ps-TRIR covers all the bases – recent advances in the use of transient IR for the detection of short-lived species in nucleic acids

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Recent developments of the picosecond transient absorption infrared technique and its ability to elucidate the nature and kinetic behaviour of transient species formed upon pulsed laser excitation of nucleic acids are described.

Introduction

The photochemical stability of nucleic acid bases is governed, at the most fundamental level, by the existence of ultrafast (picosecond and sub-picosecond) processes, which efficiently mediate excited state decay. These include base tautomerisation, the formation of dark excited states, electron or proton transfer, and/or ultrafast internal conversion (Fig. 1). Within this narrow window of time there are a large number of factors that modulate the deactivation pathways of the photochemically generated excited states. For instance, the extent to which deactivation processes are affected by the secondary structure of DNA, formed through vertical stacking interactions and lateral hydrogen bonding interactions, is still an open question. Understanding the communication between the bases in these overall processes is also a challenging aim. Until recently the main spectroscopic methods used to probe excited state species were transient UV/visible absorption[1–5] and fluorescence methods (especially using upconversion methods).[6–9] However, these approaches have limitations, particularly since they do not directly provide information about structural modifications occurring during the rapid processes associated with the ultrafast electronic and energetic perturbations that follow photon absorption. Monitoring such changes is vital to distinguish between the various possible relaxation processes. These include intramolecular reorganisation involving bond rearrangements within a particular excited nucleobase and intermolecular processes involving base–base coupling and/or solvation or other

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environmental effects. Ultrafast time-resolved infrared (TRIR) measurements have the potential to provide such insight and in this article we summarise recent work in this area.

The ps-TRIR method

The ability of TRIR to study nucleic acid bases has been driven forward in recent years by ourselves and others exploiting innovations in ultrafast laser technology. This provides a means to generate intense (mJ) ultrafast pulses (picoseconds to tens of femtoseconds) that can be used to create broadband *ca.* 200 cm^{-1} wide mid-infrared laser radiation using different frequency generation (DFG) methods. Here we describe the Rutherford Appleton Laboratory Picosecond InfraRed Absorption and Transient Excitation ('PIRATE') ps-TRIR instrument (Fig. 2). Transient infrared spectroscopy is now becoming widespread and this approach is being utilised by many groups such as those in ref. 10. The 'PIRATE' equipment generates stable broadband mid-IR pulses for TRIR using an optical parametric amplifier (OPA) approach originally described by Kaindl *et al.*[11] The mid-IR pulses are generated by difference frequency mixing in an $AgGaS_2$ crystal of the signal and idler outputs, obtained from a white light continuum seeded 800 nm pumped β-barium borate (BBO) OPA. The mid-IR pulse energy and spectral stability are obtained through careful control of the amplifier gain characteristics and the use of a stable pump laser source of good beam quality. The 267 nm and 200 nm pump beams are respectively the third and fourth harmonics of the 800 nm Ti:S laser. The 267 nm excitation is generated by mixing the 800 nm fundamental with the 400 nm second harmonic, and the 200 nm light is generated

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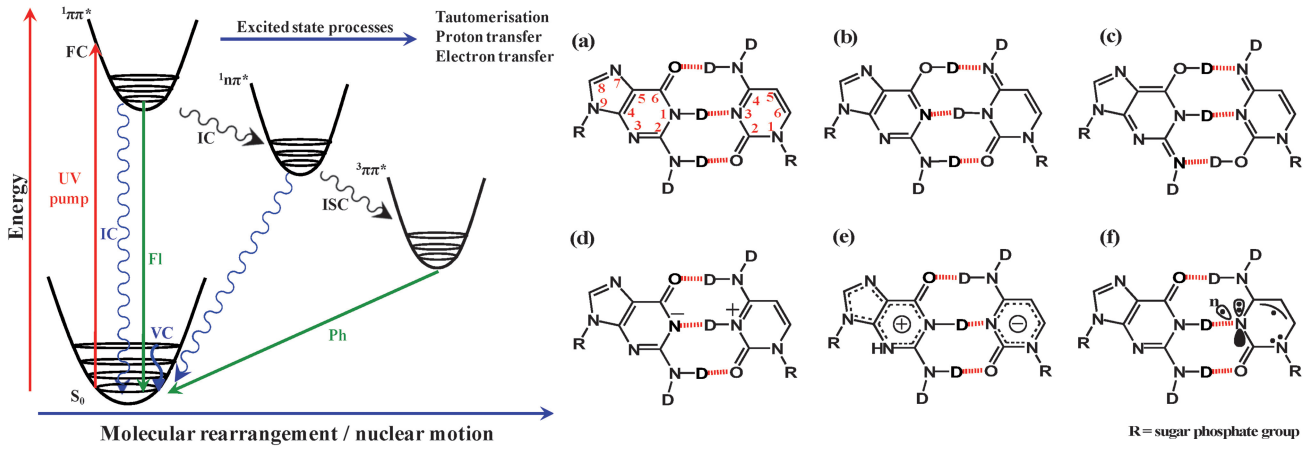
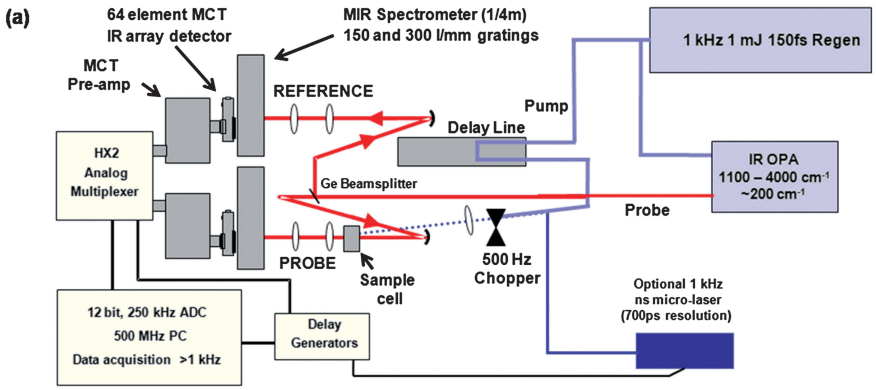


