# Probing the relative orientation of molecules bound to DNA through controlled interference using second-harmonic generation

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A method is described in which the interference of radiated second-harmonic electric fields generated by a pair of oriented molecules intercalated into double-stranded DNA is controlled and measured. The results show that the relative molecular orientation of the two molecules significantly changes the magnitude of the observed second-harmonic generation intensity, which is described by a simple model that accounts for the interferences of the radiated fields. The technique presented shows promise for future experiments investigating structural changes induced by the formation of a DNA–biomolecule complex.

alignment | daunorubicin | equilibrium binding | nonlinear spectroscopy | phase

Experiments are presented in which the relative spatial orientation of two molecules bound to a DNA duplex is systematically manipulated and subsequently probed using second-harmonic (SH) generation (SHG). Specifically, one daunomycin molecule was rotated relative to another by changing the number of base pairs separating them on a given DNA duplex that was affixed to a colloidal microparticle. Each DNA base pair on translation up or down the helix to the location of the adjacent base pair imparts a 36° rotation to the helical DNA structure (1); thus, the relative orientation of DNA intercalators with defined recognition sites can be controlled by simply changing the number of bases pairs separating them on a given DNA duplex, as sketched in Fig. 1. The interference of SH fields generated from the intercalated species is exploited to map the measured SH intensity to the intercalated molecules' relative orientation. The interference of radiated SH fields generated from bound daunomycin molecules on a DNA duplex was measured and found to be modulated by the relative spatial orientation of the two drugs. The interference of the SH radiation was described in a simple model discussed below. Taking advantage of the tailored interference, the SHG method presented has the potential to serve as a solution phase "molecular protractor" to measure DNA unwinding angles for a broad range of protein–DNA and drug–DNA complexes.

SHG is a coherent surface specific spectroscopy where intense light at frequency $\omega$ induces a second-order polarization that oscillates at twice the driving laser frequency, and thereby generates light at twice the fundamental frequency, $2\omega$ (2, 3). Because SHG is coherent, the radiated SH intensity from the $k$th particle in solution is given by the following:

$$I_{2\omega,k} \propto \left| \sum_i E_{2\omega}^i \right|^2, \qquad [1]$$

where $E_{2\omega}^i$ is the SH field from a given interfacial molecule, which add up, with its respective phase, with other interfacial molecules to produce the overall SH intensity from an individual particle. The total SHG intensity measured in experiments is the incoherent sum of the coherent SH light generated by the daunomycin pair bound to the DNA on the individual particles in solution. The isotropic bulk medium does not produce coherent

SH radiation because the SH field generated by one randomly oriented molecule is, in principle, canceled out by another molecule that has the opposite orientation, leading to an overall destructive interference of the bulk SH signal; although incoherent SH light is generated in the bulk medium by density and orientational fluctuations of the species in bulk solution (hyper-Rayleigh scattering). Experiments have previously found that bulk water is responsible for the incoherent SH background signals (4). In the work reported here, the observed SH intensity generated by a pair of molecules bound to DNA, whose orientations are well defined and controlled, is shown to contain information on the relative orientation of the two molecules, allowing for detailed solution phase investigations of relative molecular orientation.

## Experimental

The experimental apparatus has been described previously (4, 5). Briefly, a KM Labs Ti:sapphire oscillator running at 80 MHz, center wavelength of 840 nm, producing 300 mW of average power with a pulse width of ~60 fs was focused into a 2-mm cuvette. The generated SH radiation was collected with a lens, telescoped, and filtered to remove much of the fundamental laser frequency before focusing into an Acton 300i spectrograph coupled to a Princeton Instruments Spec-10 CCD camera. The exposure was set to 1 s; several hundred exposures per titration step were collected to obtain statistics. Each experiment was carried out in triplicate to ensure reproducibility and reduce uncertainties. All quoted uncertainties are given at the 95% confidence interval.

Biotinylated DNA (Integrated DNA Technologies) was coupled to 1-μm NeutrAvidin-coated silica amine beads according to previously published procedures (5–7). In all experiments, the total concentration of DNA was 420 nM, to ensure complete surface coverage; the particle density was $2.5 \times 10^8$ particles per mL, and the samples were suspended in 50 mM Tris buffer at pH 7.5. The SH intensity measured from the beads and excess DNA in solution (i.e., not attached to the particle) was the same as neat water, within the experimental uncertainty. Hyper-Rayleigh scattering from bulk water was found to be responsible for the background SH signal, which was subtracted from the measured SHG intensity in the data shown below. Excess DNA in solution was previously found to not influence the binding isotherms (4).

