Femtosecond Spectroscopy

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Detection of DNA–Ligand Binding Oscillations by Stokes-Shift Measurements**

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Low-frequency structural vibrations of proteins and DNA are important for their physiological functions, and these motions are altered when biomolecules interact with each other and with small molecules. As a model, we examine the DNA duplex d(CGCAAATTTGCG)$_2$ together with the bis(benzimidazole) dye Hoechst 33258 (H33258), which recognizes and binds to A:T-rich regions in the minor groove.[1,2] Dynamic aspects of recognition are increasingly seen to be essential for inter- and intracellular signaling networks,[3] enzyme catalysis,[4] and allosteric regulation.[5] Improved strategies for drug discovery also consider the flexibility of the biomolecular target.[6] But despite its importance, vibrational motion of ligand-dye binding has not yet been observed because infrared spectroscopy is not selective for the binding coordinate and stationary Raman spectroscopy at low frequencies (ca. THz or 30 cm^{-1}) is dominated by Rayleigh scattering. Herein we show that binding oscillations can be seen with the help of ligand fluorescence. Frequency modulation of the time-dependent Stokes shift (TDSS) may reflect coherent in/out motion of the ligand, synchronous with breathing[7] of the minor groove. The observation method is spatially selective and sensitive, compared to THz absorption measurements, and may become useful through advances in fluorescence gating.[8,9]

Figure 1 shows the X-ray structure of the DNA:H33258 complex.[1] The imido HN1 and HN3 groups of the H33258 ligand form bifurcated hydrogen bonds with nucleobase edges T O2 and A N3 in the groove floor, and they play an important role in biomolecular recognition.[1,2] We excite the ligand with short optical pulses and monitor the entire fluorescence band as it evolves over several picoseconds.

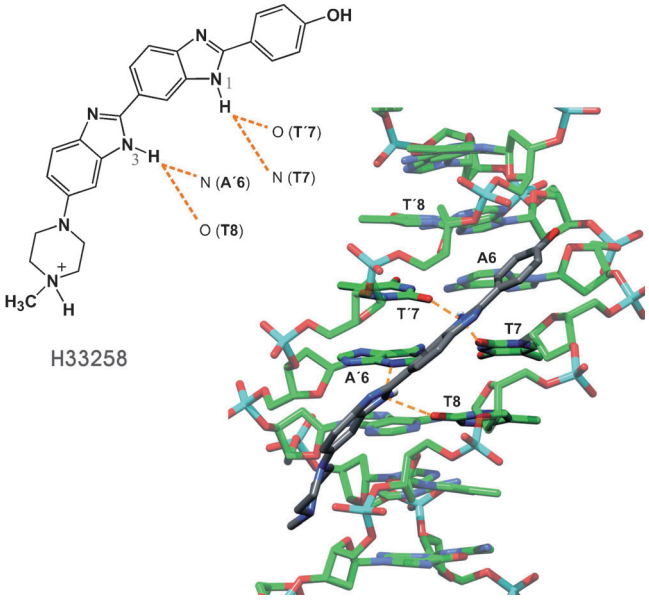

Figure 1. Central view of H33258 bound to d(CGCAAATTTGCG)$_2$, looking onto the minor groove. The ligand (black) extends bifurcated hydrogen bonds (dashed lines) from its two imido groups to nucleobase edges in the groove floor (for nucleobase indices, see Ref. [23]). We show that these hydrogen-bond distances and the groove width oscillate coherently when the chromophore is optically excited. This movement is observed through fluorescence, as frequency modulation of the time-dependent Stokes shift.

For this purpose the emission is time-gated by broadband upconversion with 85 fs resolution.[10] In general, the TDSS reflects the response of the local environment to a sudden electrostatic perturbation, induced by the electronic excitation of a molecular probe. For an oxyquinolinium betain dye in various solvents it was shown that oscillations in the TDSS encode not only intramolecular probe vibrations but also coherent motion of the solvent.[11] If the fluorescent probe is a specifically binding ligand, even its distance to the substrate could conceivably be seen as oscillating. But in all previous studies of proteins and natural DNA, only multiexponential or similar Stokes shifts were observed, corresponding to diffusive reorganization of the environment.[12–21] The debate has therefore focused on the time scales 10 ps–100 ns and on the extent to which the biomolecule or water are responsible for the diffusive reorganization.[22–25] Meanwhile, experimental methods have been improved and an oscillatory TDSS contribution has been found, albeit with unnatural DNA in which nucleobases had been replaced and where a ligand coordinate is not defined.[26] Herein we report such oscilla-