Fig. 1 Schematic state diagram (left) illustrating ultrafast processes in DNA following UV excitation and examples of transient photoproducts that may form (right) following UV excitation of Watson–Crick G–C base-pairs. (a), (b) and (c) Tautomers, (d) proton transfer product, (e) charge transfer product, and (f) $^1n_N\pi^*$ form for the excited state. Key: FC – Franck–Condon, IC – internal conversion, ISC – intersystem crossing, VC – vibrational cooling, Fl – fluorescence and Ph – phosphorescence.



(b)

$$\Delta T_N = \frac{\left[\left(\frac{I_{probe}}{I_{ref}}\right)_{pump\ on} - \left(\frac{I_{probe}}{I_{ref}}\right)_{pump\ off}\right] + \Delta T_{N-1}(N-1)}{N} \quad (1)$$

$$\Delta A_N = -\log\left(1 + \frac{I_R}{I_P}\Delta T_N\right) \quad (2)$$

Fig. 2 (a) Experimental setup of the broadband ps-TRIR instrument,[28] and (b) equations used in calculating the difference absorption spectra.

by mixing of the 800 nm fundamental with the 267 nm third harmonic, all within 1 mm β-barium borate non-linear optical crystals. The resultant time response of the PIRATE system is *ca.* 500 fs. The routine shot-to-shot energy stability and spectral stability of PIRATE are, separately, at the ±4% level. Therefore normalization of the IR probe to the pulse energy does not compensate for spectral fluctuations. To do this two linear arrays are employed, the first to monitor the laser/sample interactions and a second array as a reference detector to monitor and correct for changes in spectral profile on a shot-by-shot basis. This leads to a more than four times improvement in the signal-to-noise over pulse energy referencing alone, leading to sensitivity *ca.* 2×10^{-4} ΔA in one second of accumulation. Routinely, mid-IR band-widths of *ca.* 200 cm^{-1} are obtained on the PIRATE instrument using this design with a pulse energy of 100 nJ.

Another important development in technology for TRIR measurements has been the availability of mid-IR sensitive arrays based on mercury cadmium telluride (MCT) material. Such arrays coupled with fast read-out systems are able to make pump–probe measurements at high repetition rates and to normalize each individual IR probe pulse in a dual beam arrangement (Fig. 2(a)). The wavelength-dispersed probe and reference beams are detected by 64-element MCT arrays and the signals fed simultaneously into HX2 analog multiplexer boards based on a Rutherford Appleton Laboratory design. These were carefully engineered to minimise emf noise pickup from laboratory sources that degrades signal-to-noise. Two channels of a 4-channel 12-bit ADC capture the analog multiplexer signals and a third takes a pump on–off status signal and transfers it to PC RAM. A fast computer is used to analyse data in real time to

provide signal discrimination and normalization shot-by-shot and to average for archiving and displaying the spectral profiles of pump on–off difference signals. The change of absorbance ΔA is calculated using eqn (1) and (2) in Fig. 2(b) (where I_R and I_P are the final averages of the pump-off spectra on the reference and probe side, respectively, and N is the total number of acquisitions).

The signal discrimination is essential to remove occasional large fluctuations in the signal due to laser 'drop outs', or scattering of the probe beam by particulates and gas bubbles in the sample. This combination of probe and reference normalization and real-time pulse-to-pulse signal processing has enabled us to achieve high sensitivity with our apparatus, on the order of 2×10^{-5} ΔA with 1 min of acquisition time. This corresponds to an effective shot-to-shot stability of *ca.* 1%.

To minimise attenuation of the IR probe and transient dynamic and fixed-pattern noise in the TRIR signals due to fluctuations from air currents in the relative signal strengths along the different beam paths, all IR probe and reference beams and optics are enclosed within dry nitrogen-purged beam pipes and boxes to reduce absorption by atmospheric water vapor.

