VIP Single-Molecule Studies

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Direct Monitoring of Formation and Dissociation of Individual Metal Complexes by Single-Molecule Fluorescence Spectroscopy**

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Dedicated to Professor Peter Hofmann on the occasion of his 60th birthday

Single-molecule fluorescence spectroscopy (SMFS) has proven a valuable tool for investigating complex structures and processes in biochemistry and molecular biology.[1] Current applications of SMFS to chemical systems have been limited to polymers, to photophysical properties of chromophores, and diffusion of organic molecules in mesoporous materials, aside of its use as an analytical tool.[2–6] The observation of chemical reactions is generally hampered because of the lack of established methods for sensing single-molecular events as compared to samples in biochemistry and molecular biology. It is thus very promising that recently the field of heterogeneous catalysis has been entered with a study by SMFS of the spatial heterogeneity of ester cleavage rates on catalytically active crystal surfaces.[7]

Metal complexes play an important role in chemistry, including the field of homogenous catalysis. Their capability to interfere with spectroscopic properties of organic dyes triggered our idea to use photochemical switches based on metal coordination for the observation of chemical reactions on the single-molecule level. As a first step, we designed a metal-sensitive fluorescent system which can be attached to glass slides without adsorption to the surface and allows independent variation of the metal-binding ligand and reporter dye. Herein we demonstrate that this system can be used for the direct observation of individual association and dissociation events of a copper(II)–bipyridine chelate complex by using a confocal fluorescence microscope. Our strategy makes use of intramolecular fluorescence quenching of tetramethylrhodamine (TMR) by copper(II)–bipyridine complexes, similar to the concept of the fluorescein-based copper sensors reported earlier.[8,9]

To enable the time-resolved observation of the metal complex association and dissociation we developed a dye–ligand conjugate based on the hybridization of two modified oligonucleotides (see the Supporting Information). Strand **1** carries the bidentate ligand 2,2′-bipyridine-4,4′-dicarboxylic acid (dcbpy) at the 5′ end as a binding site for Cu^{2+} ions. The complementary strand **2** is biotinylated with a hexaethylene glycol (HEG) linker at the 5′ end and labeled with TMR at the 3′ end. Upon hybridization of the two complementary oligonucleotides the ligand and the dye label get in close vicinity such that the fluorescence of TMR is quenched when a Cu^{2+} ion binds to dcbpy (see the Supporting Information).[9] For single-molecule studies, the oligonucleotides were immobilized on a glass surface by biotin/streptavidin binding as drawn schematically in Figure 1. The glass surface was treated with bovine serum albumin (BSA) doped with about 10% biotinylated BSA, allowing for binding of recombinant streptavidin. An optimum concentration of 50 pM of the biotinylated TMR–dcbpy conjugate in aqueous solution was used for preparing a surface density of about 10–20 individual conjugates per 100 μm^2 (Figure 2). Acting as a linker, the oligonucleotides keep the ligand and dye in a solvated state and avoid adsorption to the surface, a complication observed with short or less hydrophilic linkers. To confirm the free

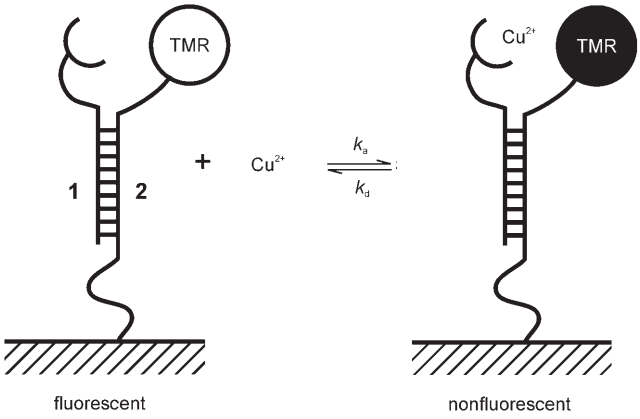

Figure 1. The TMR–dcbpy conjugate is formed by hybridization of two modified DNA oligonucleotides. Strand **1** carries the dcbpy ligand at the 5′ end as a metal ion binding site. The complementary strand **2** is labeled at the 3′ end with TMR and coupled to a hexaethylene glycol linker to biotin for immobilization on streptavidin-coated surfaces. Formation and dissociation of the $[Cu(dcbpy)]^{2+}$ complex was investigated in solution by steady-state spectroscopy as well as by single-molecule fluorescence spectroscopy with the complex immobilized on a glass surface.