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tions for DNA:H33258 whose interpretation, in terms of specific and coupled motions, is guided by molecular dynamics (MD) simulations.[27–29]

Steady-state spectra of H33258 are shown in Figure 2a. Upon binding to the duplex the absorption band shifts to the red and broadens compared to aqueous solution. For technical reasons we use a relatively high concentration (0.7 mM) of the complex. In this case the stationary emission band is red-shifted from the position at low concentration because of heterogeneity (dye loading) and self-absorption.[30] But regardless of these complications, femtosecond optical pumping at the red absorption edge (400 nm) promotes a bound H33258 chromophore to the first excited singlet state S_1. We are interested in the first few picoseconds of spectral relaxation after this excitation.

The time-resolved fluorescence spectra $S_1 \rightarrow S_0$, as measured, are shown in Figure 2b. The emission was monitored simultaneously at all relevant wavelengths and compared to that of standard dyes, to give photon distributions over fluorescence frequency directly.[10] A dip at 530 nm is caused by strong excited-state absorption.[31] This kind of "inner filter effect" can be corrected by knowledge of its shape (Figure 2c) and amplitude (from comparison with steady-state spectra at late time). Similarly the inner filter effect of ground-state absorption is estimated. After corresponding corrections, we obtain the "true" molecular quantum distributions in Figure 2d.

The fluorescence frequency as a function of time is shown in Figure 3. Figure 3a shows the peak position $\nu_P(t)$ for 20 °C

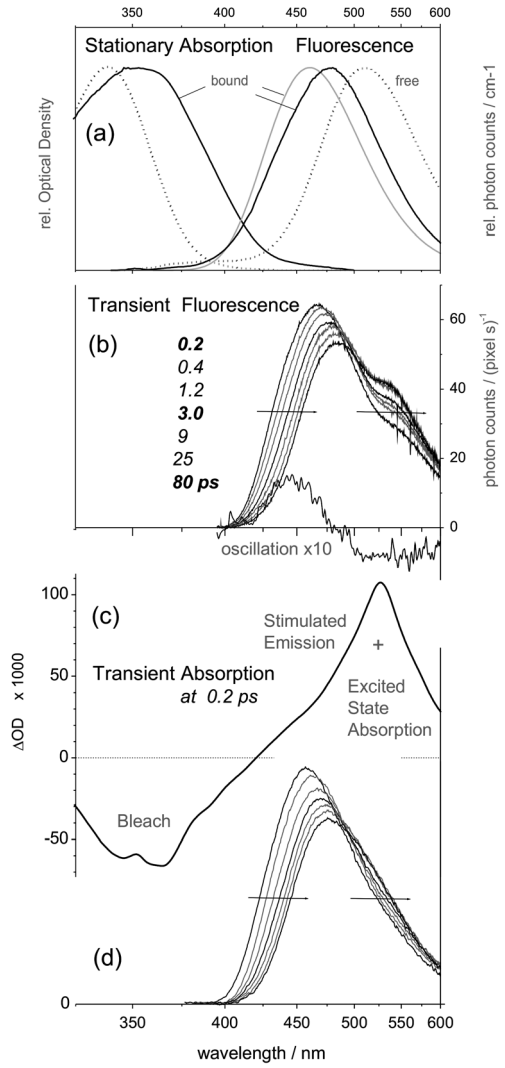

Figure 2. Optical spectra of the DNA:H33258 complex in water (pH 7.4). a) Stationary absorption and fluorescence at 20 °C. Gray line: fluorescence at low concentration. Black solid lines: fluorescence at duplex concentration 0.7 mM upon excitation at 400 nm. b) Transient fluorescence after femtosecond (fs) laser excitation at 400 nm, 1 °C, 0.7 mM, with 85 fs time resolution (full width at half maximum of instrument response function). Shown are the recorded spectra directly after photometric correction by comparison to standard dyes. The spectrum of a frequency modulation, with recurrences at 0.6 and 1.2 ps, is also shown (see text). c) Transient absorption after fs excitation.[31] Excited-state absorption at 530 nm is responsible for a dip in the recorded transient fluorescence spectra ("inner filter effect"). d) Transient fluorescence spectra at 1 °C obtained from (b) after smoothing over three pixels and correction for the inner filter effect. The dynamic red shift of the band (arrows) is measured by the peak frequency ν_P or average emission frequency $\bar{\nu}$ as a function of time.

