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Spatial pH Jump Measures Chemical Kinetics in a Steady-State System

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We measure chemical kinetics in a steady-state solution where we create a microscopic open region with conditions different from the bulk. Individual reactant molecules spontaneously diffuse through this "reaction volume". We measure the changes which take place within their short residence time in the volume. The advantage of this approach is that the time resolution is limited only by the residence time τ_D of the molecules in the reaction volume (which can easily be $<50\ \mu$s), while the time taken to average the data can be arbitrarily long. In addition, if the chemical changes are reversible, the system is always in a steady state, and no replenishment of the reactants is necessary. Also, the total specimen volume required can be very small ($<20\ \mu$L). We demonstrate the scheme by measuring the protonation induced changes of the fluorescence properties of fluorescein. We first show that a pH jump of >1 unit can be achieved by multiphoton excitation of ortho-nitro benzaldehyde (*o*-NBA). We then perform fluorescence correlation spectroscopy (FCS) to show that the residence time τ_D of fluorescein in this low-pH region is $\sim 30\ \mu$s. Subsequently, we use time correlated single photon counting (a widely used probing technique with an inherently long averaging time), and show that the data can be averaged for an arbitrarily long time, yet it captures the fluorescence lifetime of the low-pH species which exists only for the short time τ_D. Finally, we show that the time resolution can be tuned by over 3 orders of magnitude, by changing the focal volume and by changing the viscosity of the solution. The latter experiment also shows that small chemically induced changes in the fluorescence lifetime can be resolved by our technique.

The kinetics of a fast chemical process is usually studied by rapidly switching the chemical conditions of a system, and measuring its rate of approach to equilibrium. As an alternative, we measure the kinetics in a steady-state solution where we create a microscopic open region with conditions different from the bulk. Individual reactant molecules spontaneously diffuse through this "reaction volume", and change their properties accordingly. We selectively probe these molecules, and measure the changes which take place within their short residence time in the volume.

This approach has several advantages. The time resolution is limited only by the residence time τ_D of the molecules in the reaction volume (which can easily be $<50\ \mu$s), while the time taken to average the data can be arbitrarily long. In addition, if the chemical changes are reversible, the system is always in a steady state, and no replenishment of the reactants is necessary. Also, for the optical technique employed here, the total specimen volume required can be very small ($<20\ \mu$L). Moreover, the reaction can take place far away from any surfaces, enabling the study of surface-sensitive reactions. These features make it rather attractive for studying a range of phenomena, such as protein folding or protein aggregation.

In the results presented here, focused and continuous multiphoton excitation of a proton donor helps create a small open reaction volume with a pH lower than the bulk.[1] The same light beam also allows the interrogation of the reactant molecules exclusively from this reaction volume. The measurement therefore reports whatever chemical changes that can occur in the reactant molecules within the time τ_D of a pH jump. τ_D can be tuned, as required, over a large range by altering the focus parameters and/or the viscosity of the solution.

We demonstrate the scheme by measuring the protonation induced changes of the fluorescence properties of fluorescein. We first show that a substantial pH jump can be achieved by continuous multiphoton excitation of ortho-nitro benzaldehyde (*o*-NBA), by using the fluorescence of fluorescein as a pH calibrant. We then perform fluorescence correlation spectroscopy (FCS)[2−4] to show that the residence time τ_D of fluorescein in this low-pH region can be very short. Subsequently, we use time correlated single photon counting (TCSPC, a widely used probing technique with an inherently long averaging time), and show that the data can be averaged for an arbitrarily long time, yet it captures the fluorescence lifetime of the low-pH species which exists only for the brief time period τ_D. Finally, we show that the time resolution can be tuned by over 3 orders of magnitude, by changing the focal volume and by changing the viscosity of the solution. The latter experiment also shows that small chemically induced changes in the fluorescence lifetime can be resolved by our technique.

In order to calibrate the pH jump achieved by photoexcitation, we first record the steady-state fluorescence of 100 μM fluorescein as a function of pH in an aqueous medium containing 12 mM *o*-NBA (Figure 1, open squares). We use 488 nm

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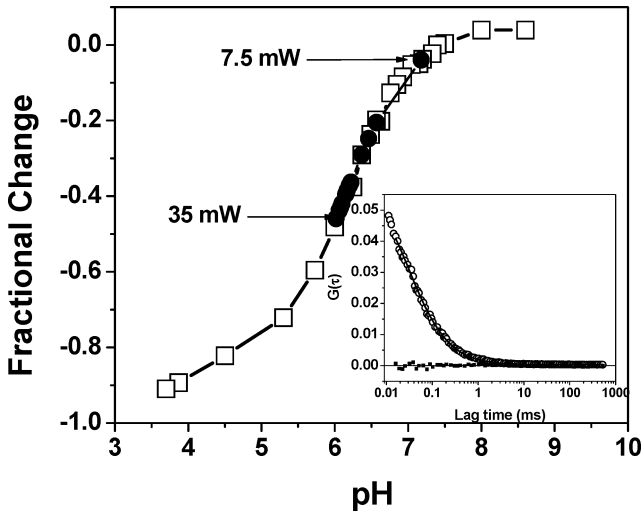

