Base-specific Ionization of Deprotonated Nucleotides by Resonance Enhanced Two-photon Detachment.

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ABSTRACT

The intrinsic ionization energy of a base in DNA plays a critical role in determining the energies at which damage mechanisms may emerge. Here, a two-photon resonance-enhanced ionization scheme is presented that utilizes the $^1\pi\pi^*$ transition, localized on the DNA base, to elucidate the base-specific ionization in a deprotonated nucleotide. In contrast to previous reports, the scheme is insensitive to competing ionization channels arising from the sugar-phosphate backbone. Using this approach, we demonstrate that for all bases except guanine, the lowest electron detachment energy corresponds to detachment from the sugar-phosphate backbone and allows us to determine the lowest adiabatic ionization energy for the other three bases for the first time in an isolated nucleotide.

Keywords: photoelectron spectroscopy, femtosecond spectroscopy, electrospray ionization, DNA, ionization energies, two-photon ionization,

Introduction

The interaction of electrons or high-energy photons with DNA is one of its main causes of damage which can ultimately lead to mutations and cell death.[1-5] From a molecular perspective, it is the ionization potentials of the DNA sub-units that are central to developing an understanding of how such damage may arise. In particular, the lowest (adiabatic) ionization energy of the nucleobases is of key importance as this is the lowest oxidation site in DNA and is an essential parameter in the conduction of holes through the polymer.[6,7] This importance is reinforced by the burgeoning applications of DNA in molecular nanotechnology.[8-10] Developing robust methods to predict the ionization energy in any environment is therefore a major scientific goal, but this is reliant on experimental benchmark data. Here, we present the experimental determination of the adiabatic ionization energies of the nucleobases in all 4 nucleotides in the gas phase.

To help elucidate the ionization mechanisms, and the threshold at which these are observed, a 'bottom-up' approach has been employed, starting with the ionization of isolated nucleobases in the gas-phase, which have been studied using photoelectron (PE) spectroscopy,[11-16] electron attachment[17-19] and computational chemistry.[20-25] In the solution phase, ionization potentials have been determined using PE spectroscopy on liquid microjets[26] and with *ab initio*[27-30] or semi-empirical calculations.[31,32] Building on the gas phase work, the PE spectroscopy of isolated nucleotides and oligonucleotides has recently been studied by the Wang group with the aim of understanding how the ionization energy changes with the introduction of the sugar and phosphate backbone.[33] Deprotonation of the nucleotide, which occurs on the phosphate,[34,35] conveniently enables mass-selection prior to PE spectroscopy, and provides a protonation state analogous to that found in the natural environment. Although the gas phase is an alien

environment for a biomolecule, isolated nucleotide anions are important model systems providing intrinsic electronic structure information, which is an essential starting point for comparisons with both theoretical calculations and experimental solvation studies, whether in bulk liquid or gas phase solvent clusters. In solution, it has been shown that in all cases the first ionization site is located on the base,[26,31] as the solvent stabilizes the phosphate. Hence, the detachment energy of the phosphate is of little significance. In contrast, the determination of the intrinsic ionization energies of the *nucleobases* within nucleotides is significant and allows one to develop an understanding of the factors affecting the stabilization of the base in solution and ultimately in DNA. In the gas-phase, single-photon PE spectroscopy of nucleotides can lead to ionization from other parts of the molecule including the phosphate and sugar. As a result, ionization from the base could not be discerned from these additional ionization channels (see Fig. 1(a)). This was the case for 2'-deoxythymidine 5'-monophospate ($dTMP^-$), 2'-deoxyadenosine 5'-monophospate ($dAMP^-$), and 2'-deoxycytidine 5'-monophospate ($dCMP^-$). For 2'-deoxyguanosine 5'-monophospate ($dGMP^-$), electron loss from the base could be identified by a feature at low electron binding energy (*eBE*) which was also observed in all G containing oligonucleotides.[33] Here, we overcome the limitations of single photon PE spectroscopy and experimentally determine the ionization energy of the base in the remaining deprotonated nucleotides.