Sample preparation is also critically important. Most nucleic acid studies are carried out in D_2O, as H_2O absorbs strongly in the region of most interest (1450–1750 cm^{-1}). (Note therefore that exchangeable H atoms are replaced by D in the ground state and hence in the transient species.) Typical nucleotide concentrations are of the order of 10 mM. For such samples conventional IR cells with CaF_2 windows have been used (Harrick Scientific) with variable pathlengths (5–50 μm). In order to prevent sample degradation on the windows of the cell, it is randomly oscillated in the plane perpendicular to the direction of the laser beams using motors and smooth ball slides mounted on an *x*–*y* stage. Both the pump and probe laser beams are focused to around 100 μm diameter and pump laser energy set below the threshold for sample degradation through sample window interface effects and non-linear processes.

ps-TRIR studies of nucleic acids

Bases, nucleosides and nucleotides

It has been known for many years that the fluorescence quantum yield of each of the four natural deoxynucleotides is very low ($<10^{-4}$), implying a sub-picosecond lifetime for the singlet excited states of these complexes.[1] In recent years the lifetimes of the excited states of the nucleosides and nucleotides have been determined directly by both visible light transient absorption studies and by fluorescence lifetime measurements using upconversion methods.[1–9] These show that the lifetimes of the $\pi\pi^*$ states are indeed very short (460 fs for guanosine; 290 fs for adenosine; 720 fs for cytidine; 540 fs for 5′-thymidine).[3] It might therefore be questioned whether studying these compounds with the PIRATE instrument, using only picosecond time resolution, would yield any useful information. However, our initial studies[12] did indeed show that after 267 nm excitation both ground state bleaching and transient absorption could be observed in the spectral region 1580–1750 cm^{-1} for each of the nucleotides, with lifetimes ranging from 2 to 5 ps, which are too long to be assigned to the electronic excited states.[3] The transient absorption shows features, which are characteristic of the decay of 'hot' electronic ground states, namely the 'tracking' of the IR absorption band to higher wavenumber as the relaxation of the vibrationally excited molecule proceeds. This shifting of the transient bands at early times in each case is believed to be due to vibrational cooling – that is vibrational relaxation observed by IR bands shifting as the populations of specific higher vibrational modes formed as a consequence of the rapid conversion of the upper to ground electronic state cool to $v = 0$ and/or anharmonic coupling of the higher vibrational modes with lower vibrational modes as energy cascades through to the lower modes. The similarity of the spectral features with those obtained by 2D-IR experiments[13] strongly suggests that 267 nm excitation and subsequent internal conversion results in population of the $v = 1$ state. However, the early time dynamics associated with the bands' shifting remains to be fully explained and will require observations made across the lower spectral region. A temperature-dependent study might also be useful to see if temperature can induce similar spectral changes. This was previously done in experiments investigating the anti-Stokes Raman and non-Boltzmann equilibrated electronic excited states in *trans*-stilbene.[14] Although evidence for vibrational cooling of the ground states has previously been deduced from transient broadening of UV absorption bands,[3] ultrafast infrared measurements should allow a more detailed study and provide greater insights into this process. For example, in a recent study[15] it was found that there were mode-specific kinetics associated with 5′-dGMP where the vibrational relaxation of the hot ground state is slower (4.7 ps) for the predominantly ring-based vibration (centred at 1581 cm^{-1}) than for the 1669 cm^{-1} centred carbonyl stretch vibrational band (3.1 ps). This observation has been attributed to environmental perturbations, as one would expect the C═O to be more influenced by surrounding solvent molecules through H-bonding. This permits a more dominant intermolecular energy migration process either directly through this mode or *via* lower (<400 cm^{-1}) modes that are coupled through vibronic coupling or cascade energy transfer to the lower modes.

In the case of 5′-dAMP and 5′-dGMP the decays are highly reversible (Fig. 3), consistent with very rapid internal conversion as expected from the visible transient absorption and fluorescence measurements. Also with 5′-TMP the dominant process is cooling of the hot ground state, although in these TRIR experiments the system was found to be less reversible with a significant amount of bleaching having a lifetime of greater than 1 ns, possibly due to the formation of photoproduct and/or triplet state (see later). However, the most interesting results were found for 5′-dCMP. Here, in addition to the formation of a hot ground state (as found for the other bases), a new entity with a lifetime of 33 ps was specifically characterised.[16] This clearly indicates that there are at least two deactivation routes for the initially formed $\pi\pi^*$ state – one involving sub-picosecond formation of the ground state (presumably following very rapid internal conversion) and the other leading to this longer-lived species. Of the various possibilities considered for this transient (*e.g.* tautomers, excited states), some are unlikely on the basis of the strong IR transient absorption band and the authors favoured its assignment as a $^1n_N\pi^*$ excited state.[16] Advanced detailed computational studies of its IR spectrum will be required to confirm this assignment. The very low probability of the radiative transition

