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Binding of the Anti-Cancer Drug Daunomycin to DNA Probed by Second Harmonic Generation

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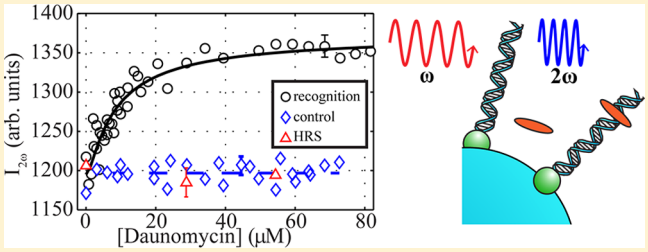
Supporting Information

ABSTRACT: Second harmonic generation (SHG) was used to selectively probe DNA–drug interactions without the need for chemical labels or invasive detection methods. In particular, the binding constant of the anticancer drug daunomycin to a recognition triplet sequence in a 33-mer of double stranded DNA was determined. The SHG method, which is interface selective, probed the binding of daunomycin to DNA that was tethered to the surface of colloidal microparticles suspended in aqueous solution. Probing biomolecule coated colloids is expected to yield larger SH signals and provides experimental flexibility as compared to experiments performed at planar interfaces. The change in SHG intensity as daunomycin was added to the microparticle solution was fit to a Langmuir binding model, which yielded an equilibrium constant of 2.3 (±0.7) × 10^5 M^{-1}; the corresponding Gibbs free energy change at 20 °C is −7.2 ± 0.2 kcal/mol. Control experiments established that daunomycin preferentially binds to DNA at the recognition sequence. The equilibrium was found to be unaffected by the presence of free DNA in solution, and hyper-Rayleigh scattering from bulk molecules did not change with increasing daunomycin concentration. The extracted equilibrium constants are in agreement with the range of reported values found in the literature.



INTRODUCTION

This work presents a sensitive method for determining equilibrium binding constants for biomolecular interactions in a label free and noninvasive way. The experiments used second harmonic generation (SHG) to probe the binding of a drug to DNA tethered to the surface of colloidal microparticles suspended in aqueous solution. Biomolecule coatings on nano- and microparticle surfaces have opened up a broad field of self-assembling superstructured materials;[1−7] some biomolecule coated particles have found applications in drug delivery systems and in vivo detection schemes.[8,9] The present work also builds on interface specific nonlinear optical experiments that have measured the electronic and vibrational spectra of DNA covalently bound to planar fused quartz surfaces immersed in aqueous media,[10−13] and recent work that tracked in real time the cleavage of DNA by the restriction enzyme EcoR1 and the subsequent rehybridization of DNA attached to colloidal polymer microparticles without labeled reporter molecules or invasive detection methods.[14] Using SHG to study biomolecular reactions, with one of the reactants attached to a colloidal interface, can yield larger signals than analogous experiments performed at planar interfaces due to the larger number of molecules that can be accommodated in the laser focus. Additionally, the number of particles in solution can be changed without changing the density of the DNA that is attached to the individual particles, allowing for flexibility in experimental design.

The class of anthracycline chemotherapeutic drugs has long been studied due to their potent anticancer properties.[15−20] Daunomycin (structure given in Figure 1a) is a member of this class of drugs that is used to treat acute leukemia.[15] The mechanism by which the chemotherapeutic behavior of daunomycin is manifested on the molecular level has been investigated by a wide variety of methods including X-ray crystallography,[21−25] optical absorption and fluorescence,[26−37] surface plasmon resonance,[38] calorimetery,[39−41] restriction enzyme foot printing,[42−44] cyclic voltammetry,[45] NMR,[46−52] scanning force microscopy,[53] and various theoretical approaches;[54−62] among these methods, restriction enzyme foot printing and scanning force microscopy are invasive. It is generally accepted that the anticancer properties arise from the intercalation of the aromatic rings of daunomycin into the DNA double helix while simultaneously stabilizing the complex through H-bonding of its amino sugar in the DNA minor groove.