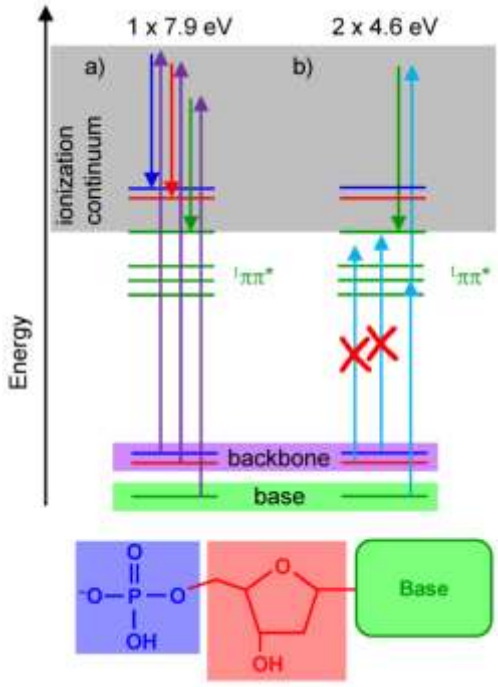

Figure 1. Schematic energy level and ionization scheme for a) one-photon vacuum ultraviolet PE spectroscopy, and b) resonance enhanced two-photon detachment (R2PD). The one-photon scheme leads to ionization from the base moiety and sugar-phosphate backbone. The R2PD scheme, on the other hand, leads to ionization specifically from the base moiety, and is insensitive to the backbone of the nucleotide.

Several techniques have been employed in an attempt to determine the site of the lowest binding energy in isolated oligonucleotides. The Kappes group has performed careful chemical substitution and PE spectroscopy of mass- and isomer-selected oligonucleotide anions to determine the binding sites in these polymeric systems.[36,37] For the mononucleotides, however, theory has been the primary tool for settling this ambiguity. The Wang group carried out density functional theory (DFT) studies with their single-photon PE spectra, which gave the lowest binding site as the phosphate group for all nucleotides except $dGMP^-$.[33] More sophisticated

theory has since been performed, which has called this assignment into question. Both the partial third order self-energy approximation of the electron propagator (P3),[21,38,39] and complete active space with a perturbation theory correction (CASPT2)[40,41] methods suggest that the first ionization is from the base in $dTMP^-$ and $dGMP^-$, with P3 also indicating that the phosphate is responsible for the first ionization from $dCMP^-$ and $dAMP^-$, although CASPT2 has not been performed on these systems. Here, we present a simple experimental methodology that provides direct and unique insight into the photodetachment energy of the base in nucleotides. Specifically, a resonance-enhanced two-photon detachment (R2PD) scheme is used that confirms the assignment of the low *eBE* feature in $dGMP^-$ and reveals, for the first time, that in $dCMP^-$, $dTMP^-$ and $dAMP^-$, the phosphate has the lowest ionization energy.

Single-photon PE spectroscopy is unable to determine *a priori* from where in the nucleotide the electron is detached, as the vacuum ultraviolet photon is sufficient to detach from the base, sugar and phosphate and all of these will contribute to the final PE spectrum (see Figure 1(a)). For $dXMP^-$ (where X = A, G, T or C), this leads to broad and structureless bands. To circumvent this limitation, we exploit the fact that all four bases have a strong $^1\pi\pi^*$ transition in the UV around 4.6 eV (270 nm) and employ a two-photon excitation scheme. As shown in Figure 1(b), a single 4.6 eV photon is insufficient to detach an electron from the base, sugar or phosphate moieties, but will excite it to the optically bright $^1\pi\pi^*$ states localized on the base. A second photon can subsequently remove the electron from the excited state, ionizing *only* the base. The time between the absorption of both photons must be minimized because the lifetimes of the $^1\pi\pi^*$ states in isolated bases have been shown to be very short.[42-45] Hence, the use of femtosecond (fs) pulses is critical to the experimental scheme as they minimize detachment occurring from photo-products that can be formed following internal conversion.[46-49]

