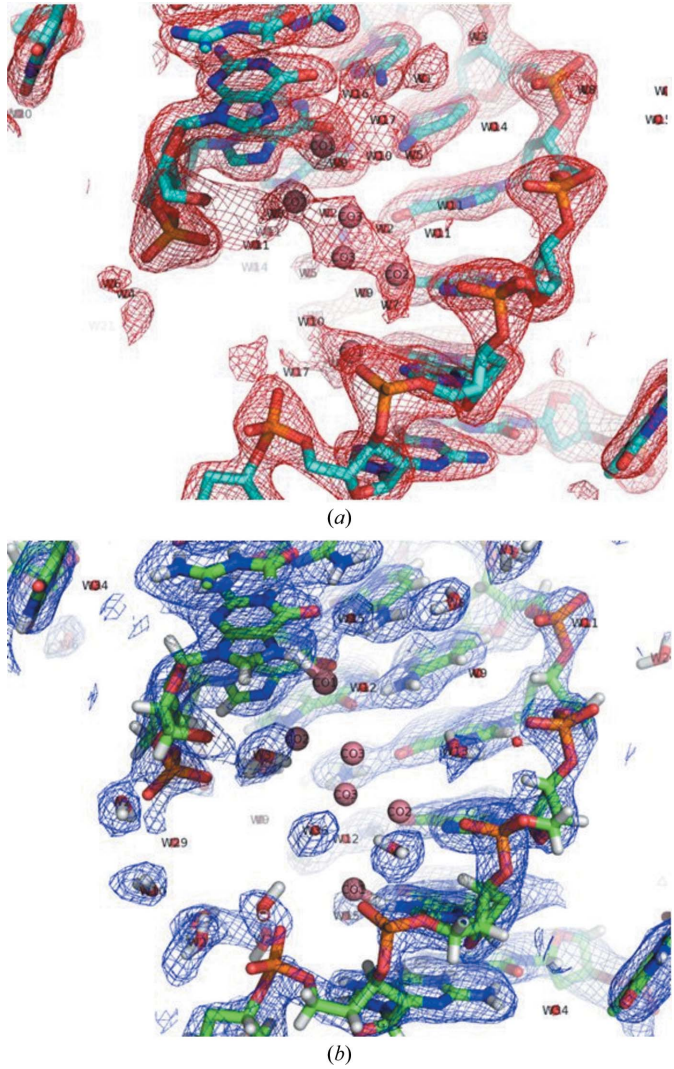

Figure 2
A view into the major groove of d(AGGGGCCCCT)$_2$ showing $2F_o - F_c$ density maps of (*a*) electron density (red) and (*b*) nuclear density (blue). The electron-density map clearly shows the Co atoms present in the structure. The neutron scattering density map shows water molecules located in a manner that is consistent with cobalt hexammine interactions, but the cobalt hexammine itself is only weakly visible (see text).

The concentration of the samples was determined by UV spectroscopy. The samples were annealed from 368 K prior to crystallization. Crystals were obtained at room temperature in hydrogenated buffer using the sitting-drop vapour-diffusion method. Crystallization conditions were optimized from a previously published protocol (Gao *et al.*, 1999) using vapour-diffusion techniques and a layer of Al's oil (a 1:1 mixture of paraffin and silicone oil) on the surface of the MPD in the reservoir solution against which the crystallization drop was equilibrated. Crystals were mounted in quartz capillaries and left to equilibrate by vapour diffusion against deuterated crystallization buffer to reduce neutron incoherent scattering from the exchangeable hydrogen in the crystal sample. Samples were tested by X-ray diffraction and found to have $P6_122$ symmetry, with unit-cell parameters $a = b = 33.02$, $c = 78.82$ Å. For further information, see Leal *et al.* (2009).

2.1.2. DNA quadruplex preparation and characterization. The DNA oligonucleotide d(AGGGTTAGGGTTAGGGTTAGGG) was purchased from Eurogentec (Belgium). The dried oligonucleotide was used without any further purification and was dissolved in deionized water to a 2 m*M* strand concentration. Aliquots were dialysed overnight against either 200 m*M* KCl or 200 m*M* NaCl solution (as required). After dialysis, quadruplex formation was induced by heating the aliquots to 368 K for 5 min and slow-cooling to 277 K overnight. The presence of DNA quadruplexes was verified by 1D H-NMR. The quadruplex-binding ligand used in this study is shown in Fig. 1.