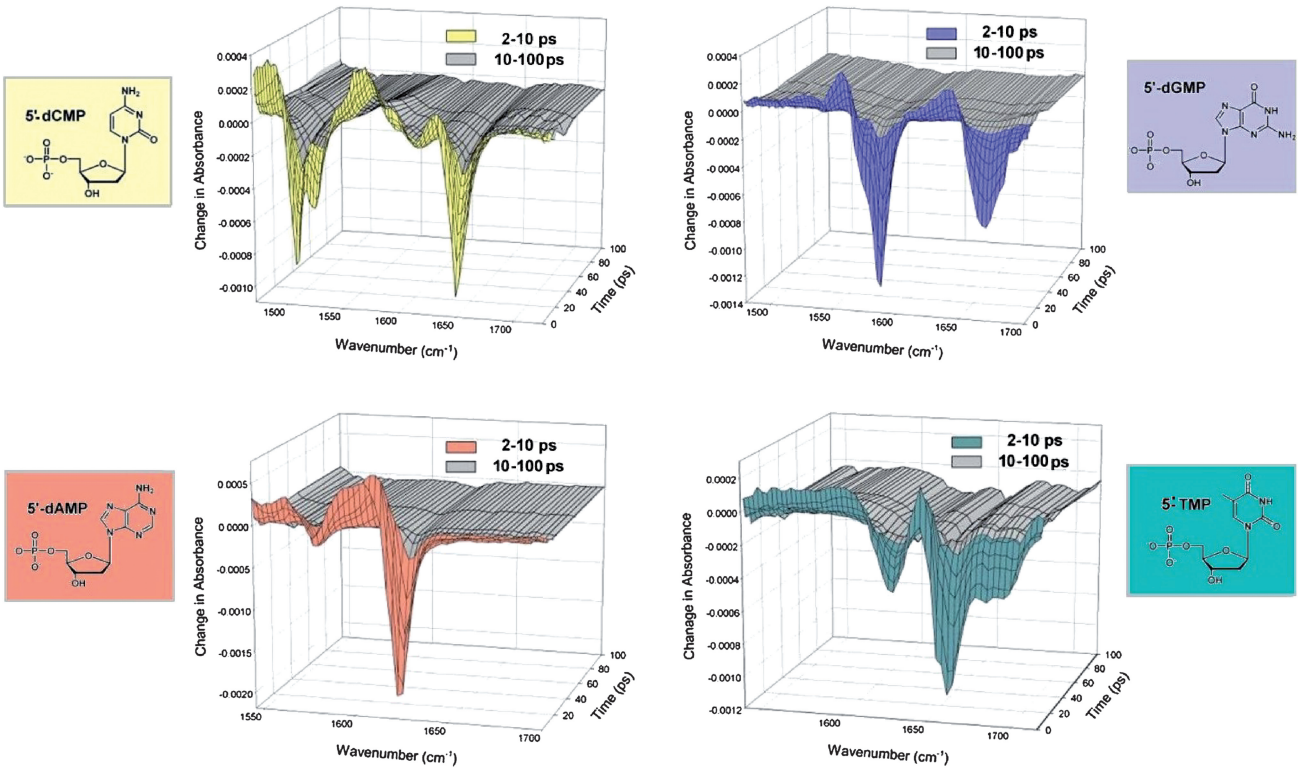


Fig. 3 DNA building blocks: ps-TRIR spectra observed after 267 nm excitation of the four mononucleotide bases (10 mM) in 50 mM phosphate buffer in D_2O (pH 7). Plots show the evolution of the TRIR profile between 2 and 100 ps.[12,15,16]

from this state to the ground state explains why this electronic excited state could not be observed by fluorescence methods. While the nucleoside cytidine (dCyd) gave very similar results to the nucleotide (5′-dCMP), the behaviour of the nucleobase cytosine (Cyt) was strikingly different (as only a 'hot' ground state was observed) demonstrating that the attachment of the ribose ring to the N1 position of the pyrimidine ring has a major influence on the photophysics. Whether this is a consequence of electronic or steric effects remains to be elucidated.

The thymine moiety is the most photo-reactive of the nucleic acid bases and cyclobutane dimers are the most abundant photolesions in UV-irradiated DNA.[17] The role of the triplet excited state in their formation is still uncertain. To learn more about this species Kohler and co-workers[18] have used both ns- and ps-TRIR to study the triplet state of thymine and of thymidine in deuterated acetonitrile, where its yield is about 10 times that in water. The $\pi\pi^*$ triplet state was shown in the ns-TRIR study to have a characteristic band at 1603 cm^{-1} and to decay with a lifetime of 560 ns. In their ps-TRIR measurements, as well as a species exhibiting a broad band initially centred at 1640 cm^{-1}, which was assigned to the vibrationally excited ground state, another species was observed at 1602 cm^{-1}. Most of the signal at this wavenumber decayed within 10 ps but about 20% remained to longer times and was assigned to the triplet state. It was proposed that the triplet state is most probably formed from a vibrationally non-relaxed $\pi\pi^*$ singlet state or less likely it may be populated from the $^3n\pi^*$ excited state. It should be noted, however, that this behaviour may be quite different in aqueous solution where the ordering of the excited states may vary.