To establish the mechanism by which a drug functions for use in drug design, it is necessary to determine the equilibrium binding constant, K, of the drug to a target receptor site and to extract the related thermodynamic properties to elucidate the

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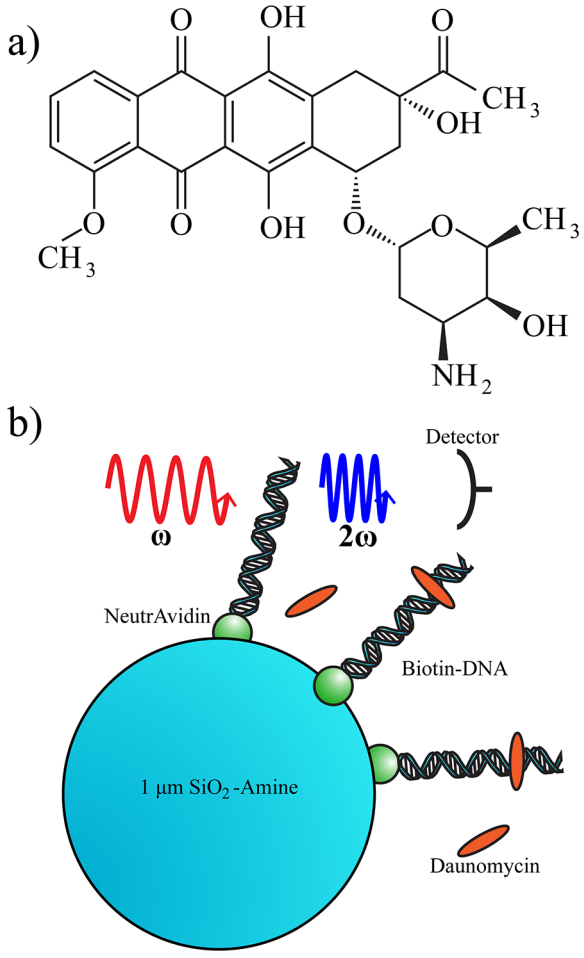

Figure 1. The chemical structure of daunomycin is given in part a. A sketch of the DNA coated beads is given in part b. The DNA sequence used in the recognition experiments is biotin-5′-CTCAGTGAACTC-AAGTGAATCCAA**TCG**AAGTT-3′; the recognition sequence is bolded and underlined. The control DNA sequence differs only by replacing the three bases of the recognition sequence with TTC bases. The complementary strand is implied for succinctness.

inherent driving forces. For the binding of daunomycin to double stranded DNA (dsDNA), the relevant reaction is

$$\text{dsDNA} + \text{Daunomycin} \overset{K}{\leftrightarrow} [\text{dsDNA}\cdot\text{Daunomycin}]$$

In the drug−DNA binding experiments carried out, a sequence of dsDNA was designed such that it contained one "recognition sequence", which is a sequence of three base pairs that are known to preferentially bind daunomycin. Previous studies have shown that daunomycin most favorably binds to 5′-(TCG)-3′, (ACG), (AGC), and (TGC) triplet sequences[19,42,43] and, more generally, indicate that daunomycin favors adjacent GC nucleotides as binding sites. As crystal structures have shown, it is at the location of these bases that the fused rings of daunomycin are intercalated into the DNA double helix.[21−25] A sketch of the DNA coated microparticles and the relevant reaction/detection scheme is given in Figure 1b; the DNA sequences that are used are given in the caption of Figure 1. To serve as a control, a dsDNA sequence was specified that substituted the three base pair recognition sequence with TTC bases such that adjacent GC base pairs were intentionally omitted.

SECOND HARMONIC GENERATION

Incident electromagnetic radiation at a frequency, ω, interacts with molecules, inducing a polarization that oscillates at the incident frequency and at multiple orders of the incident frequency. Of interest are those interactions that generate a coherent second order polarization at twice the fundamental laser frequency, 2ω, which is SHG. Coherent signals originating from the second order polarization, including sum frequency, difference frequency, and second harmonic generation, are forbidden in centrosymmetric and isotropic media.[63−65] Although coherent SHG is forbidden in centrosymmetric and isotropic bulk media for reasons of symmetry, it can be generated by a centrosymmetric structure, e.g., a sphere, provided that the object is centrosymmetric over roughly the length scale of the optical coherence, which is a function of the particle size, the wavelength of the incident light, and the refractive indices at ω and 2ω.[63] The radiated SHG field, $E_{2\omega}$, from an individual particle is proportional to the second order polarization, $P^{(2)}_{2\omega}$, at 2ω and is given by