2.1.3. Neutron crystallography. Neutron diffraction experiments were carried out using the new LADI-III Laue diffractometer at the Institut Laue–Langevin (ILL). The instrument is based on a large cylindrical neutron image-plate detector and has been used to study a wide range of macromolecular crystals (Petit-Haertlein *et al.*, 2009, 2010; Leal *et al.*, 2009; Novak *et al.*, 2009; Teixeira *et al.*, 2008; Gardberg *et al.*, 2009; Weiss *et al.*, 2008; Oksanen *et al.*, 2009; Blakeley *et al.*, 2010; Tomanicek *et al.*, 2010). The improved design of LADI-III provides a threefold gain in neutron detection by comparison with its predecessor at the ILL (Wilkinson *et al.*, 2009; Blakeley *et al.*, 2009) and exploits improved signal-to-noise and decreased spatial overlap. The incident beam is defined by a nickel/titanium multi-layer bandpass filter which selects a neutron wavelength range extending from 3.1 to 4.2 Å and centred at 3.7 Å. Data were recorded as a series of Laue images with a defined step separation about the vertical rotation (φ) axis of the detector. The Laue data were processed using the *LAUEGEN* program (Campbell, 1995; Helliwell *et al.*, 1989) modified for the cylindrical geometry of the LADI-III detector (Campbell *et al.*, 1998). The program *LSCALE* (Arzt *et al.*, 1999) was used to derive the wavelength-normalization curve using the intensities of symmetry-equivalent reflections measured at different wavelengths. *SCALA* (Collaborative Computational Project, Number 4, 1994) was used to combine and merge the observed data. There was no appreciable radiation damage to the sample and no explicit absorption correction was applied. Data were recorded to a resolution of 2.4 Å with a completeness of 82%.

2.1.4. X-ray crystallography. X-ray data sets collected in connection with this work were recorded using an in-house diffractometer (Cu $K\alpha$ radiation; $\lambda = 1.5418$ Å) equipped with a MAR 345 image-plate detector. Data were recorded at room temperature in such a way as to maximize their comparability with the neutron data and hence their viability in the context of refinement against a joint neutron/X-ray target function. X-ray data were reduced using *MOSFLM* (Leslie, 2006) and *SCALA* and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Observations deviating by more than five standard deviations from the mean

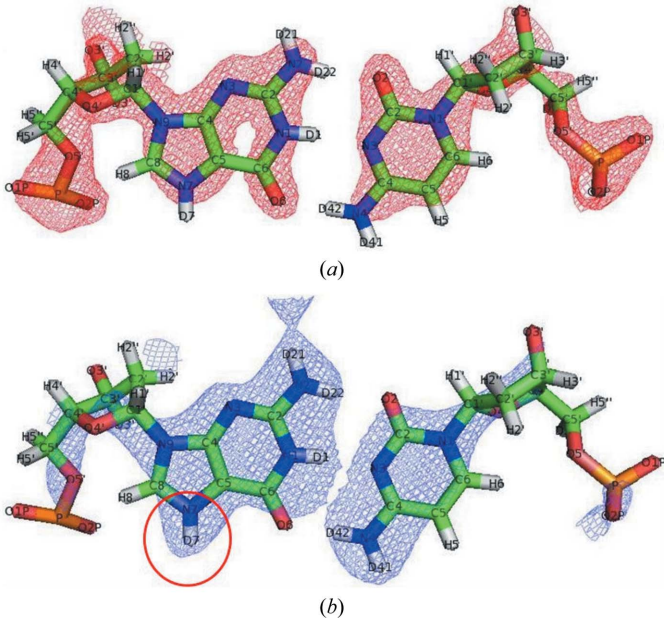

Figure 3
$2F_o - F_c$ density maps determined on the basis of observed data recorded from the same crystal of the oligonucleotide duplex d(AGGGGCCCCT)$_2$ in an A conformation: (*a*) electron-density map, (*b*) nuclear density map. Note that the N7 position of guanine (circled) is deuterated, implying that this position would be protonated in hydrogenous solvent conditions.