Protonated GMP

The properties of nucleotides are sensitive to the pH of the medium. Thus in acid solution guanine is protonated on the 7-position of the base, which causes a marked lengthening of the singlet state lifetime (τ = *ca.* 200 ps). Fig. 4 shows the ps-TRIR spectra of the H_2GMP in unbuffered D_2O solution, where both protonated and unprotonated GMP are present. The characteristic signals of both the protonated excited state (*e.g.* bleaching at 1690 cm^{-1} and transient absorption at 1635 cm^{-1}; $\tau = 214 \pm 20$ ps) and the unprotonated GMP (*e.g.* bleaching at 1677 cm^{-1} and transient absorption at 1635 cm^{-1}; $\tau = 3.5 \pm 0.3$ ps) are readily distinguishable. This example also nicely illustrates the power of ps-TRIR in unravelling the behaviour of complex systems, such as those which might be found in natural DNA samples.[19]

Oligo- and poly-nucleotide systems

In DNA systems the interaction between the nucleobases is dominated by base-stacking and/or hydrogen-bonding. Such processes also occur at high concentrations of the nucleotides. For example, guanine is well-known to self-associate, yielding stacks of tetramers where the bases are linked through Hoogsten-type base-pairing. A ps-TRIR study of a 540 mM solution of the sodium salt of GMP gives a spectroscopic signature and kinetic behaviour quite different from that of the isolated nucleotide.[15] Similar behaviour is found for polyguanylic acid (polyG) where the high local concentration of the guanine again promotes the formation of the stacked tetrad. Two distinct transient species

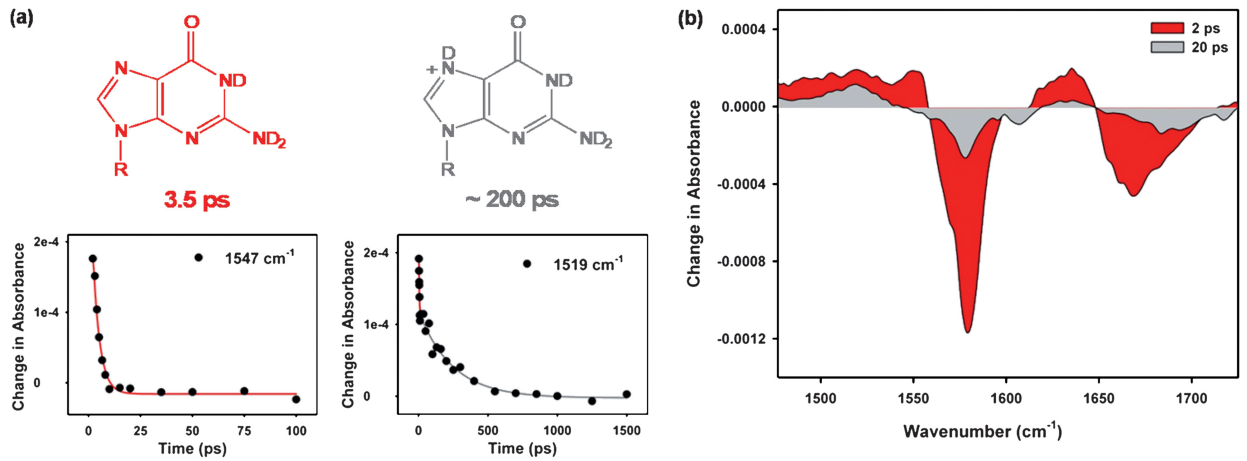


Fig. 4 (a) Kinetics displayed by GMP under neutral and acidic conditions. (b) ps-TRIR spectra of a mixture of species in unbuffered solution D_2O at 2 and 30 ps after excitation at 267 nm.[19] The spectrum at 2 ps is characteristic of GMP, while the spectrum at 20 ps is characteristic of the excited state of $GMPD^+$.

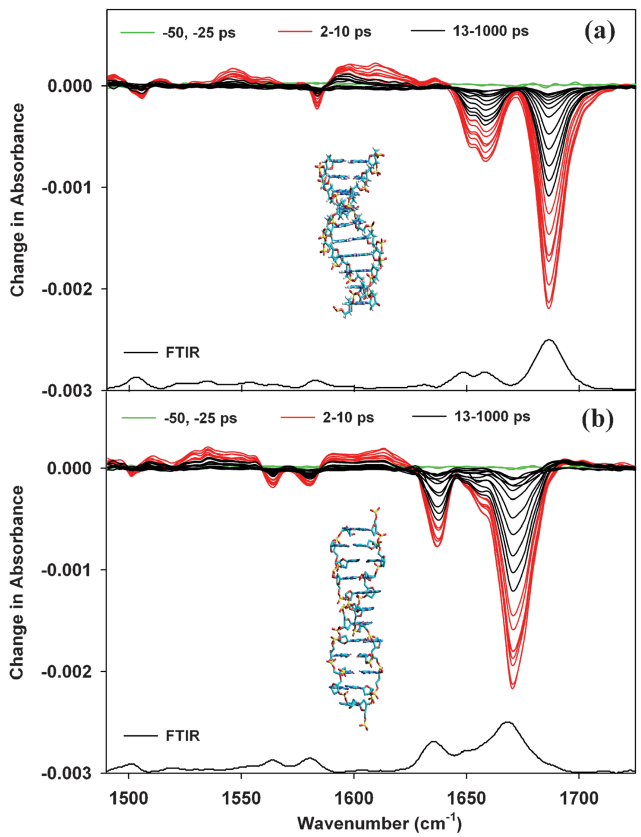


Fig. 5 ps-TRIR spectra recorded after 267 nm excitation in 50 mM potassium phosphate D_2O buffer at pH 7 of (a) B-form $[poly(dG\text{-}dC)]_2$ (10 mM) and (b) Z-form $[poly(dG\text{-}dC)]_2$ (10 mM) with 4 M NaCl.[20] FTIR spectra are shown below the ps-TRIR. Delays are at −50, −25 (green), 2–10 (red) and 13–1000 (black) ps.

