DNA Damage by OH Radicals Produced Using Intense, Ultrashort, Long Wavelength Laser Pulses

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We probe femtosecond laser induced damage to aqueous DNA, relying on strong-field interaction with water wherein electrons and free radicals are generated *in situ*; these, in turn, interact with DNA plasmids under physiological conditions, producing nicks. Exposure to intense femtosecond pulses of 1350 and 2200 nm light induces single strand breaks and double strand breaks (DSBs) in DNA. At the longer wavelength (and at higher intensities), rotationally hot OH radicals induce DSBs, producing linear DNA. Strand breaks occur due to single or multiple OH hits on DNA. With 2200 nm light, DSBs are formed mostly by the action of two OH radicals; use of OH scavengers establishes that the probability of a two-hit event reduces much faster than a one-hit event as scavenger concentration is increased. Thermal effects do not induce DSBs with 2200 nm light.

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DNA is a highly stable, naturally occurring, long-chain polymer whose lifetime, under physiological conditions, is ~130 000 years before it spontaneously hydrolyzes [1]. DNA's double-stranded, double-helical structure accounts for its mechanical robustness. Concomitantly with this stiffness, DNA must also withstand large conformational changes—bending, compression, and twisting [2]—to facilitate effective packing into chromosomes. Damage to this robust polymer upon exposure to radiation occurs when single or double DNA strands break; such breakage constitutes the most lethal damage that occurs at the cellular level. Strands break when the sugar-phosphate backbone is ionized upon exposure of living matter to high-energy radiation. About a decade ago, experiments on DNA (in dry form) established a new paradigm: even electrons possessing a few electron volts of energy induced strand breakages via formation of dissociative temporary negative ion states [3]. More recently, experiments on DNA in its native, aqueous state showed that damage is caused by both slow electrons and OH. generated, *in situ*, in strong-field interactions with H_2O (in which DNA plasmids were suspended) using 45 fs pulses of intense (1–12 TW cm^{-2}) 820 nm light [4]: supercoiled DNA transformed into relaxed DNA in these interactions, as quantified by gel electrophoresis. We report here the use of intense light pulses of longer wavelength, 1350 and 2200 nm, at intensities in the TW cm^{-2} range to delineate the role played by OH radicals in inducing strand breaks in DNA close to physiological conditions. At these wavelengths we show that electrons do not directly induce DNA damage. Unexpectedly, and potentially importantly, we find not only that a higher percentage of native supercoiled DNA structure becomes relaxed, compared to earlier work at 820 nm [4], but that there is also a propensity to form linear DNA [Fig. 1(a)]. Linearization of DNA is an unambiguous signature of double strand breaks (DSBs), which are important as they are not readily amenable to repair and which were hitherto thought to be caused only by high-energy radiation. We show that strand breaks result from interactions of OH. with DNA, either in a single hit or via multiple hits. Systematic measurements at longer wavelengths and different intensities reveal that wavelength effects dominate the damage dynamics, with rotationally excited OH$^{\cdot}$ being pivotal in inducing DSBs.

An optical parametric amplifier pumped by 800 nm, 40 fs, 4 mJ pulses from a Ti:sapphire amplifier provided ultrashort pulses of 1350 and 2200 nm light at 1 kHz repetition rate. The optical parametric amplifier's output was filtered using RG850 color glass; a set of dielectric mirrors separated signal and idler pulses of duration ~56 and ~64 fs, respectively, measured using a homemade autocorrelator. Lenses of different focal lengths were used to ensure that our DNA sample was irradiated with almost identical peak intensities (25–75 TW cm^{-2}) at different wavelengths [5]. The M^2 value was 2.8. Our DNA plasmid (pBR322), obtained commercially (Merck-Millipore), was suspended in 2 l of deionized water in dialysis bags with 2 kDa size cutoff. After two changes every 3 h, they were dispensed into convenient volumes and stored at −20 °C with concentration spectrophotometrically determined at 260–280 nm. We standardized the DNA quantity that yielded maximum nicking, establishing a working range of $2–6 \times 10^{11}$ molecules in 300 μl; the lower end of this range yielded the best percentage of relaxed species post laser exposure. The plasmid concentration was $1.9–3.8 \times 10^{11}$ cm^{-3}, corresponding to 0.9–1.8 μg