$$E_{2\omega} \propto P^{(2)}_{2\omega} = \chi^{(2)}_{\mathrm{T}} E_\omega E_\omega \tag{1}$$

where the total second order susceptibility, $\chi^{(2)}_{\mathrm{T}}$, is the sum of the second order susceptibilities of all adsorbed species on an individual particle and E_ω is the incident electromagnetic field. The second order susceptibility for adsorbate species i can be written as

$$\chi^{(2)}_i \propto N_i \sum_{k,e} \frac{\mu_{gk}\mu_{ke}\mu_{eg}}{(\omega_{gk} - \omega - i\Gamma)(\omega_{eg} - 2\omega - i\Gamma)} \tag{2}$$

where μ_{gk}, μ_{ke}, and μ_{eg} are the transition dipole matrix elements between electronic states of the molecules, ω_{gk} and ω_{eg} are the transition frequencies between states, Γ is the line width for the transitions, and N_i is the number density of the ith species at the interface of an individual particle. Equation 2 shows that, when an electronic transition of an interfacial molecule is resonant with ω or 2ω, there is an enhancement of the SHG signal. Since daunomycin has an absorption tail at 2ω = 420 nm (see the Supporting Information text), it dominates the SHG response. At the low density of particles used in these experiments, 2.5×10^8 particles/mL, the SHG field from each particle is independent of other particles and has random phases. The total SHG intensity, $I_{2\omega}$(total), is an incoherent summation of the intensity generated by each particle, $I_{2\omega}$, and is expressed as

$$I_{2\omega}(\text{total}) \propto \sum_j^n |E_{2\omega}|^2(j) = nI_{2\omega} \tag{3}$$

where n is the number density of particles and $|E_{2\omega}|^2(j)$ is the absolute square of the radiated SHG field from the jth particle. It is the changing magnitude of the SHG signal from daunomycin as it binds to dsDNA attached to the silica-amine beads that tracks the binding. In the absence of daunomycin, the SHG intensity is due to nonresonant SHG from the microparticle and incoherent SHG, i.e., hyper-Rayleigh scattering (HRS), which arises from density and orientational fluctuations of the bulk molecules. It was determined that bulk water is largely responsible for the observed HRS background signal.

If the interface is charged, there can be an additional source of SHG radiation originating from the third order polarization, $P^{(3)}_{2\omega}$, oscillating at 2ω.[63,66,67] The contribution to the SHG

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signal from the third order polarization is described as a product of two oscillating electric fields and an electrostatic, zero frequency field that extends from the interface into the bulk media and polarizes the bulk molecules (predominantly water molecules).[63,66,67] The magnitude of the signal from the third order polarization is strongly dependent on the pH and the electrolyte concentration of the solution, which serve to neutralize charges at the interface and to screen bulk molecules from the electric field generated by the charged interface. Given that the present experiments are carried out in a relatively concentrated electrolytic solution (50 mM Tris buffer), the contribution from the third order polarization is negligible.

EXPERIMENTAL DETAILS

To probe the binding of daunomycin to dsDNA attached to colloidal microparticles, a second harmonic generation apparatus previously used to monitor the reaction of EcoR1 with dsDNA was employed.[14] Briefly, a KM laboratories Ti:sapphire oscillator running at a repetition rate of 80 MHz, with a center wavelength of 840 nm, producing approximately 300 mW average power with a pulse width of 60 fs was focused into a 2 mm quartz cuvette containing the sample. The generated SHG radiation was collected with a short focal length lens, telescoped, and filtered to remove much of the residual fundamental laser radiation before it was focused to an Acton SpectraPro 300*i* spectrograph coupled to a Princeton Instruments Spec-10 CCD camera. The camera exposure was set to 1 s; several hundred exposures were collected per concentration step to acquire statistics. Measurements were reproduced at least three times and averaged to establish reproducibility and improve error bars. Quoted uncertainties are given at the 95% confidence level.