are observable by ps-TRIR with a short-lived transient (lifetime *ca.* 5 ps) assigned to vibrationally excited ground state and a longer-lived species (*ca.* 36 ps) to an excimer-type excited state of the stacked G-tetrads. Very similar transient decays were also recorded for the G-regions of the oligonucleotide characteristic of the human telomere sequences $(TTAGGG)_n$.[15]

These studies are useful and relevant to the investigation of double-stranded polynucleotides such as DNA, where the structures are well-defined and the importance of both hydrogen-bonding and base-stacking has been established. In natural DNA there are of course many combinations of stacked pairs of different bases, so it is initially sensible to look at simpler systems where only two types of bases are involved. An example of this is $[poly(dG\text{-}dC)]_2$, which forms regular B-DNA double-stranded structures in solutions at moderate ionic strengths. ps-TRIR experiments again reveal evidence for at least two quite distinct processes with the first having a lifetime of *ca.* 7 ps and the second of *ca.* 30 ps, occurring with approximately equal amplitude (Fig. 5).[20] The first, which is characterised by broad transient absorption bands, is attributed to the vibrationally excited ground state with the excitation apparently delocalised over several base-pairs. By contrast the second transient is characterised by a sharper absorption band, peaking at 1597 cm^{-1}, indicative of a localised excited state. There has recently been intense theoretical interest[21–24] in the processes following excitation of G–C base-pairs, with reactions such as electron- and/or proton-transfer (see Fig. 1) between the two nucleobases being proposed to explain the fact that the excited state fluorescence is extremely short-lived and shorter than that of the constituent bases. It is possible therefore that the 1597 cm^{-1} transient species could be the product of one such process. However, it has been tentatively proposed that this transient is the cytidine-based $^1n_N\pi^*$ state.[20] Full elucidation of the nature of this species will require calculation of the spectra for a range of possible intermediates.

Preliminary data have also been presented for double-stranded B-DNA, containing only alternating A and T bases, *i.e.* $[poly(dA\text{-}dT)]_2$.[12] As with the case of $[poly(dG\text{-}dC)]_2$ the return to the ground state can be analysed in terms of two principal processes. The first is assigned to the 'hot' ground state and the second to an exciplex (**exci**ted state com**plex**).[25] The spectrum of this excited

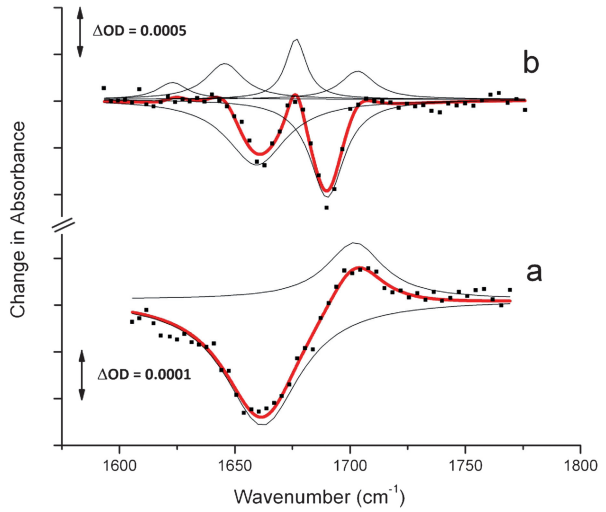


Fig. 6 TRIR spectra generated using 200 nm excitation for (a) 5′-dGMP (10 mM) averaged between 30 and 500 ps; (b) poly(dG-dC)–poly(dG-dC) (10 mM)/bp, averaged between 500 and 1000 ps.[26] Both samples are in 50 mM phosphate D_2O buffer. Solid lines: (black) individual Lorentzian curves and (red) the summed multicurve fit.

state shows similarities to the spectrum of the exciplex spectrum of the dinucleotides dApT and TpdA, although the lifetime is significantly longer for the polynucleotide.[25]

Photoionisation

Irradiation of 5′-dGMP with 200 nm excitation is known to generate both electronic excited states and radical cations. This was confirmed in a ps-TRIR study, where both the vibrationally hot ground state and another longer-lived ($\tau > 1$ ns) species with an infrared marker band at *ca.* 1700 cm^{-1} were observed (Fig. 6a).[26] The intensity of this longer-lived transient is *ca.* 20 times smaller than that of the hot ground state band at *ca.* 1638 cm^{-1}. While this species is assigned to a product of photoionisation, the exact nature of the transient remains controversial at this time because the guanine radical cation produced immediately after photoionisation may undergo rapid deprotonation[27] (eqn (3)), where G(–H)$^{\bullet}$ represents a guanine which has lost a hydrogen atom:

$$G^{\bullet+} \rightarrow G(-H)^{\bullet} + H^{+} \quad (3)$$

Evidence for the formation of similar transient species were also found upon the photoionisation of $[poly(dG\text{-}dC)]_2$ (Fig. 6b).