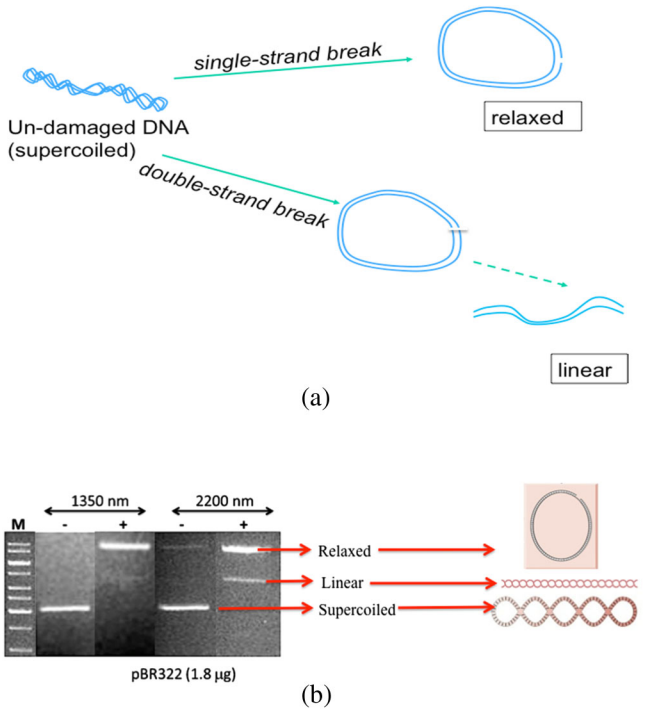

FIG. 1 (color online). (a) Schematic depiction of single strand breaks and double strand breaks induced upon laser irradiation. Linear DNA results from DSBs and is usually not amenable to repair. (b) Gel images obtained on irradiating DNA plasmid pBR322 with 1350 and 2200 nm light. The negative and positive signs above the panels indicate, respectively, no laser exposure and laser exposure for 3 min. The panel marked M shows an image of the DNA ladder containing fragments of known length. Laser intensities were ~25 TW cm^{-2}.

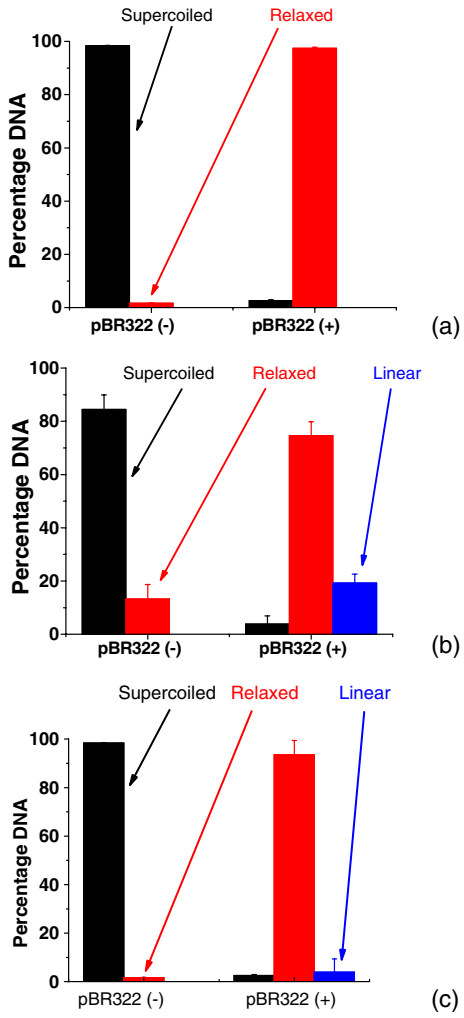

FIG. 2 (color online). Percentage of supercoiled and relaxed DNA in normal conditions (−) and after irradiation (+) at (a) 1350 and (b) 2200 nm at ~25 TW cm^{-2} intensity. Note the formation of a linear structure when irradiation is by 2200 nm light. This is indicative of double strand breaks occurring. (c) Results obtained using 1350 nm light of ~72 TW cm^{-2} intensity.

per 300 μl, out of which $\sim 3 \times 10^8$ lay within the laser focal volume (constituting 0.03% of plasmids). Related work [6] has established that strong thermal gradients are induced as our laser beam propagates through water, causing convective flow such that DNA molecules within the interaction volume are constantly replenished. After laser irradiation, DNA fragments were separated using electrophoresis; post separation, the gel was stained with ethidium bromide, a DNA binding fluorescent dye, to facilitate imaging using a gel documentation system in conjunction with standard gel-analysis software (ImageJ) and DNA ladders containing linear fragments of known length.