## Results and Discussion

Daunomycin is an anticancer drug that is commonly used to treat acute leukemia (8–10), and has a somewhat unique property among small-molecule DNA binding agents in that it preferentially binds to triplet sequences in double-stranded DNA (10–14). The work here makes use of the specificity of daunomycin to the sequence TCG to spatially orient the two intercalated drugs on a given DNA duplex. Daunomycin has the effect of lengthening the DNA helical axis by ~3.4 Å and imparting a small unwinding of the DNA helix (~8°) (15–17) relative to non-complexed B-DNA. The sequence of the DNA 33-mer duplex that was used in the experiments was referenced to the sequence

**Fig. 1.** The relative orientation of two daunomycin molecules (orange with black arrows) bound to DNA is sketched here (omitting the amino sugar for clarity).

used where only one recognition site was present: 5′-CTC AAG TGA ACT CAA GTG AAT CCA A**TC G**AA GTT-3′; the recognition site is highlighted and the complementary strand is implied for succinctness. To change the orientation angle between the two drugs on a DNA duplex, the distance between two recognition sites was varied by changing the number of base pairs separating the two recognition sites. For instance, the DNA used where the spacing was 20 bases apart was 5′-CTC AA**T CG**A ACT CAA GTG AAT CCA A**TC G**AA GTT-3′.

Representative binding isotherms collected in the experiment are shown in Fig. 2. It was observed that the SH intensity at the maximum density of bound daunomycin, which is the plateau of the isotherm, changed as the spacing between the two recognition sequences was varied. The orientation angle between the two daunomycin molecules is given by $\Phi = n * 36°$, where $n$ is the number of base pairs separating the two recognition sites. Therefore, when $n = 5, 15, 25$, etc., the two binding sites are antiparallel and the SHG will destructively interfere; similarly, when $n = 10, 20, 30$, etc., the intercalated drugs are aligned parallel and will constructively interfere, as observed from the SH binding data in Fig. 2.

To quantify the modulated SHG intensity, the signal intensity at the isotherm plateau was averaged and plotted vs. number of base pairs separating the two binding sites in Fig. 3. This plot more clearly illustrates the oscillatory behavior that was expected to occur by systematically rotating one daunomycin relative to another by changing the number of base pairs separating them, and collecting the resulting SH light. The relative orientation is depicted in Fig. 3 as arrows representing the individual drug molecules.

A simple model was implemented to quantify the interference of radiated SH fields from the daunomycin molecules on a DNA duplex, which is given below. A coordinate system is defined

according to Fig. 4 where two generated SH fields, $E_a$ and $E_b$ are defined relative to the $x$ axis by the angles $\phi_a$ and $\phi_b$ for $E_a$ and $E_b$, respectively (bold type indicates vector quantities). Similarly, the angle out of the $x$–$y$ plane is defined as $\theta_a$ and $\theta_b$. The SH intensity due to the intercalated daunomycin molecules, $a$ and $b$, is the coherent sum of their individual SH fields and is written as follows:

$$I_{2\omega} \propto |E_a + E_b|^2 = |E_a|^2 + |E_b|^2 + 2E_a \cdot E_b, \qquad [2]$$

where each SH field can be written explicitly in terms of vector components to give the following:

$$E_a = E_a\left(\sin\theta_a \ \cos\phi_a \ \hat{x} + \sin\theta_a \ \sin\phi_a \ \hat{y} + \cos\phi_a \ \hat{z}\right). \qquad [3]$$

The DNA's helical axis is defined along the $z$ axis in this coordinate system so the DNA bases lie parallel to the $x$–$y$ plane. Based on the crystal structure of the daunomycin–DNA complex, daunomycin is known to intercalate parallel to the DNA's bases (15–17) yielding the approximation, $\theta_a \sim \theta_b \sim 90°$. Applying this approximation to Eq. **3** and substituting into Eq. **2** yields the following:

$$I_{2\omega} \propto |E_a|^2 + |E_b|^2 + 2|E_a||E_b|(\cos\phi_a \ \cos\phi_b + \sin\phi_a \ \sin\phi_b). \qquad [4]$$

Simplifying and noting that the intensity is proportional to the orientational average of the intercalated daunomycin–DNA complex on a given particle gives the following:

$$I_{2\omega} \propto |E_a|^2 + |E_b|^2 + 2|E_a||E_b|\langle\cos(\phi_a - \phi_b)\rangle. \qquad [5]$$