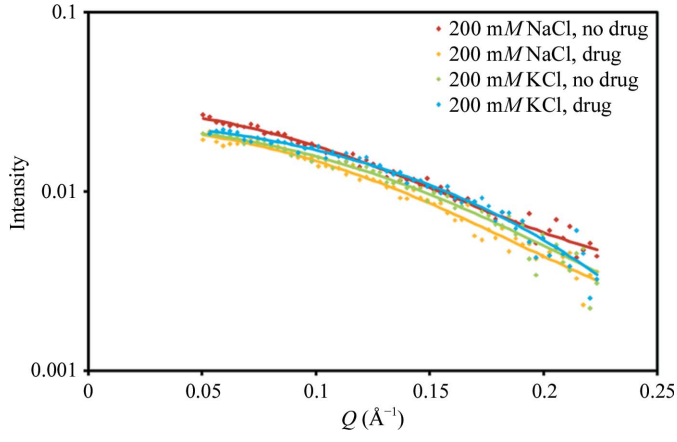

Figure 4
SANS data recorded using the D22 instrument at ILL for 200 m*M* NaCl and KCl samples of ligand (I) with the oligonucleotide d(AGGGTTAGGGTTAGGGTTAGGG).

were rejected during scaling. Data were collected to a resolution of 2.02 Å with a completeness of 98%. Joint X-ray/neutron refinement was carried out using *PHENIX* (Adams *et al.*, 2009).

2.1.5. Small-angle neutron scattering (SANS). SANS measurements were performed on instrument D22 at the ILL. The sample-to-detector distance was 2.8 m and covered a q range of 0.01–0.25 Å^{-1}. Samples of ∼200 µl were loaded into 1 mm path-length Hellma cells. The sample temperature was controlled by a water bath circulating through the sample holder. Data reduction and radial averaging of the solution scattering data were performed using *GRASP* (Dewhurst, 2008). Analysis was performed using the *ATSAS* software package (Konarev *et al.*, 2006). Molecular models were visualized using *PyMOL* (DeLano, 2002).

3. Results

3.1. Crystallographic studies of an A-DNA-forming oligonucleotide reveal unusual base-protonation states

The X-ray structure, as shown in Fig. 2(*a*), shows the presence of four cobalt-binding sites. Three cobalt sites are situated in the interior of the major groove and the remaining cobalt is located in the interstitial space created by the symmetry-related strands. Interestingly, these positions showed up only weakly in the $2F_o - F_c$ neutron maps (Fig. 2*b*). This situation arises in part from the relatively low neutron scattering length of cobalt, but is mainly because cobalt hexammine $\{[Co(NH_3)_6]^{3+}\}$ is a classic example of an exchange-inert metal complex and there has obviously been little, if any, H/D exchange in the amine groups even after prolonged exposure to D_2O. As a result, given the resolution of this study (2.4 Å) and the fact that there are 18 H atoms in the vicinity of each of these Co atoms, the negative scattering length of hydrogen results in serious density cancellation in the major groove of the molecule.

There are also a number of interesting observations relating to the bases of the structure. In this crystal symmetry, the central GGGCCC core of the self-complementary sequence forms the duplex, which is largely recognisable as an A-DNA-type structure. The terminal AT part of the sequence is flipped out from the duplex, forming interactions with symmetry-related molecules. While the X-ray data suggest nothing out of the ordinary at the termini of the molecules, the neutron data show protonation at N7 of guanine 2 and guanine 3, and N3 of adenine 1 (Fig. 3).

3.2. Small-angle solution scattering studies of the DNA quadruplex d(AGGGTTAGGGTTAGGGTTAGGG) and the effect of NaCl and KCl

Data were recorded for a range of NaCl and KCl ionic strengths extending from 10 to 200 m*M*, with the goal of identifying the effect of the nature and strength of the ionic environment. As an example, Fig. 4 shows a selection of SANS data recorded from both NaCl and KCl samples at 200 m*M*.

Fig. 5 shows dummy-atom modelling carried out for data recorded over the whole range of ionic strengths for each of the NaCl and KCl systems. For NaCl and KCl concentrations up to 100 m*M* essentially the same sample 'dumbbell'-shape profile is extracted from the data in both the absence and the presence of the ligand. Beyond 100 m*M*, however, the behaviour of the two systems changes, with an obvious difference in the behaviour of the KCl–quadruplex system at KCl concentrations higher than 100 m*M*.