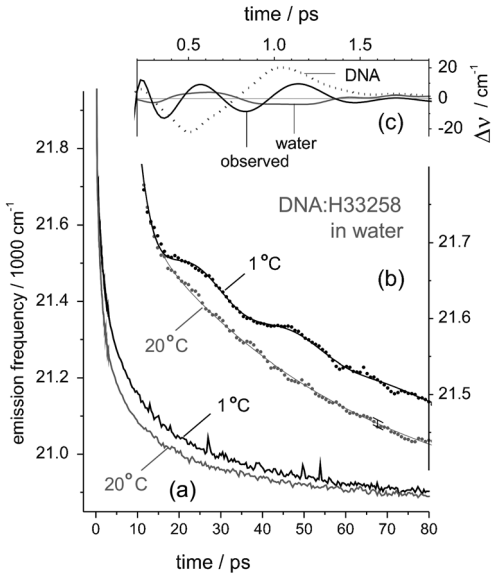

Figure 3. Time-dependent Stokes shift (TDSS) of DNA:H33258 in water (0.7 mM). a) Peak frequency ν_p, from fitting a spectral lognorm shape to the fluorescence bands as in Figure 2d. Note that noise is smaller before 10 ps owing to better statistics. b) Average frequency $\bar{\nu}$ (points) calculated directly, avoiding spectral fits, from the raw spectra as in Figure 2b. A constant was added to align with ν_p. On lowering the temperature to 1 °C, weak oscillations appear on a diffusive spectral relaxation curve. $\bar{\nu}$ curves calculated from a global (t, ν) fit of the transient spectra are also shown (lines), and the confidence interval is indicated. c) Residuals of the black curve in (b) to a triexponential fit (black solid line). Also shown are residuals for simulated time-correlation functions $C_{DNA}(t)$ (dotted) and $C_{water}(t)$ (gray). They are scaled by the observed Stokes shift (cf. Figure S8 in the Supporting Information) to indicate the oscillation amplitudes that are expected from the two relaxation channels. Multiexponential fits were restricted to $t = 0.1$–10 ps. For (c), the ordinate scale of (b) was expanded 2×.

and 1 °C (from lognorm fits of spectra as in Figure 2d). Similarly the average position $\bar{\nu}(t)$ could be used; there is no reason why one should be preferred. With red-edge excitation the two measures yield the same dynamics. The $\nu_P(t)$ curve for 20 °C is described by a Gaussian function plus exponential time functions (see the Supporting Information). This result agrees qualitatively with upconversion data on the (different) diluted system.[20]

Discovery of frequency modulation (FM) during the first few picoseconds is at the center of this work. For this purpose, we prefer to calculate the average position $\bar{\nu}(t)$ directly from the measured transient spectra (as in Figure 2b). The early behavior is shown in Figure 3b. Upon lowering the temperature from 20 to 1 °C, oscillations appear on a diffusive relaxation curve (because friction is reduced for coherent nuclear motion, which is responsible, see below). Comparison of the two curves shows that the oscillations at 1 °C are significant. The spectral change beneath $\bar{\nu}(t)$ is found with a global fit (i.e., for all wavelengths) by one Gaussian function plus three exponentials. To model the oscillatory part at 1 °C, we add (after some trials) the function $s(t) = \xi\exp[-\gamma_1 t]\cos[\omega_1 t] + \exp[-\gamma_2 t]\cos[\omega_2 t]$. Optimal values are $\xi = 0.2$, $\omega_1 = 5.4\ \mathrm{ps}^{-1}$, $\gamma_1 = 0.85$, $\omega_2 \approx 2\omega_1$, $\gamma_2 = 1.71\ \mathrm{ps}^{-1}$. We also tried a single frequency, but in this case neither the observed damping nor the behavior in ethylene glycol can be described (see below). The spectrum associated with $s(t)$ is also shown in Figure 2b; it has a dispersive form passing through zero near the fluorescence peak, as expected for FM. Very likely, the oscillations reflect coherent supramolecular motion, as will be argued in the remainder of this work.