As well as causing direct photoionisation, 200 nm UV radiation can cause damage to DNA indirectly by producing radical species from other molecules, especially from the solvent. In the case of water $^{\bullet}OH$, $^{\bullet}H$ and the solvated electrons are produced and these species can then attack DNA. To study such indirect processes a nanosecond laser was electronically synchronised to the ps-TRIR instrument.[28] Doing this provides a way to work beyond the limits imposed by the optical delay lines and onto much longer timescales (microseconds), thus allowing diffusional processes to be directly observed. This was demonstrated by the generation of the oxidised guanine species *via* the biomolecular reaction of 5′-dGMP with carbonate radicals, formed by nanosecond laser pulse (267 nm) excitation of $[Co(NH_3)_4CO_3]NO_3$.[24] Using the ultrafast IR probe beam the same characteristic transient at *ca.* 1704 cm^{-1} is observed with this indirect method as for the photoionisation experiments when exciting with 200 nm radiation.

This ability to investigate redox processes of DNA across ps- to μs-timescales provides the means to study the direct oxidative damage to DNA over a wide timescale in its natural environment without the need for secondary reagents.

2-Aminopurine (2-AP). Further insight into photoionisation can be obtained by a study of 2-AP, which is an isomer of adenine (6-aminopurine). It can readily be incorporated into DNA where it mimics A and G by participating in Watson–Crick base-pairing with T or C respectively forming unperturbed double helical DNA structures.[29] However, unlike the natural DNA bases, 2-AP has a strong electronic absorption band at 305 nm (allowing its selective excitation within DNA). It is fluorescent with a long lifetime (*ca.* 10 ns)[30] and is easily ionised by 308 nm laser light in a two-photon process.[31] This latter property creates a convenient method of injecting charge into synthetic polynucleotide sequences. Kuimova *et al.* have performed ps-TRIR studies using 267 nm to ionise 2-AP, *via* a two-photon process.[32] The ps-TRIR spectra obtained in neutral and acidic conditions show significant differences that are assigned to the formation of distinct species, namely the 2-AP radical cation, 2-AP$^{\bullet+}$, in acidic conditions and the uncharged radical, 2-AP-(–H)$^{\bullet}$, in neutral conditions. The spectra of the various species have been modelled by DFT calculations (EDF1/6-31 + G*) to support the assignments of the intermediates and indicate that deprotonation of 2-AP$^{\bullet+}$ in neutral solution takes place within 2 ps following photoionisation. Future ps-TRIR studies should involve a study of the reactions of the guanine radical cation in DNA following its generation by electron transfer from photoionised 2-AP.

Photodimerisation

One of the most important photochemical reactions of DNA is the cycloaddition of two neighbouring thymine groups to form cyclobutane dimeric species. It had generally been supposed that this reaction, which occurs with a yield of *ca.* 3% following UV excitation in poly(dT) or $(dT)_{20}$, proceeds through the triplet state.[33] However, an elegant examination by Schreier *et al.* has demonstrated that the photoproduct is formed in less than 1 ps.[34] This conclusion was reached by comparison of the behaviour of 5′-TMP (where photodimers cannot form on an ultrafast timescale) with that of $(dT)_{18}$ in the 1300–1500 cm^{-1} spectral region. The small differences between the transient spectra of the mononucleotide and oligonucleotide showed the presence of characteristic photodimer bands at 1465, 1402 and 1320 cm^{-1} in the latter. Although at the shortest times some of these marker bands were obscured by the signals due to vibrational cooling, the authors were able to confidently predict that the dimerisation occurs on a femtosecond timescale. It is proposed that the process occurs when the pyrimidine bases are already present in the correct conformation to allow the formation of the *cis-syn* dimer upon excitation.

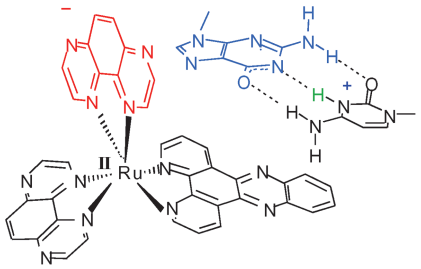


Fig. 7 Proposed product of proton-coupled electron transfer formed from excited $[Ru(TAP)(dppz)]^{2+}$ bound to double-stranded poly(dG-dC).