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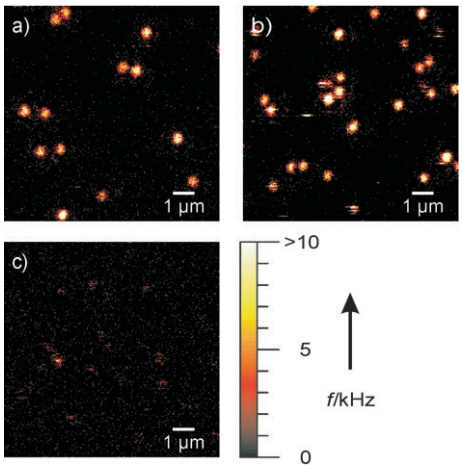

Figure 2. Scan images of the immobilized TMR–dcbpy conjugate in 10 mM MOPS buffer (pH 7; excitation: 532 nm, 5 μW; resolution: 50 nm pixel^{-1}; scan rate: 2 ms pixel^{-1}) in the absence of Cu^{2+} ions (a) and after addition of 2 μM (b) and 50 μM (c) of $CuSO_4$.

rotation of the probe we used modulated polarization excitation (see the Supporting Information).[10,11]

To localize the individual probes on the glass surface, raster scan images of the samples were recorded with circular polarized light for isotropic excitation. Figure 2 shows fluorescence intensity images of the immobilized TMR–dcbpy conjugate at three different concentrations of $CuSO_4$ in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffered solution. Since the scanning proceeds line by line (from the upper-left to the lower-right corner), single spots in the images carry information on fluorescence intermittency as indicated by some striped point-spread functions (PSFs) in Figure 2b. At 2 μM $CuSO_4$ only few probes show a decrease in fluorescence intensity and an appearance of long off states (Figure 2b, dark stripes within some PSFs) as compared to the control sample in the absence of $CuSO_4$ (Figure 2a). Fluorescence is almost completely quenched at 50 μM $CuSO_4$ (Figure 2c). The observed changes in molecular brightness and the PSF patterns at different concentrations of $CuSO_4$ indicate that the underlying dynamic process must be related to the association and dissociation of copper(II) complexes.

To access the kinetics of the observed dynamics, the fluorescence intermittencies of single-probe molecules were investigated. After imaging and addition of $CuSO_4$ the molecules were individually positioned in the laser focus for time-resolved recording of their fluorescence emission. Representative traces of different samples under varying $CuSO_4$ concentrations are plotted in Figure 3. In the absence of Cu^{2+} the TMR–dcbpy conjugate exhibits a fluorescence emission of about 3 kHz until the dye is photobleached (Figure 3a). Fluctuations in the signal arising from triplet blinking dynamics are not resolved in Figure 3 because they occur on a much faster timescale. At 0.5 μM and 4 μM $CuSO_4$ (Figure 3b and c, respectively) stochastic fluorescence fluctuations with off states lasting several seconds can be observed. Variations in the intensity levels are due to imperfect positioning of the molecules in the laser focus. Shorter on states at higher $CuSO_4$ concentrations (Figure 3c) indicate that fluorescence intensity fluctuations are correlated with the association kinetics of the $[Cu(dcbpy)]^{2+}$ complex. This interpretation is further supported by studies on TMR–DNA conjugates in which the dcbpy ligand is missing (Figure 3d and e). Here the fluorescence emission of TMR apparently remains unchanged at concentrations of 2 μM $CuSO_4$ (Figure 3d), while at higher $CuSO_4$ concentrations short off times in the millisecond range can be observed (Figure 3e). Since these samples did not contain an explicit binding site for Cu^{2+} ions, we attribute the short off times to collisional quenching of TMR by Cu^{2+} ions. This interpretation is in accordance with ensemble studies on the quenching of ligand-free TMR–DNA conjugates by Cu^{2+} and $[Cu(bpy)]^{2+}$, which is two orders of magnitude less than quenching of the dcbpy–DNA conjugate by $CuSO_4$ (see the Supporting Information). Comparison of the different conjugates (Figure 3a–c versus 3d–e) clarify together with our ensemble studies that a direct interaction between the Cu^{2+} ions and TMR starts becoming prominent at high concentrations of $CuSO_4$. Since the fluctuation pattern of the long off times observed for the TMR–dcbpy conjugate occurs on the timescale of seconds, it can be assumed that they correspond to individual binding events of Cu^{2+} ions to dcbpy. Hence, data analysis of the duration of the on states and the off states by statistical means should yield information about the respective association and dissociation kinetics of the $[Cu(dcbpy)]^{2+}$ complex.