Molecular dynamics simulations have been used by some of us[27–29] to decompose the calculated solvation relaxation function of DNA:H33258, $C(t)$, into contributions from the environment (DNA, water, ions) and from the intramolecular conformation of the H33258 solute: $C(t) = C_{\mathrm{DNA}}(t) + C_{\mathrm{water}}(t) + C_{\mathrm{ions}}(t) + C_{\mathrm{conf}}(t)$. High-frequency oscillations of the partial $C_i(t)$ are caused by intramolecular motion (see Figure S7 in the Supporting Information). The underlying slow behavior for $C_{\mathrm{DNA}}(t)$ shows a local maximum around 1.05 ps. For $C_{\mathrm{water}}(t)$ the slow behavior has a very weak shoulder around 0.6 ps. Oscillations are seen better when a multiexponential fit is subtracted from each smoothed curve and the resulting $\Delta C_i(t)$ are scaled to match the experiment (Figure 3c). Modulation of the fluorescence frequency is predicted for solvation by DNA (dotted line) and, to a lesser extent, by water (gray). The ion contribution is featureless for the time range of interest, and the conformational contribution is too small to be observed.

Comparison with experiment (black points and lines in Figure 3b,c) shows that amplitude and time scale of the simulated FM agree reasonably with the observed FM. The qualitative features appear to have been captured by MD even though the exact simulation results depend on the force field. More specifically, the DNA contribution is predicted to be the same size as or larger than that of water, and its recurrence at 1.05 ps matches the experimental recurrence at 1.1 ps. When H33258 is simulated free in water, no oscillations are found. Thus the wave $\exp[-\gamma_1 t]\cos[\omega_1 t]$ should reflect coherence in a 29 cm^{-1} structural mode of the complex.

Looking into the set of coordinates we find that the "docking" distances of the two bifurcating H-bonds also oscillate with a 1.05 ps period. It makes sense to associate this motion with the oscillation in the calculated $C_{\mathrm{DNA}}(t)$ curve (and therefore with the observed frequency modulation), because the emission of the chromophore shifts upon binding (Figure 2a). Similarly, the width of the minor groove (Figure S10 in the Supporting Information) could be involved. It is defined as the distance between phosphorous atoms at positions four base pairs away on the opposite strand. Ignoring the terminal base pairs, there are six minor-groove widths for the dodecamer studied: A6–C11′, T7–G10′, T8–T9′, T9–T8′, G10–T7′, and C11–A6′. Their time correlation functions (TCFs, Figure S10 in the Supporting Information) exhibit a recurrence near 1 ps, implying coherent motion across the entire binding region close to the frequency which is observed experimentally. A comparison of the groove-width TCFs with and without the dye molecule attached to DNA reveals several distinct differences. Most notably, the recurrence at approximately 1 ps is absent from the TCFs when the dye is not bound. Moreover, the TCFs decay more rapidly when the dye is bound to DNA, which suggests that the intrinsic flexibility of DNA is changed upon formation of the complex. Taken together, we may conclude that the observed oscillatory motion is essentially that of biomolecular recognition.

The observation of several FM terms could be due simply to the presence of several dye species. This possibility can be ruled out because complex oscillatory response is also seen with diluted complexes (see the Supporting Information). Our MD simulations suggest that the 0.6 ps recurrence is due to water. One may object that the calculated amplitude is too small, compared to that of the DNA contribution, for both to add up to the double-peak structure of Figure 3c (black line). But the imbalance could be due to inadequacies in the simulation force fields or in the approximations necessary to compute the response.[29] Also, the experimental curve could be inaccurate, as it was taken from a strongly sloping background (cf. Figure 3b). Thus coherent, collective water motion remains a viable explanation which will be discussed below. First an alternative interpretation: that the 0.6 ps feature is an overtone of the DNA fundamental, will be explored with the help of Figure 4.

Optical excitation $S_1 \leftarrow S_0$ of the H33258 ligand is generally accompanied by a change of equilibrium structure for the entire complex. The change along the 29 cm^{-1} "recognition" coordinate is measured by displacement Δ of the potential-energy minima. If the mode is displaced upon excitation ($\Delta \neq 0$, Figure 4a), then a simple wavepacket is launched on S_1, leading to modulation of the $S_1 \rightarrow S_0$ emission energy at the fundamental frequency ω_1. Another situation is sketched in Figure 4b. Here the mode is not displaced upon excitation ($\Delta = 0$) but the force constant is changed instead. The initial Gaussian distribution in the excited state S_1 evolves into a bimodal distribution and back, causing a modulation of the emission energy at the overtone $2\omega_1$, which could also explain the 0.6 ps recurrence.