Photosensitised oxidation

It is well known that the excited states of many compounds when intercalated between the base-pairs of DNA are quenched, a process that has been ascribed to electron transfer from (primarily) a guanine base to the excited state. One such intercalating system is the metal complex $[Ru(TAP)_2(dppz)]^{2+}$ (TAP = 1,4,7,10-tetraazaphenanthrene; dppz = dipyrido-[3,2-*a*:2′,3′-*c*]-phenazine). Here a comparative study was made of the complex bound to double-stranded $[poly(dG\text{-}dC)]_2$ using transient visible spectroscopy (to monitor the formation of the reduced metal complex by its absorption at $\lambda > 500$ nm) and ps-TRIR (to follow changes in the IR spectrum of the nucleic acid).[35] It was observed that the formation of the reduced complex caused a simultaneous bleaching of both the guanine and cytosine carbonyl bands. Interestingly, a weak band at *ca.* 1700 cm^{-1} was also observed. This was tentatively assigned to an oxidised guanine transient species, as it is similar to that formed upon direct photoionisation (see above). In the case of the photosensitised reaction the process was believed to proceed *via* proton-coupled electron transfer from the metal complex excited state (eqn (4) and Fig. 7).

$$[Ru^{III}(TAP)(TAP^{\bullet -})dppz]^{2+\star} + \text{'G}\equiv\text{C'} \rightarrow [Ru^{II}(TAP)(TAP^{\bullet -})dppz]^{1+} + \text{'G(–H)}^{\bullet}\equiv\text{CH}^{+}\text{'} \quad (4)$$

Conclusions

As reported above, the last few years have demonstrated the power of ps-TRIR to probe ultrafast processes in nucleic acids. By providing functional group-specific information these studies considerably extend the insights already gained from transient UV/visible absorption and fluorescence techniques. In the case of the nucleotides the process of ground state vibrational relaxation is directly monitored (and in the case of GMP different rates for the ring and carbonyl modes observed). Dark excited states have been monitored for the pyrimidine nucleotides.

Increased sub-picosecond time resolution will be necessary in order to study the short-lived excited nucleobase $\pi\pi^*$ excited states. Of course, the infrared bands associated with these very short-lived excited states will broaden due to Heisenberg uncertainty making resolution and assignment of their IR spectra more challenging. On the other hand, faster time resolution opens up a new window onto the intramolecular state-to-state mode coupling by resolving coherence effects. These may provide information on the excited and ground states and how they couple so as to enhance and/or perturb intra- and inter-energy relaxation processes.

An important area for future study is the determination of the distribution of vibrational energy after relaxation of the initially formed excited states. In particular, the involvement of high- and low-energy modes and the energy distribution amongst the stacked base-pairs needs to be defined. Currently reported experiments can only provide limited information on these topics and it is likely that further insights will only be realised through advanced computation and possibly 2D-IR experiments.

A major future challenge is to fully characterise the longer-lived species (see Table 1) which are apparent in polynucleotides. In particular it will be important to determine whether these excited states are localised on a particular base [as has been suggested for the B-form of $[poly(dG\text{-}dC)]_2$] or on pairs of stacked bases (as in classic excimers/exciplexes) or alternatively delocalised over several base-pairs. Such characterisation will

Table 1 Transient species (lifetime > 10 ps) observed by ps-TRIR of nucleic acids

System	Ref.	Band/cm^{-1} [a]	Lifetime/ps	Assignment/comment
Bases and mononucleotides				
5′-dCMP	16	1574	33 ± 4 (20%)	$^1n_N\pi^*$ state
5′-GMP in 0.132 M H_3PO_4	19	1514	239 ± 9	GD^+ $\pi\pi^*$ singlet state
		1631	240 ± 20	
Thymine in CD_3CN	18	1603	560 000 ± 30 000	$\pi\pi^*$ triplet state
Photoionised 5′-dGMP	26	1700	>1000	$G^{\bullet +}$ or $G^{\bullet}(-D^+)$
Polynucleotides				
$[Poly(dA\text{-}dT)]_2$	12	1530–1605	150 ± 20 (35%)	Exciplex
		1633	162 ± 27 (54%)	
		1642	207 ± 41 (56%)	
$[Poly(dG\text{-}dC)]_2$ B-form	20	1546	38 ± 17 (43%)	$^1n_N\pi^*$ state
		1595–1605	21 ± 4 (100%)	
$[Poly(dG\text{-}dC)]_2$ Z-form	20	1520–1560	16 ± 2 (100%)	Exciplex
		1587–1605	17 ± 2 (100%)	
		1616	10 ± 2 (100%)	
Poly(G)	19	1553	35 ± 9 (28%)	Excimer
		1599	36 ± 4 (41%)	
		1648	36 ± 8 (28%)	

[a] ± 5 cm^{-1}.

require that experimental studies go hand-in-hand with computational investigations. If the excited state is indeed localised on particular bases or base-pairs then it will be intriguing to see whether it is possible to distinguish such features in mixed sequence natural DNA and to find how the properties of such excited states depend on base-stacking, H-bonding and conformation within the polynucleotide. Excitation of drug–DNA complexes is also expected to yield new insights into the nature of the binding of pharmaceuticals to nucleic acids.

Another area where we may expect ps-TRIR to have a major impact is in the characterisation of transient species responsible for photochemical reactions of DNA, building on the work already reported for thymine dimer formation and guanine oxidation. As mentioned above, the possibility of directly monitoring the guanine radical cation provides the opportunity to study oxidative damage in DNA, not only in the naked polynucleotide but also when protein-bound and in other biologically relevant conditions. As such, the technique may be expected to contribute greatly to our understanding of the role of such species in mutagenesis, genetic modification and errors in protein transcription.

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