Figure 1(b) shows typical gel images obtained at 1350 and 2200 nm [7]. With 1350 nm light, some of the initially supercoiled DNA converts to a relaxed form. For 2200 nm light of the same intensity, almost the entire supercoiled structure disappears and both relaxed and linear forms are observed. We systematically varied irradiation time, plasmid concentration, and incident laser energy, to quantify our gel observations. As seen in Fig. 2, unexposed pBR322 samples were almost entirely (98%) supercoiled; on exposure to 1350 nm light (25 TW cm^{-2}), the conformational change was dramatic: 97% became relaxed. Figure 2 also shows results with 2200 nm light on a different sample where the initial plasmid conformations were 84% supercoiled and 16% relaxed [7]. Upon irradiation, less than 5% maintained their supercoiled structure: 76% of the plasmids became relaxed while, most unexpectedly, ~20% became linear. Our observations of the supercoiled → relaxed transformation are qualitatively consistent with results obtained in 820 nm experiments at about the same intensity [4] but, quantitatively, the relaxation is much more pronounced at longer wavelengths. DSBs are seen to occur more readily with 2200 nm light. Are these conformational changes indicative of strand breakages induced by electrons and OH radicals? If so, how are electrons and OH radicals formed upon strong-field interactions with H_2O? Can the effects of wavelength and intensity be delineated? These physics issues are addressed in the following.

There are many models of solvation and thermalization of electrons produced upon multiphoton ionization of aqueous H_2O, but there has been limited success in rationalizing observations. The consensus is that photoelectrons undergo different thermalization and recombination dynamics (see Ref. [8] and references therein). Multiphoton excitation of H_2O with low-energy (< 9 eV) photons yields electrons that are ~1 nm away from their parent holes while at energies > 11 eV, the photoelectrons can be 3 nm away [8], with photoionization involving a series of proton and electron transfers to preexisting traps. With even higher energy photoexcitation, electrons are ejected directly into the conduction band. Under our strong-field conditions, tunnel ionization dominates and the latter scenario most likely governs electron dynamics. Water's high polarity ensures that a large percentage of these electrons are solvated wherein proximate H_2O molecules form a cage that "traps" electrons by acting as a screen of the free charge; solvated states live for ~500 ps [8]. Shadowgraphy has been used to characterize the spatial and temporal variation of refractive index and transient absorption induced by a filament generated as an intense 120 fs laser pulse propagates in H_2O [9]: electron densities of $\sim 10^{18}$ cm^{-3} are obtained. We note that $H_2O^{-\cdot}$ states are also formed with ~7 eV electrons [10] but their lifetime (a few hundred attoseconds) precludes any role in inducing DNA damage.

Strong optical fields interact with aqueous water ($[H_2O]_n$) to yield excited (H_2O^*) and ionized ($H_2O^{+\cdot}$) molecules; $OH^\cdot$ radicals (with lifetimes in the microsecond range [11]) are subsequent products of collisions: $H_2O^* + H_2O^{+\cdot} \rightarrow OH^\cdot + H_3O^+$ [12]. Figure 3 shows how the percentage of relaxed species varies as $OH^\cdot$ and electron scavengers are added to the DNA-water suspension. Pronounced quenching of relaxed DNA occurs with the OH scavenger, sodium acetate, but little or no effect is discernible as the electron scavenger, 5-bromouracil (5BrU), is added. The supercoiled → relaxed transition with 1350 nm light is, consequently, driven almost exclusively by OH radicals. Similarly with 2200 nm light, there is effective quenching of relaxed and linear species upon addition of sodium acetate, but addition of 5BrU produces little or no effect: both single strand breaks (SSBs) and DSBs are induced essentially by OH radicals.