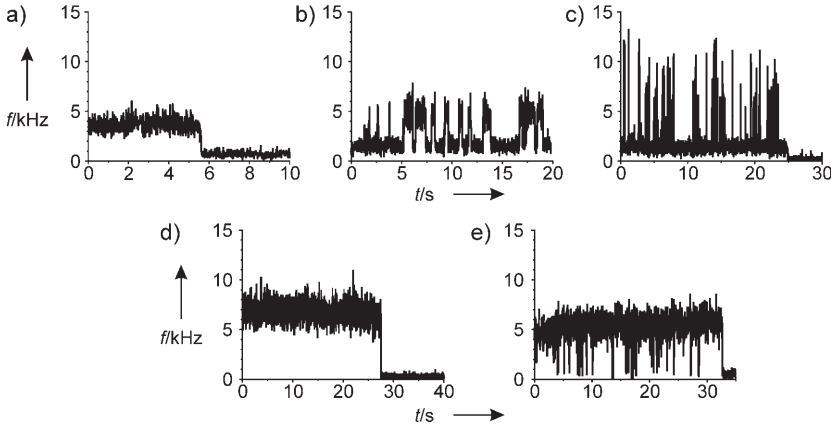

Figure 3. a–c) Fluorescence traces of the TMR–dcbpy conjugate at different concentrations of $CuSO_4$: 0 μM (a), 0.5 μM (b), and 4 μM (c). Control experiments with a TMR conjugate missing the dcbpy ligand are shown for $CuSO_4$ concentrations of 2 μM (d) and 6 μM (e). For data presentation and analysis a time resolution of 20 ms was used.

To determine the reaction rates, the durations Δt of individual on and off states of multiple molecules at given concentrations of $CuSO_4$ were collected in histograms and scaled to probability density functions $P(\Delta t)$ (see the Sup-

porting Information). While no correlation between the off times and the $CuSO_4$ concentration is visible (Figure 4a), the on times decrease steadily with $CuSO_4$ concentration (Figure 4b). According to Equation (1) and (2) the probability

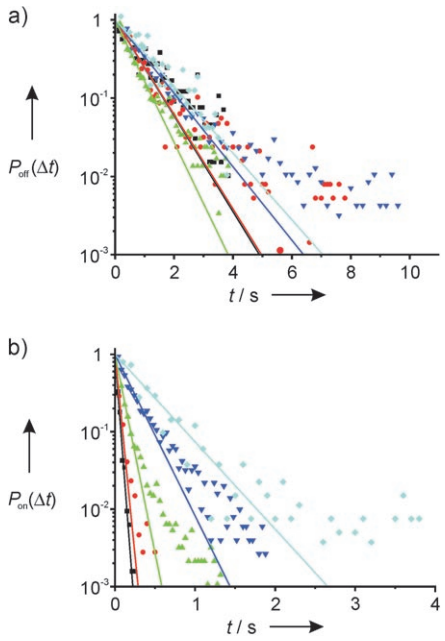

Figure 4. Probability density functions of the off times (a) and on times (b) for all five different $CuSO_4$ concentrations (black 0.5 μM, red 2 μM, green 4 μM, blue 6 μM, cyan 10 μM). At short off/on times the applied monoexponential model fits well to the data, while a deviation from the monoexponential behavior can be observed for longer off/on times on some histograms.

$$P_{on}(\Delta t) = e^{-k_a c(Cu^{2+})\Delta t} = e^{-\nu_{on}\Delta t} \quad (1)$$

$$P_{off}(\Delta t) = e^{-k_d c(Cu^{2+})\Delta t} = e^{-\nu_{off}\Delta t} \quad (2)$$

density functions $P_{off}(\Delta t)$ and $P_{on}(\Delta t)$ were subjected to a monoexponential model fit for estimating the desired off and on rates ν_{off} and ν_{on}. The deviations from the monoexponential model at longer off and on times in some, but not all, of the probability density functions indicate a higher complexity underlying the observed switching between off and on states, which might be due to slow conformational changes of the probe itself. Yet the monoexponential approximation seems sufficient for the presented description of complex association and dissociation because the deviations show only low occurrences (logarithmic axis in Figure 4) and more complex fit models do not give a clear trend.