Other solution structural work on d(AGGGTTAGGGTTAGGGTTAGGG) (Callow *et al.*, 2010) suggests that the dumbbell shape being modelled in this work corresponds to an end-on-end dimer of the quadruplex. The structure observed in the presence of KCl and (I) at concentrations of 150 and 200 m*M* is consistent with a monomeric complex.

4. Discussion and conclusions

The results emphasize the differing scopes and the complementarity of crystallographic and solution scattering methods for the study of nucleic acid systems and for an understanding of their behaviour in different environments. X-ray single-crystal studies offer the possibility of high-resolution or even ultrahigh-resolution information, although, as has been found in protein crystallographic work (Howard *et al.*, 2004), it is difficult to locate H atoms with high B factors and/or low occupancy even when very high-resolution data are available.

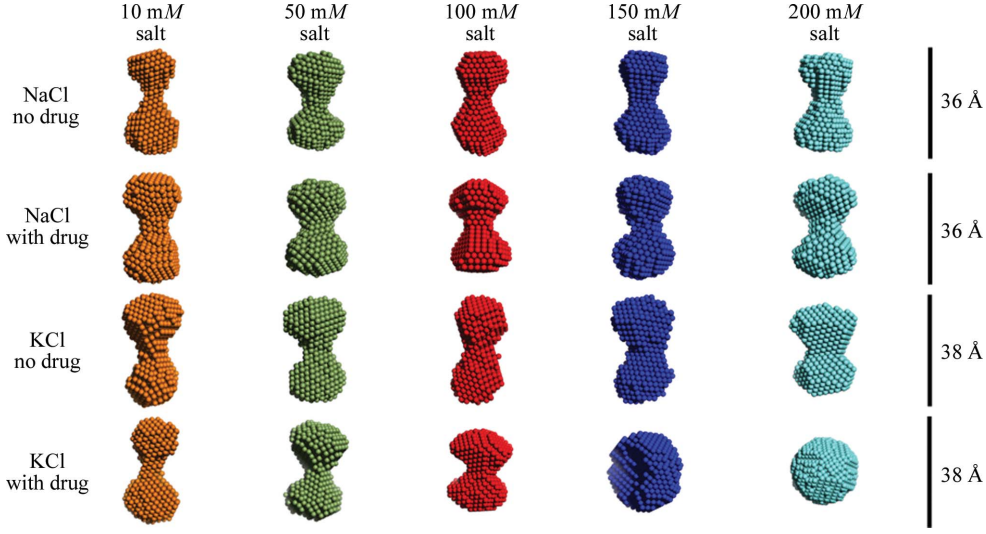

Figure 5
Dummy-atom modelling of SANS data recorded from ligand–quadruplex systems as a function of ionic strength of NaCl and KCl.

The importance of this for the study of DNA oligonucleotides is well illustrated by the preliminary work on the A form of d(AGGGGCCCCT) described in §3.1. Unexpected base protonation is observed in some of the purine bases of this oligonucleotide, suggesting a significant influence of the local environment. Such a possibility has been suggested (Lamm & Pack, 1990), but to our knowledge this is the first time that it has been observed experimentally in this type of system. More extended data have recently been recorded for two different crystal symmetries and a full account is being prepared for publication (Leal *et al.*, in preparation). The results raise the possibility of protonation shifts being a significant aspect of DNA polymorphism and one that could be of importance in recognition and regulation. The occurrence of such shifts might not be that surprising given the major changes in hydration and ionic strength that are associated with conformational transitions in DNA. A systematic study involving as wide a range of approaches as possible is needed to pursue this. However, the reality is that only neutron approaches will be able to provide unambiguous information on the location of H atoms on the bases of DNA.

The solution studies of DNA quadruplexes illustrate how a completely different approach can be taken to the study of ligand interactions with DNA, especially for situations in which the ligand induces changes in the solution structure of the DNA. The SANS modelling studies summarized in §3.2 show that the nature of the chemical environment and its effects on DNA structure are identifiable in solution studies. While the resolution is limited, it is important to note that solution studies provide important complementary information relating to a completely different type of environment.

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