Might multiphoton absorption (MPA) be the cause of DNA damage? DNA has maximum linear absorption around 260 nm; excitation causes lesions including DSBs [13]. Use of 1350 nm and 2200 nm light would entail 6- and 9-photon excitation, respectively. At fixed photon flux, more multiphoton induced damage would be expected with 1350 nm light than at 2200 nm; the higher order process would ensure that MPA efficiency at 2200 nm is much less than at 1350 nm. Our results [Figs. 2 and 3] show that, at 25 TW cm^{-2} intensity, DSBs occur only with 2200 nm light. We, therefore, postulate that MPA is unlikely to be the precursor of the DNA damage we

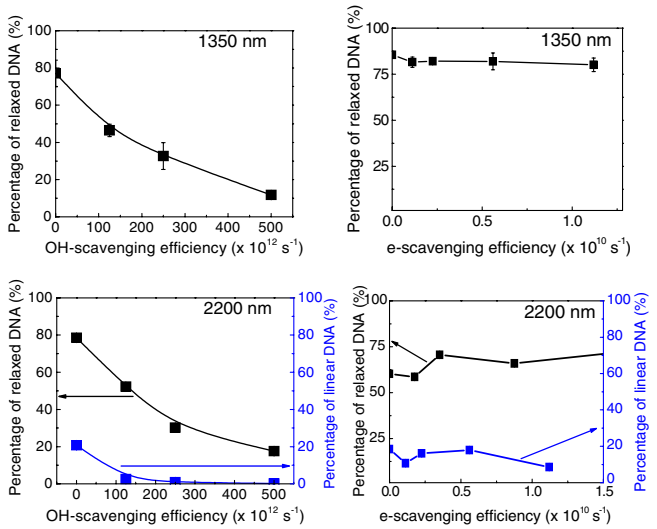

FIG. 3 (color online). Percentage of relaxed DNA as a function of *e*- and OH-scavenging efficiencies upon addition of sodium acetate (left-hand panels) and 5-bromouracil (right-hand panels). The former is an OH scavenger while the latter is primarily an electron scavenger. The upper panels show results obtained at 1350 nm; the lower panels show results at 2200 nm. The incident laser intensities were ~25 TW cm^{-2} for all panels. The blue data points obtained with 2200 nm light pertain to linear DNA resulting from double strand breaks. The upper and lower right-hand panels show that there is no reduction in either relaxed or linear DNA upon addition of the electron scavenger: damage to DNA is, consequently, primarily OH induced. Moreover, the upper and lower left-hand panels show that, at large OH-scavenging efficiencies, relaxed DNA quenches to ~10% levels for 1350 nm while it quenches to ~20% for 2200 nm light; this indicates larger thermal effects playing a role at 2200 nm. For linear DNA, however, data in the lower right-hand panel indicate that thermal effects play no role whatsoever.

observe. Our scavenger data also allow assessment of whether thermal effects cause strand breakages. At 1350 nm and high scavenging efficiency values (500×10^{12} s^{-1}), SSBs quench to ~10% levels while at 2200 nm they quench to ~20%. This underlines the increased importance of thermally induced damage at 2200 nm compared to that of 1350 nm, in line with higher absorption by water at 2200 nm. These λ-dependent values also confirm that thermal gradients are not a major contributor to strand breaks. However, most noteworthy is the observation that there is complete quenching of DSBs (to 0% levels) at 500×10^{12} s^{-1} scavenging efficiency. In other words, thermal effects are certainly not responsible for DSBs we observe.

Strand breaks are, therefore, indirectly caused by strong field-water interactions that form $OH^\cdot$, which, as is known from x-ray and γ-ray studies, account for the majority of radiation damage to cellular systems [14]. Strands break when H atoms are abstracted from one of the five C atoms of the deoxyribose pentose ring [15]. Although DNA cleavage apparently has no base or sequence specificity,

early experiments in which specific H atoms were substituted by D atoms [16] established that OH. preferentially attacked the sugar H atoms in the order $5'H > 4'H > 3'H \sim 2'H \sim 1'H$. Subsequent computer modeling [17] confirmed that "hits" by OH· on sugar H atoms yield SSBs. However, insights are still awaited on details of how OH· induces DSBs. Such breaks may be induced either directly or indirectly [18]. In the former instance, a DSB would occur via radical transfer between complementary DNA strands [19], a "single hit" process. Indirect effects, on the other hand, require inhomogeneous deposition of energy in water such that clusters of OH· radicals form. Each DSB would then result from two proximate SSBs induced by two separate OH· radicals from such a cluster, a "two-hit" process. Our measurements (Fig. 3) show that DSBs are scavenged much more readily than SSBs, providing unambiguous evidence of a two-hit process (the probability for a two-hit event is seen to reduce must faster than a single-hit event).