Experimental

The experimental set up has been described elsewhere[50,51] and only details pertaining to this work are provided here. $dXMP^-$ is generated via electrospray ionization from a ~ 0.5 mM solution of dXMP or dXMP sodium salt (Sigma-Aldrich) in methanol. Deprotonated $dXMP^-$ ions are stored in a trap for ~200 ms and pulsed collinearly into a time-of-flight mass spectrometer. The ion packet is intersected by a laser pulse at its temporal focus and emitted photoelectrons are analyzed according to their electron kinetic energy (*eKE*) using a velocity map imaging arrangement.[50,52] Light at 4.64 eV (267 nm), resonant with the lowest $^1\pi\pi^*$ transition in all four bases, is generated by 3rd harmonic generation of the fundamental of a commercial chirped-pulse amplified femtosecond laser system, and loosely focused into the interaction point. Pulses are on the order of 120 fs duration and the typical intensity in the interaction region is ~ 5×10^{10} W cm^{-2}, which is below the onset of non-resonant processes. PE spectra are reconstructed from the images using polar onion peeling.[53] PE images are collected both in the presence and absence of ions, and are subtracted every 10 camera frames, removing background PE noise. PE spectra have been calibrated to the known spectrum of I^- and the overall resolution at *eBE* ~6 eV is 0.15 eV.

Results and Discussion

The solid lines in Figure 2(a)-(d) show the R2PD spectra taken at 4.64 eV for $dXMP^-$ with X = A, T, C and G, respectively, with the total photon energy corresponding to 9.28 eV (*i.e.* 2 × 4.64 eV). The R2PD spectra have broad asymmetric features with an onset of *eBE* ~ 5.7 eV observed for detachment from the base in $dAMP^-$, $dTMP^-$ and $dCMP^-$. In contrast, the onset in $dGMP^-$ is significantly lower at *eBE* = 4.65 eV, indicating that base-specific ionization in $dGMP^-$ occurs at significantly lower energy than in the other three nucleotides.

Also shown in Figure 2, as dashed lines, are the single-photon PE spectra taken by the Wang group, with 7.866 eV photons.[33] For the four nucleotides, the single photon PE spectra show an intense, broad, and unresolved peak, with an onset of ~5.4 eV (see Table 1). The PE spectrum of $dGMP^-$ additionally shows a smaller feature separated from these intense features at lower binding energy with an onset at *eBE* = 4.61 eV. This PE feature was assigned to direct detachment from G in $dGMP^-$, with the large feature onset at 5.8 eV assigned to detachment from the backbone. Similarly, the onset of the broad features in $dAMP^-$, $dTMP^-$, and $dCMP^-$ were assigned to detachment from the backbone, with ionization of the base presumably convoluted in this peak. However, individual contributions due to the backbone and base cannot be unraveled in these spectra. This is exacerbated by the fact that the ionization cross-sections of the base and backbone may differ dramatically. For example, in $dGMP^-$ the feature assigned to detachment from the base is about 7 times smaller than the features believed to arise from ionization of the backbone. Hence, no experimental insight into the relative detachment channels could be gained.

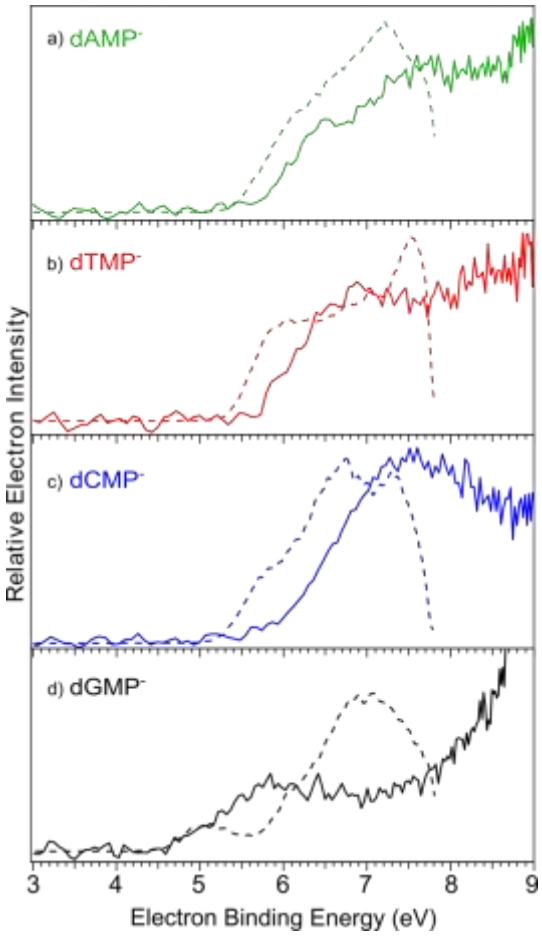

Figure 2. Comparison of one- and two-photon detachment spectra for a) $dAMP^-$ (green), b) $dTMP^-$ (red), c) $dCMP^-$ (blue) and d) $dGMP^-$ (black). One-photon spectra by Wang and co-workers are represented by dashed lines, whilst the solid lines present the two-photon detachment (R2PD) from the present work. The higher *eBE* onset in a) to c) indicate that phosphate detachment is the lowest ionization for these bases, whilst for $dGMP^-$ it is the base which detaches first. Adapted with permission from ref. 33.