Silica-amine microparticles (diameter = 1 μm) were purchased from Polysciences, Inc.; NeutrAvidin was covalently attached to the surface of the beads using a procedure supplied by Bangs Laboratories, Inc. The positively charged silica-amine particles were chosen in order to limit electrostatic binding of positively charged daunomycin to the surface of the particle. A 33-mer of biotinylated dsDNA was purchased from Integrated DNA Technologies, dissolved in 50 mM Tris buffer at pH 7.5, and added to the solution containing the particles according to previously published procedures.[4,5,14] The DNA surface coverage on the microparticles was estimated to be $\sim 1.6 \times 10^4$ DNA/μm^2, as determined from particle centrifugation measurements. The concentration of the microparticles used in all of the SHG experiments was 2.5×10^8 particles/mL, which was found to yield optimal signal while minimizing scattering losses. All reactions were carried out in 50 mM Tris buffer at pH 7.5. Daunomycin was purchased from Sigma-Aldrich and used without further purification. The bulk concentration of daunomycin was determined from optical absorption measurements using the extinction coefficient at $\lambda = 480$ nm, $\varepsilon_{480} = 11\,500$ M^{-1} cm^{-1}.[68]

RESULTS AND DISCUSSION

The SHG intensity is proportional to the square of the number of interfacial daunomycin−dsDNA complexes; a good fit of the data is obtained by utilizing the Langmuir binding model

$$I_{2\omega}(\text{total}) \propto \left[\frac{Kc}{(1 + Kc)}\right]^2 \quad (4)$$

As before, $I_{2\omega}$(total) is the observed SHG intensity that changes as the available binding sites on DNA are filled, K is the equilibrium constant for the reaction of daunomycin with dsDNA, and c is the bulk concentration of daunomycin in solution. It should be noted that, since the DNA recognition sequence has only one preferential binding site, complicated models that account for the exclusion and cooperativity of bound daunomycin molecules to a single dsDNA duplex are not necessary.[69] From the measurements of the SHG signal as a function of daunomycin concentration, one obtains the equilibrium constant of daunomycin binding to the 33-mer of DNA having a specific recognition sequence using eq 3.

The SHG binding isotherm given in Figure 2, and fit with the relationship in eq 3, yields an equilibrium binding constant of

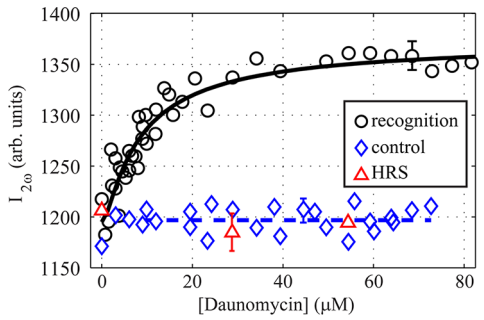

Figure 2. The binding isotherm from SHG measurements in the presence of excess DNA is the same as the isotherm collected under stoichiometric coverage conditions (see the Supporting Information). The solid black line is a fit to eq 3 given in the text. The dashed blue line represents the mean of the SHG signal observed in the control experiment. For clarity, one representative error bar for each set of data is given.

$2.3\ (\pm 0.7) \times 10^5$ M^{-1}, and thereby a Gibbs free energy of -7.2 ± 0.2 kcal/mol at 20 °C. The presence of excess DNA in solution was found to not influence the binding isotherms (see the Supporting Information), which is expected, since the concentration of DNA is sufficiently low that there is negligible depletion of bulk daunomycin. To establish that daunomycin binds specifically to the recognition site in the dsDNA, and that the measured equilibrium constant describes the propensity of daunomycin to this sequence, control experiments were performed where the three base pairs of the recognition sequence were replaced with TTC bases, such that the control dsDNA does not contain adjacent GC nucleotides. The results of the control experiments are given in Figure 2; the data shows that daunomycin does not bind to the control DNA. The lack of binding was expected, since the control DNA does not include the preferential daunomycin intercalation sites. Therefore, the equilibrium binding constants measured in the recognition DNA experiments describes the binding of daunomycin to the TCG sequence. This also implies that only one daunomycin is bound to each recognition DNA duplex. The control results agree with surface plasmon resonance findings in which nonspecific binding of daunomycin to control sequence DNA aptamers was not observed.[38]