With a final experiment we explore the other hypothesis: that water is behind the 0.6 ps recurrence of the time-resolved

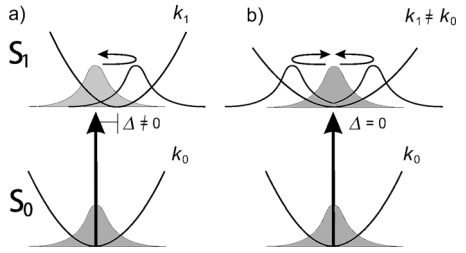

Figure 4. Modulations of the emission frequency, expected from coherence (curved arrows) in the 29 cm^{-1} "recognition" mode. a) When the structure is changed upon optical excitation, a single wavepacket is generated, leading to modulation at the fundamental frequency. b) When the structure remains (displacement $\Delta = 0$) but the force constant is changed instead, the wavepacket becomes bimodal, leading to a modulation at twice the fundamental frequency.

emission frequency. Would this feature disappear if water is removed? In ethylene glycol, hybridization of 21-mer oligonucleotides has been shown at reduced temperature.[32] Our association studies in ethylene glycol (Figure S12, Figure S13 in the Supporting Information) show sharp change at 23 °C, both for ligand absorption and for hyperchromism of natural bases, indicating that an ordered DNA:H33258 complex exists below this temperature. The spectral relaxation curve of the complex at −10 °C (black line in Figure 5) has a broad local maximum around 1 ps, almost identical to simulated

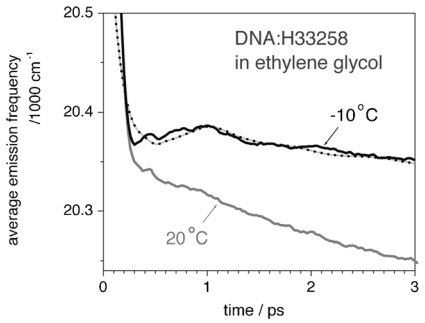

Figure 5. Stokes shift of DNA:H33258 fluorescence in ethylene glycol (average of spectra as in Figure 2b). At −10 °C the chromophore, bound to the duplex, shows a broad oscillation peak at 1.2 ps. The curve strongly resembles simulated $C_{DNA}(t)$ which, after scaling, is represented by the dotted line. At 20 °C the complex is partly dissociated.

$C_{DNA}(t)$ (dotted line, scaled from Figure S7 in the Supporting Information). The significance of the oscillation is again seen by comparison with the curve for 20 °C. (In this case not only is friction larger, as before, but also the complex is partly dissociated.)

In summary for the 58 cm^{-1} FM, there are two interpretations: 1) water is involved directly, by coherent collective motion which affects the ligand; 2) water acts indirectly, by reducing a structural change for biomolecular recognition upon electronic excitation. Simulations by mixed quantum mechanics/molecular mechanics, for example, would resolve which mechanism is responsible for the 0.6 ps recurrence. Also, the groove width coordinates should be correlated with the solvation energy of the ligand. Most importantly, the dynamics of biomolecular recognition appears to be reflected in a 29 cm^{-1} (1.1 ps) frequency modulation of the fluorescence band, owing to coherent oscillation of the ligand–substrate distance and groove breathing. The molecular mechanics of ligand–substrate binding may thus be observed directly, with the potential to become ultrasensitive.[8,9]