Comparing the $dGMP^-$ spectra following one- and two-photon excitation, several important similarities and differences are apparent. Firstly, the onset at *eBE* = 4.65 eV in the R2PD scheme is in excellent agreement with the *eBE* = 4.61 eV measured with one photon. As the R2PD

scheme ionizes only from the base moiety (the sugar and phosphate are optically dark), the original assignment of the lower binding energy peak to ionization of the G base has been confirmed experimentally. Moreover, the agreement in *eBE* onsets demonstrates that our methodology is a viable way of determining the onset of ionization of the base in a nucleotide. We recognize however, that a number of final electronic states are potentially accessible following photodetachment. Specifically, we assume that the ground state of the neutral deprotonated nucleotide corresponds to one in which a hole resides on the base and an electron on the phosphate. As the phosphate anion has a considerably lower electron detachment energy than the ionization energy of a neutral base, this assumption may not necessarily hold. Regardless, we are still measuring the adiabatic detachment energy of the base itself.

Secondly, the R2PD spectrum for $dGMP^-$ shows a very intense signal at high *eBE* (> 8 eV), which is not present for the other nucleotides. This feature has been truncated above 8.8 eV, where it continues to rise sharply, dwarfing the R2PD base-selective ionization (see Supporting information). This feature arises from single-photon ionization of the base; although the adiabatic ionization energy of $dGMP^-$ is 4.65 eV, both the initial internal temperature of the ions ($\geq$ 300 K)[54] and the bandwidth of the femtosecond laser pulses will lead to a small fraction of molecules to be ionized with a single 4.64 eV photon. The relative intensity indicates that this process has a much larger cross section, as may be expected for a single-photon process compared to a R2PD process. Note that, with respect to the *eBE* axis shown in Figure 2(d), the location of this feature is misleading, as this axis assumes a total absorbed energy of 9.28 eV (two photons of 267 nm).

Finally, the spectral profile of the PE spectra from $dGMP^-$ differs substantially between the one-photon and R2PD spectra. Specifically, the features due to detachment from the base have a

maximum around 5.1 eV and 5.9 eV in the one-photon and R2PD spectra, respectively. There are two probable causes for this discrepancy. Firstly, the R2PD spectrum is dependent on both the cross section for excitation to the $^1\pi\pi^*$ state, and the subsequent ionization from this state. In contrast, direct one-photon ionization is solely dependent on the ionization cross section of the base. Thus, the Franck-Condon factors for the two processes will be different, leading to different maxima observed in the PE spectra. Secondly, for the R2PD spectrum, the two photons must be absorbed within ~120 fs of each other (the laser pulse duration). Albeit short, this timescale is comparable to the fastest dynamics observed in neutral DNA bases by Stolow and co-workers using time-resolved PE spectroscopy.[43] For A, C and T, the decay was observed to be bi-exponential with a sub-50 fs component and a slower picosecond component. The initially excited $^1\pi\pi^*$ state rapidly decays to lower lying states, believed to be $^1n\pi^*$ states. In the PE spectra, this manifests as a shift to higher *eBE* by ~1 eV in adenine, with similar shifts observed in the other bases. Unfortunately, no time-resolved PE data has been acquired for the isolated guanine base, although the lifetimes of integrated ion yield experiments show similar timescales and bi-exponential dynamics as those observed for the other bases.[45]

To gain additional insight into the early-time dynamics of the $^1\pi\pi^*$ state in $dGMP^-$, we have performed preliminary femtosecond time resolved PE experiments[55-58] with a 4.64 eV pump and a 1.55 eV probe (see Supporting information). Our results show a ~50 fs decay component and a rapid red-shift in *eKE* of the signal maximum, which correlates to an increase in *eBE*. Hence, detachment from both the initial $^1\pi\pi^*$ state and lower-lying states are likely to contribute to the R2PD spectrum. Our observed shift of 0.8 eV in the R2PD PE spectral maximum in $dGMP^-$ relative to the single-photon spectrum is in fair agreement with the shift observed in the time resolved PE spectra of adenine.[59] Unfortunately we cannot extract this information quantitatively

from our preliminary time resolved data because the probe pulse has insufficient energy to detach the electron from these low-lying states. It is important to note that the presence of excited state dynamics, occurring on a timescale shorter than the experiment affords, invalidates an assignment of the vertical binding energy of the base in the nucleotide. In principle, using a much shorter excitation pulse would circumvent this issue and allow the vertical binding energy to be extracted. The ~50 fs dynamics, however, would require pulses that are significantly shorter than this, which are non-trivial to generate with our current experimental setup.