It should also be noted that the increase in SHG signal is not due to incoherent hyper-Rayleigh scattering of daunomycin nor daunomycin aggregates in the bulk solution. This is shown in Figure 2 where the SHG signal did not change, within experimental error, as the daunomycin concentration increased in the absence of DNA coated microparticles. Aggregation of

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Table 1. A Summary of Equilibrium Constants Obtained for the Binding of Daunomycin to dsDNA Using Different Methods of Analysis

method	DNA type	buffer	K (M^{-1})
SHG (present work)	33-mer	Tris	2.3 (±0.7) × 10^5
circular dichroism[36]	fish sperm	Tris	1.98 × 10^5
absorption/fluorescence[20,26−28,32,34]	calf-thymus	phosphate	1.96 × 10^5 to 7.0 × 10^5
absorption[37]	chicken blood	McIlvain	1.8 × 10^5
cyclic voltammetry[45]	calf-thymus	phosphate	2.35 × 10^5
H^1-NMR[46,47,51,52]	4- and 6-mers	deuterated phosphate	4.3 × 10^5 to 1.7 × 10^6
scanning force microscopy[53]	pBluBacHis b	ammonium acetate	1.2 × 10^5

the daunomycin molecules does not influence the observed binding isotherms because the equilibrium constants for self-association are approximately 100 times smaller than the binding of daunomycin to dsDNA.[27] This means that, at a daunomycin concentration where aggregation must be considered, at ∼80 μM daunomycin, the available binding sites on the dsDNA are already saturated, as can be seen from the plateau in Figure 2 reached at ∼40 μM daunomycin. The equilibrium constant obtained from the SHG experiments is in the range of literature values obtained in bulk media using different detection methods and various lengths and sequences of DNA, as summarized in Table 1.

The selectivity of SHG provides a high sensitivity to the population of daunomycin−DNA complexes relative to linear optical methods because only the drug−DNA complexes at the microparticle interface generate coherent SHG signals. In linear optical methods, all of the absorbing species in solution contribute to the signal, which includes the free daunomycin, daunomycin aggregates, and the daunomycin−DNA complexes, and must be addressed in the data analysis. The SHG binding isotherms selectively monitor the drug−DNA complexes at the microparticle interface and are straightforward to construct and analyze using simple models to describe the binding equilibrium.

CONCLUSION AND OUTLOOK

The work presented here demonstrates the capability of second harmonic generation to selectively probe the binding of drugs to DNA without the need for labeled reporter molecules or invasive detection methods. The binding of the anticancer drug daunomycin to a 33-mer of double stranded DNA tethered to colloidal microparticles was measured using SHG. It is expected that SHG experiments on colloidal particles will yield larger signals than similar measurements at planar interfaces, while providing a flexible platform for experimental designs. The binding data was fit with a Langmuir binding model, which yielded an equilibrium constant of 2.3 (±0.7) × 10^5 M^{-1} and a Gibbs free energy of −7.2 ± 0.2 kcal/mol at 20 °C. The equilibrium constants obtained from the SHG measurements at two different bulk concentrations of DNA were the same within the experimental uncertainty, indicating that excess DNA in solution does not affect the binding of daunomycin to the dsDNA attached to the particles. Control experiments established that daunomycin preferentially binds to the TCG sequence in the recognition DNA experiments and that the binding constants describe the affinity of daunomycin to these nucleotides. The measured equilibrium constants are in agreement with the range of values found in the literature. Aggregation of daunomycin molecules does not affect the obtained binding curves because the DNA binding sites were saturated before aggregation of daunomycin occurred; i.e., above 40 μM daunomycin all of the available binding sites are occupied. The intensity of incoherent SHG (hyper-Rayleigh scattering) signals originating from species in the bulk media, including aggregates, did not change as a function of daunomycin, and it was therefore not necessary to explicitly address these bulk signals in the analysis. SHG, a label free and noninvasive detection method, complements more traditional methods of detection and is expected to have broad applicability to the study of biomolecular equilibrium characteristics and reaction dynamics.

ASSOCIATED CONTENT

Supporting Information

Additional information on the optical absorption spectrum of daunomycin and the SHG isotherm at stoichiometric DNA particle coverage. 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 interest.

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