As noted, hydrogen abstraction leads mainly to SSBs, which are amenable to repair. DSBs, on the other hand, are not readily repairable; they constitute an important subset of DNA damage that, our results indicate, are OH induced in strong fields generated with 2200 nm but not 1350 nm light of equal intensity. We have extended earlier measurements [4] at 820 nm to intensities up to 100 TW cm^{-2}; we found no evidence for linear DNA. By systematically varying intensities at 820, 1350, and 2200 nm we conclude that wavelength, not intensity, is primarily responsible for inducing DSBs [20]. Higher electron energies are obtained at 2200 nm than at 1350 nm (and 820 nm). At 25 TW cm^{-2}, U_p values are 1.5 eV (820 nm), 4 eV (1350 nm), and 11 eV (2200 nm). Collisions with these electrons can electronically excite H_2O: at electron energies ≥ 9.13 eV, direct dissociation of $H_2O^\star$ is adiabatically correlated to OH· fragments in the excited $A^2\Sigma^+$ state [21]. H_2O's electronic configuration $1a_1^2 2a_1^2 1b_2^2 3a_1^2 b_1^2$ yields the X^1A_1 ground state. A $1b_1 \rightarrow 4a_1$ excitation requires ~7.3 eV and yields the A^1B_1 excited state, which dissociates to yield OH· in its ground $^2\Pi$ state. A $3a_1 \rightarrow 3sa_1$ excitation, requiring only 1.6 eV more, leads to the B^1A_1 $H_2O^\star$ state that adiabatically dissociates [22] to excited OH* ($A^1\Sigma^+$), but dominantly, it nonadiabatically dissociates into OH· ($^2\Pi$) that is rotationally very hot [23], with as much as ~4 eV of rotational energy. The high rotational angular momentum in such OH· results from the large torque that acts near a conical intersection between potential energy surfaces of the B and X states in the collinear H-O-H geometry [23]. Rotationally hot OH· will have a high propensity for abstracting sugar H atoms via simultaneous collisional interactions at two different sites, giving rise to DSBs. We have tested this conjecture in experiments at 1350 nm but at a high enough intensity (75 TW cm^{-2}) to ensure that $U_p = 11$ eV. Under these conditions our gel data do, indeed, reveal the presence (at ~4% level) of linear DNA. Electronic excitation of H_2O so that dissociation produces rotationally excited OH. seems to be critical to DSB formation. At low intensities of 2200 nm light (5 TW cm^{-2}), no linear DNA was observed while at 25 TW cm^{-2} intensity up to 20% of the signal was due to linear DNA. In the former case the electron energy is insufficient to electronically excite H_2O, precluding formation of rotationally hot OH.

In summary, we have experimentally probed strand breakages in aqueous DNA, relying on strong-field interactions with H_2O wherein electrons and OH· generated *in situ* interact with DNA plasmids under physiological conditions, producing nicks. Experiments with electron and OH scavengers indicate that at long wavelengths, SSBs and DSBs are induced by OH radicals. At 2200 nm formation of linear DNA occurs, which is evidence of OH-induced DSBs; similar breaks are observed with 1350 nm light at much higher intensities (when rotationally hot OH. can be formed). Thermal effects also give rise to SSBs, more with 2200 nm light than at 1350 nm. However, they have no role to play in inducing DSBs. In our experiments a single optical cycle takes either 4.5 or 7.4 fs, too short a time for the DNA to react directly to the high field magnitudes at the peak of the cycle. Contrary to an earlier finding [24] that IR irradiation causes fragmentation and cell death, we find no gel evidence of plasmid fragmentation. The strong-field physics that underpins our results has implications beyond studies of DNA *per se*. Intense laser beams of wavelengths >1300 nm are currently characterized as eye safe in industry. Our findings that OH. produced upon strong irradiation of water at such wavelengths can induce DSBs in DNA raise concerns about how safe is "eye safe." Furthermore, *in situ* generation of slow electrons and radicals within aqueous media is likely to be important in diverse situations in which the effects of low energy radiation need to be probed under physiologically relevant conditions.

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respectively; for $\lambda = 1350$ nm and $f = 10$ and 15 cm, $d = 92$ and 138 μm, respectively.

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