The respective rates ν_{on} and ν_{off} were determined by fitting a monoexponential model function to the histograms (Figure 5 and in the Supporting Information). The on rates plotted in Figure 5 (squares) reproduce nicely the linear dependency from the $CuSO_4$ concentration as predicted by Equation (1), while the off rates remain constant over the observed concentration range (circles). We obtained an association rate constant of $k_a = (3.3 \pm 0.3) \times 10^6\,M^{-1}s^{-1}$ for

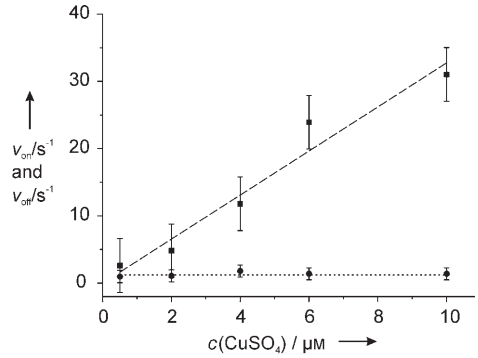

Figure 5. The obtained on rate ν_{on} (squares) plotted against the applied $CuSO_4$ concentrations shows a linear behavior corresponding to the association rate of $[Cu(dcbpy)]^{2+}$. Since the dissociation of the complex is independent from the $CuSO_4$ concentration, the corresponding off rate ν_{off} (circles) remains constant.

the complex formation at ambient temperature and pH 7.0 by a linear fit to the on rates ν_{on}. Averaging of the off rates ν_{off} yielded a dissociation rate constant of $k_d = (1.2 \pm 0.4)\ s^{-1}$.

Table 1 compares our results obtained by SMFS with data on $[Cu(bpy)]^{2+}$ complexes acquired by the temperature-jump or the stopped-flow methods.[12–15] The stability constant of

Table 1: Comparison of the kinetic rate constants for $[Cu(dcbpy)]^{2+}$ determined by time-resolved SMFS and $[Cu(bpy)]^{2+}$ reported in the literature.

Method	k_a [$\times 10^6\,M^{-1}s^{-1}$]	k_d [s^{-1}]	K_S [$\times 10^6\,M^{-1}$]
Stern–Volmer analysis[a]	–	–	4.2 ± 0.3
single-molecule experiments[b]	3.3 ± 0.3	1.2 ± 0.4	2.7 ± 0.9
literature[c]	50	0.09	500

[a] See the Supporting Information. [b] Ligand: dcbpy; $K_S = k_a/k_d$. [c] Ligand: bpy; k_a and k_d were determined by the temperature-jump method at low pH value,[9] K_S by spectrochemical titration.[10]

$(2.7 \pm 0.9) \times 10^6\,M^{-1}$ from single-molecule data is close to the quenching constant K_{SV} of $(4.2 \pm 0.3) \times 10^6\,M^{-1}$ obtained from Stern–Volmer analysis (see the Supporting Information). For a comparison of the kinetic rate constants k_a and k_d and the complex stability constant K_S with literature data, both the electron-withdrawing effect of the carboxylic groups (decreasing basicity of the ligand) and conjugation to oligo-DNA (possibly leading to intramolecular H-bonding and hydrophobic interactions of the free ligand) have to be taken into account. Hence, the corresponding $[Cu(bpy)]^{2+}$ complex is expected to be more stable than the $[Cu(dcbpy)]^{2+}$ derivative used in our experiments. Conformational dynamics related to the C_6 linkers and the oligonucleotides are expected to take place on a much faster timescale than metal exchange. This notion is supported by recent studies on conformational changes in DNA hairpins that have shown that a single base pairing introduces a fast dynamic component on the order of 10^{-7} s.[16] Taking into account that the formation of a GC base pair with three hydrogen bonds is expected to be much stronger than the Cu^{2+}–TMR coordination, interference between the observed Cu^{2+} binding dynamics and conforma-

tional changes in TMR or the dcbpy side group seems quite improbable.