Table 1 Comparison of adiabatic detachment energies (*ADE*) for nucleotide anions and corresponding neutral nucleobases, measured using one-photon PE spectroscopy and base specific R2PD on the nucleotides. In $dGMP^-$, the lowest energy for single-photon ionization is from the base, whilst in the other nucleotides it is from the backbone. In all instances, the *ADE* of the base moiety in the nucleotide anion is ~3 eV lower than in the neutral base.

Mononucleotide	**one-photon *ADE* (eV)[a]**	**R2PD *ADE* (eV)**	**nucleobase *ADE* (eV)[b]**
$dAMP^-$	5.55 ± 0.10	5.65 ± 0.15	8.26
$dTMP^-$	5.40 ± 0.10	5.60 ± 0.15	8.87
$dCMP^-$	5.30 ± 0.10	5.80 ± 0.15	8.68
$dGMP^-$	4.61 ± 0.10	4.65 ± 0.15	7.77

[a]Ref. 33, [b]Ref. 11

We now discuss the remaining nucleotides, which display a higher *eBE* onset in the R2PD spectra than in the single-photon PE spectra. Table 1 presents the onset binding energies

produced by extrapolating the steepest part of the onset, for both the single-photon and R2PD methods (see Supporting information). The base specific R2PD onsets show a clear blue shift for $dAMP^-$, $dTMP^-$ and $dCMP^-$. The higher *eBE* onsets in the R2PD spectra confirm the assignment of Wang and co-workers that the lowest oxidation site on the nucleotides corresponds to electron loss from the backbone (deprotonated phosphate).[33] Presumably, in the one-photon PE spectra, the large phosphate peak obscures features arising from ionization of the base, as it is almost an order of magnitude larger (in $dGMP^-$). We note that ultrafast decay pathways are also present in adenine, thymine and cytosine bases[42,43,60] and the spectral shape of the R2PD spectra do not reflect the vertical Franck-Condon profile between the ground state bases and the ionized base.

The observed experimental results agree with DFT calculations performed by the Wang group, which assigned a phosphate moiety as the site of first electron detachment in all nucleotides except $dGMP^-$. The more recent P3 calculations,[21,38,39] as well as calculations using CASPT2,[40,41] disagree with this and assign the first ionization to the base π system in $dTMP^-$ as well as $dGMP^-$. It is worth noting that these computational efforts have been to determine vertical, not adiabatic, detachment energies. It is feasible that, as the base and phosphate detachments are close together in energy (~0.1 eV in the case of $dAMP^-$), the ordering will switch between vertical and adiabatic detachment energies, especially as we may expect the geometric response to electron loss to be substantially different between the base and phosphate moieties. Thus, theory and experiment do not necessarily disagree and it would be particularly interesting to determine the adiabatic ionization energies of nucleotides using higher levels of theory. The discrepancy could also arise from the conformational distribution that will be present in the experiments, which are performed at or above room temperature.[34] The introduction of an ion-mobility cell prior to R2PD spectroscopy will be able to address these shortcomings.[61]