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[1] M. C. Vega, I. G. Saez, J. Aymami, R. Eritja, G. A. Vandermarel, J. H. Vanboom, A. Rich, M. Coll, *Eur. J. Biochem.* **1994**, *222*, 721.
[2] C. E. Bostock-Smith, S. A. Harris, C. A. Laughton, M. S. Searle, *Nucleic Acids Res.* **2001**, *29*, 693.
[3] R. G. Schmock, L. M. Gierasch, *Science* **2009**, *324*, 198.
[4] S. Hammes-Schiffer, S. J. Benkovic, *Annu. Rev. Biochem.* **2006**, *75*, 519.
[5] G. M. Lee, C. S. Craik, *Science* **2009**, *324*, 213.
[6] R. V. Mauldin, M. J. Carroll, A. L. Lee, *Structure* **2009**, *17*, 386.
[7] T. Ambjörnsson, S. K. Banik, O. Krichevsky, R. Metzler, *Biophys. J.* **2007**, *92*, 2674.
[8] P. Fita, Y. Stepanenko, C. Radzewiccz, *Appl. Phys. Lett.* **2005**, *86*, 021909.
[9] X.-F. Ha, X.-H. Chen, Y.-X. Weng, J.-Y. Zhang, *J. Opt. Soc. Am. B* **2007**, *24*, 1633.
[10] X.-X. Zhang, C. Würth, L. Zhao, U. Resch-Genger, N. P. Ernsting, M. Sajadi, *Rev. Sci. Instrum.* **2011**, *82*, 063108.
[11] J. L. N. Lustres, S. A. Kovalenko, M. Mosquera, T. Senyushkina, W. Flasche, N. P. Ernsting, *Angew. Chem.* **2005**, *117*, 5779; *Angew. Chem. Int. Ed.* **2005**, *44*, 5635.
[12] P. Abbyad, X. H. Shi, W. Childs, T. B. McAnaney, B. E. Cohen, S. G. Boxer, *J. Phys. Chem. B* **2007**, *111*, 8269.
[13] D. Andreatta, J. L. P. Lustres, S. A. Kovalenko, N. P. Ernsting, C. J. Murphy, R. S. Coleman, M. A. Berg, *J. Am. Chem. Soc.* **2005**, *127*, 7270.
[14] E. B. Brauns, M. L. Madaras, R. S. Coleman, C. J. Murphy, M. A. Berg, *J. Am. Chem. Soc.* **1999**, *121*, 11644.
[15] E. B. Brauns, M. L. Madaras, R. S. Coleman, C. J. Murphy, M. A. Berg, *Phys. Rev. Lett.* **2002**, *88*, 158101.
[16] P. Changenet-Barret, C. T. Choma, E. F. Gooding, W. F. DeGrado, R. M. Hochstrasser, *J. Phys. Chem. B* **2000**, *104*, 9322.
[17] X. J. Jordanides, M. J. Lang, X. Y. Song, G. R. Fleming, *J. Phys. Chem. B* **1999**, *103*, 7995.
[18] T. P. Li, A. A. P. Hassanali, Y. T. Kao, D. P. Zhong, S. J. Singer, *J. Am. Chem. Soc.* **2007**, *129*, 3376.
[19] S. K. Pal, J. Peon, A. H. Zewail, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1763.
[20] S. K. Pal, L. A. Zhao, A. H. Zewail, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8113.
[21] L. Y. Zhang, Y. T. Kao, W. H. Qiu, L. J. Wang, D. P. Zhong, *J. Phys. Chem. B* **2006**, *110*, 18097.
[22] B. Halle, L. Nilsson, *J. Phys. Chem. B* **2009**, *113*, 8210.
[23] L. Nilsson, B. Halle, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 13867.
[24] S. K. Pal, A. H. Zewail, *Chem. Rev.* **2004**, *104*, 2099.
[25] D. Zhong, S. K. Pal, A. H. Zewail, *Chem. Phys. Lett.* **2011**, *503*,1.
[26] A. Dallmann, M. Pfaffe, C. Mügge, R. Mahrwald, S. A. Kovalenko, N. P. Ernsting, *J. Phys. Chem. B* **2009**, *113*, 15619.
[27] K. E. Furse, S. A. Corcelli, *J. Phys. Chem. Lett.* **2010**, *1*, 1813.

[28] K. E. Furse, S. A. Corcelli, *J. Am. Chem. Soc.* **2008**, *130*, 13103.
[29] K. E. Furse, S. A. Corcelli, *J. Chem. Theory Comput.* **2009**, *5*, 1959.
[30] G. Cosa, K.-S. Focsaneanu, J. R. N. McLean, J. P. McNamee, J. C. Scaiano, *Photochem. Photobiol.* **2001**, *73*, 585.
[31] A. L. Dobryakov, S. A. Kovalenko, A. Weigel, J. L. Pérez-Lustres, J. Lange, A. Müller, N. P. Ernsting, *Rev. Sci. Instrum.* **2010**, *81*, 113106.
[32] G. Bonner, A. M. Klibanov, *Biotechnol. Bioeng.* **2000**, *68*, 339.