By probing the presence of the $[Cu(dcbpy)]^{2+}$ complex and by quantitatively investigating its underlying association and dissociation dynamics in thermodynamic equilibrium, we have shown that it is possible to transfer concepts of single-molecule spectroscopy to coordination chemistry in solution.

We have recently observed in ensemble studies that fluorophore quenching by $[Cu(bpy)]^{2+}$ depends on the availability of free coordination sites at the metal ion and fluorescence is restored in the presence of coligands which bind to the remaining free sites of the metal ion.[8] This result offers the intriguing perspective of following individual reaction steps that are promoted by an immobilized M(L) fragment—including substrate binding and conversion, dissociation of reaction product, and coligand-dependent modulation of reactivity—with a time resolution of better than 1 μs by standard SMFS instrumentation at the single-molecule level.[5] Since fluorescence quenching also often depends on the oxidation state of a metal ion, such studies might be readily extended to metal-centered redox processes. Valuable information on the elementary steps of metal-promoted reactions might be gained by using substrates which alter their fluorescence intensity or emission wavelength when binding to or reacting at the metal site, as described recently for the $[Cu(bpy)]^{2+}$-catalyzed cleavage of fluorogenic carboxylic esters.[17,18]

Once established, such a set of methods should give completely new insights into the reaction dynamics of small-molecule coordination compounds, including metal-centered homogeneous catalysis.

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[1] P. Tinnefeld, M. Sauer, *Angew. Chem.* **2005**, *117*, 2689 – 2728; *Angew. Chem. Int. Ed.* **2005**, *44*, 2642 – 2671.
[2] D. A. Vanden Bout, W. T. Yip, D. Hu, D. K. Fu, T. M. Swager, P. F. Barbara, *Science* **1997**, *277*, 1074 – 1077.
[3] Y. J. Lee, D. Y. Kim, J. K. Grey, P. F. Barbara, *ChemPhysChem* **2005**, *6*, 2404 – 2409.
[4] H. Uji-I, S. M. Melnikov, A. Deres, G. Bergamini, F. De Schryver, A. Herrmann, K. Müllen, J. Enderlein, J. Hofkens, *Polymer* **2006**, *47*, 2511 – 2518.
[5] P. Tinnefeld, D. P. Herten, M. Sauer, *J. Phys. Chem. A* **2001**, *105*, 7989 – 8003.
[6] C. Hellriegel, J. Kirstein, C. Bräuchle, *New J. Phys.* **2005**, *7*, 23.
[7] M. B. J. Roeffaers, B. F. Sels, H. Uji-i, F. De Schryver, P. A. Jacobs, D. E. De Vos, J. Hofkens, *Nature* **2006**, *439*, 572 – 575.
[8] J. Brunner, R. Krämer, *J. Am. Chem. Soc.* **2004**, *126*, 13626 – 13627.
[9] A. Mokhir, A. Kiel, D. P. Herten, R. Krämer, *Inorg. Chem.* **2005**, *44*, 5661 – 5666.
[10] K. D. Weston, L. S. Goldner, *J. Phys. Chem. B* **2001**, *105*, 3453 – 3462.
[11] O. Piestert, H. Barsch, V. Buschmann, T. Heinlein, J. P. Knemeyer, K. D. Weston, M. Sauer, *Nano Lett.* **2003**, *3*, 979 – 982.
[12] I. Fabian, H. Diebler, *Inorg. Chem.* **1987**, *26*, 925 – 928.
[13] H. Diebler, *Ber. Bunsen-Ges.* **1970**, 74, 268 – 276.
[14] R. H. Holyer, C. D. Hubbard, S. F. A. Kettle, R. G. Wilkins, *Inorg. Chem.* **1965**, *4*, 929 – 935.
[15] T. S. Roche, R. G. Wilkins, *J. Chem. Soc. D* **1970**, 1681 – 1682.
[16] J. Kim, S. Doose, H. Neuweiler, M. Sauer, *Nucleic Acids Res.* **2006**, *34*, 2516 – 2527.
[17] R. Kierat, R. Krämer, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4824 – 4827.
[18] J. Brunner, A. Mokhir, R. Krämer, *J. Am. Chem. Soc.* **2003**, *125*, 12410 – 12411.