Calculated gas phase structures have shown that the most important factor in determining ionization energy ordering of the nucleotides is the degree of intra-molecular hydrogen bonding between the phosphate and base, which has been shown by theory and gas phase IR spectroscopy to differ greatly between $dGMP^-$ and the other bases.[34,35,39] The structure of $dGMP^-$ is considerably more compact, with a hydrogen bond between the base and phosphate, whilst the remaining three nucleotides form an open structure. This results in the G base residing significantly closer to the charge carrying phosphate and leads to a destabilization of the π system on the base through Coulomb interactions, which lowers the adiabatic detachment energy (*ADE*). The relation between phosphate-base distance and detachment energy is, to an extent, reflected in the differences between the nucleotide and neutral nucleobase *ADE*s (see Table 1), which is > 3 eV for G and T and < 3 eV for A and C. This may suggest that $dTMP^-$ also has a shorter base-phosphate distance, or samples such structures at the experimental internal temperature. It is important to note that structural considerations alone do not explain the very low base IP in $dGMP^-$; the extra destabilization induced by the nearby phosphate only accounts for a portion of the low *ADE*, and the fact that neutral G has a low *ADE* to be begin with must also be considered. Calculations have the advantage that different ionization channels can be readily identified with specific molecular orbitals, whereas in experiment, this is non-trivial. The combination of one-photon and R2PD experiments overcomes some of these limitations, granting a larger wealth of benchmark data.

We now briefly turn to the comparison between the ionization of DNA components in the gas and solution phases. Recent liquid microjet experiments by Slavíček *et al.* have measured the PE spectra of aqueous nucleosides of C and T.[26] Additionally, numerous estimates of the ionization energies in solution of DNA components have been made using either pure *ab initio*

calculations,[26-30] or by adjusting gas phase ionization energies with calculated thermodynamic parameters.[31] In aqueous solution the adiabatic ionization energy of the base in the two nucleosides studied decreases by ~2 eV relative to that of the gas phase base.[26] This shift arises almost entirely from solvent reorganzition following base ionization, both locally[29,62-65] and due to a more extensive rearrangement.[26,30] Our measurements indicate that the charged phosphate decreases the ionization energy of the base by ~3 eV (see Table 1) through strong Coulomb interactions with the base. In solution, however, calculations by Slavíček *et al.* show that this Coulombic interaction is completely screened[26,30] such that the adiabatic ionization energy of that in solution is effectively that of the base only.

It is important to recognize that neither full solvation nor full isolation accurately represents the biological environment of a nucleotide. In double helix DNA, the phosphate groups are surrounded by water and counter ions, but water is excluded from the bases which are paired through hydrogen bonding and experience extensive stacking interactions with their neighbors. Hence, the effective charge screening and the ionization energy of the base observed in solution may be very different in DNA. The *ADE* of the bases in isolated nucleotides determined here, combined with experiments on solvated nucleosides, presents key reference points towards building an understanding of the various factors that influence the lowest ionization energies DNA. In particular, gas phase data presents an important benchmark for theoretical calculations upon which solvation models can subsequently be built. This may allow for the systematic prediction of the ionization energy in any environment. Further experiments will also play an important role in this. For example: incremental solvation of the phosphate will provide insight into the screening of the charge by water; the extension to oligonucleotides can begin to probe

effects of π-stacking on the ionization energies;[36,37] and extension to base-pairs allows exploration of the effect of the unique hydrogen bond interactions within DNA.[62-64]

The R2PD scheme presented here is in principle scalable to larger and more complex systems. The intramolecular hydrogen bonded geometry of $dGMP^-$, which is partly responsible for the lowering of the base-specific *ADE*, is a unique feature of isolated nucleotides and, while it persists for small oligonucleotides as demonstrated by the low ionization energy of G in all G-containing di- and tri-nucleotides,[33] it is clearly not a feature of DNA. Hence, although gas phase spectroscopy can provide useful insights into intramolecular effects, it is important to bear in mind that some of these interactions are not relevant in real biological environment. Nevertheless, the scalability of our approach in conjunction with chemical substitution and microsolvation techniques, may offer a wealth of information about charge location and state ordering of DNA.

Conclusions

In conclusion, we have presented a simple, but powerful method for exploring base-specific ionization within anionic nucleotides. By comparison of spectra taken with nanosecond and femtosecond lasers, the first detachment energy can be assigned unequivocally as either to the base or the backbone for a given nucleotide; the lowest ionization is shown to be from the base in $dGMP^-$, but from the phosphate in the other deoxynucleic acids. Using our method, we have measured the adiabatic ionization energy of the bases in all four nucleotides. The effect of the charged backbone is to reduce the base *ADE* by around 3 eV compared to an isolated nucleobase. In principle, this technique is also applicable to oligonucleotides, allowing the ionization properties of these polymeric systems to be explored.

Supporting Information.

Full R2PD spectrum of $dGMP^-$; analysis of *ADE* values from R2PD spectra; preliminary $dGMP^-$ time resolved PE spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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