Setting $\Phi = \phi_a - \phi_b$ and noting that $|E_a| = |E_b| \equiv |E_{2\omega}|$ yields the following:

$$I_{2\omega} \propto 2|E_{2\omega}|^2[1 + \langle\cos\Phi\rangle], \qquad [6]$$

where $|E_{2\omega}|^2$ represents the generated SH intensity of one daunomycin molecule bound to DNA. The SHG signal from a single silica particle is the coherent summation of all of the individual daunomycin–DNA complexes attached to the particle. The total SHG signal is the incoherent sum of the SHG from the individual particles. The orientational average in Eq. **6** contains the variation in daunomycin orientations (e.g., tilt angles, etc.) relative to the DNA helical axis. To fit the



**Fig. 2.** Representative binding isotherms collected with different numbers of base pairs separating two daunomycin molecules bound to DNA (listed on *Right*).
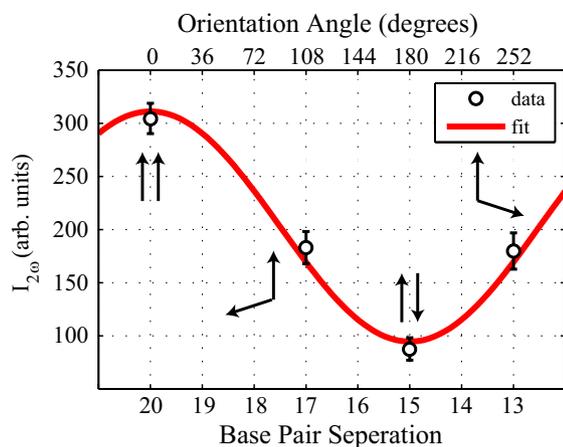
**Fig. 3.** The SH intensity is plotted vs. base pair separation on the lower axis, and orientation angle on the upper axis. The relative orientation of the bound daunomycin molecules is sketched as arrows that are associated with each data point.



**Fig. 4.** Depiction of the relative angles between SH fields generated by the *a* and *b* daunomycin molecules bound to DNA.

experimental data, the orientational average above is replaced with an empirical parameter, *g*, and summing over the particles yields the following:

$$I_{2\omega}(\text{total}) \propto \sum_k I_{2\omega,k} = 2[1 + g \cdot \cos \Phi] \sum_k \left| E_{2\omega,k} \right|^2. \quad [7]$$

The experimental data in Fig. 3 was fit to Eq. **7** to yield $g = 0.53 \pm 0.17$ and $\sum_k |E_{2\omega,k}|^2 = 101 \pm 15$ arbitrary units (arb. units). The value of $\sum_k |E_{2\omega,k}|^2$ extracted from the fit is in agreement with the SH intensity that was observed in experiments with one recognition site ($120 \pm 17$ arb. units), as expected. The agreement between the fit and the measured intensity from the binding of daunomycin to one recognition site serves as an internal consistency check to ensure the model and fit retrieve meaningful parameters.

The retrieved value of *g* indicates that the interference "efficiency" is ~53%. The deviation from ideal SH interference might be due to the ensemble distribution of orientations that the two drugs can take relative to one another, resulting in altered relative orientations, e.g., the daunomycin molecules might be twisted or tilted slightly from one another when intercalated into the DNA. Imperfect interference could also occur from structural and orientational fluctuations occurring on timescales much faster than the measurement. The slight unwinding of DNA by daunomycin could affect the interference of the SHG
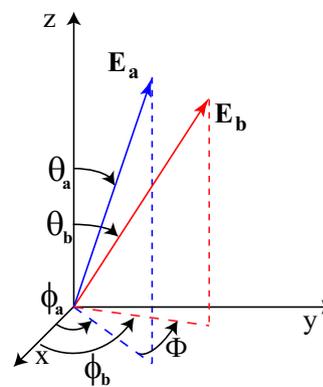
fields, which would be observed in Fig. 3 as a phase shift (i.e., the maxima and minima would not coincide with 0° or 180°, respectively). Because the data are well described by Eq. **7** (Fig. 3), it is surmised that daunomycin-induced changes in the DNA structure are within the experimental error of the results reported here. Despite its simplicity, the model successfully recovered the correct oscillation frequency of the SH intensity vs. base pair separation.

## Conclusions

The results presented here demonstrate that the interference of second-harmonic fields generated by a pair of molecules complexed to double-stranded DNA can be used to probe their relative orientation. The straightforwardness of the experimental approach to control and measure relative molecular orientation, e.g., the anticancer drug daunomycin complexed with DNA investigated in this study, presents opportunities to probe structural aspects of biomolecular complexes in solution. For instance, it could be possible to investigate structural changes in DNA due to complex formation with proteins or drugs, e.g., one would measure a phase shift in the SH intensity vs. the number of base pairs separating the intercalated daunomycin molecules, and thus, their relative orientation angle would differ before and after complex formation of DNA with the ligand of interest.

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