Figure 1. Steady-state fluorescence measurements of fluorescein. Fractional change in fluorescence counts of 100 μM fluorescein as a function of pH ranging from 3.7 to 10.4 (open squares). The change is calculated using fluorescence at pH 7.4 as the base value. Measurements from the pH jump experiment (using 12 mM of *o*-NBA as the proton donor) are plotted at different excitation powers, ranging from 7.5 to 35 mW (filled circles). Inset: Calibration of the probe volume using FCS. FCS trace of 150 nM fluorescein in aqueous solution excited with a 740 nm multiphoton beam (open circles). The data are fitted to a single species 3D diffusion model with $\tau_D = 30$ μs (solid line). The residuals are shown as filled squares.

excitation in a fluorimeter, which excites only fluorescein and not *o*-NBA. The observed titration curve (Figure 1, open squares) is in good agreement with that reported previously for fluorescein in aqueous solutions.[1,5−7] The pK_a value estimated from our result is 6.1 and is close to the value of 6.2 reported earlier.[7]

The *o*-NBA−fluorescein mixture is then irradiated with a mode-locked infrared laser (wavelength 740 nm, pulse-width 130 fs, repetition rate 76 MHz) using a 60× water dipping objective lens (Nikon, Japan). This excites both *o*-NBA and fluorescein via a two-photon mechanism. Excited *o*-NBA releases protons[1] and is expected to lower the pH of the reaction volume in an intensity-dependent manner. The fractional difference of fluorescein fluorescence between the specimen containing the *o*-NBA and a control specimen (without *o*-NBA) is calculated, as the excitation power is varied from 7.5 to 35 mW. The fluorescence counts from an *o*-NBA containing sample were corrected separately for static quenching by *o*-NBA, which is calculated from steady-state fluorimetry data. The fractional changes are mapped directly onto the calibration curve in Figure 1 (filled circles), and provide a measure of the local pH change in the excitation volume. We estimate that the pH changes from 7.2 to 6.1 as the excitation power is increased from 7.5 to 35 mW.

To verify whether the observed change of fluorescence is indeed caused by a lowering of the pH, we repeat the same experiment with 2 mM phosphate-citrate buffer present in the solution. This is expected to buffer the protons released by *o*-NBA and resist any pH change. No change in the fluorescence is observed (Supporting Information Figure 1, open squares). Separately, experiments are repeated using 920 nm of excitation light, where *o*-NBA is not excited but fluorescein is. Again, no change in the fluorescence is observed (Supporting Information Figure 1, filled squares). The experiments are also performed using rhodamine-B as a fluorescent reporter. Rhodamine-B fluorescence is not sensitive to pH in this pH region. In this case also, no change in fluorescence is observed between the control and the *o*-NBA containing rhodamine-B solution (Supporting Information Figure 1, filled circles). These results show that the observed change in the fluorescein fluorescence is indeed due to a change in the pH caused by the protons released from the photoexcited *o*-NBA.

We then measure the time resolution of the experiment which is given by the average residence time of a fluorescein molecule in this low-pH region. We employ fluorescence correlation spectroscopy (FCS) to measure this quantity. We perform FCS of a 150 nM fluorescein solution using the same multiphoton excitation (Figure 1 inset, open circles). The correlation trace is fitted with a single diffusing component[8] assuming a three-dimensional Gaussian excitation volume (Figure 1 inset, solid line), and yields an average residence time of 30 μs. This sets the basic time resolution for our experiment. Since the residence time varies as the hydrodynamic radius of the molecule, we can have a time resolution of about 100 μs even for measuring the protonation kinetics of a much more massive molecule (such as a green fluorescent protein). We note that, at these power levels for two-photon excitation, photobleaching and sample heating are negligible. In general, deviation of the autocorrelation curve from the diffusional model fit can serve as a good indicator for photobleaching artifacts. We do not observe any such deviations here (Figure 1, inset).

One important aspect of the method is that the time available to record the data is independent of the time resolution of the experiment. We show this by measuring the lifetime of fluorescein in the low-pH reaction volume with the TCSPC technique. Lifetime is a useful parameter for gauging the changes in the local environment of a fluorescent probe, but TCSPC can take on the order of a second of averaging time to yield meaningful data. It is therefore difficult to employ TCSPC to probe species that exist for much less than 100 ms. In our experiment, we measure the lifetime of fluorescein in the probe volume at several excitation powers, using a data averaging time of 2 min (data shown for a representative excitation power of 25 mW, Figure 2, inset). The fluorescence lifetime at pH 7 is 3.0 ± 0.1 ns (see the fit in Figure 2, inset), and is found to be independent of the excitation power. This null result is expected as the lifetime of fluorescein does not change as a function of pH in aqueous medium.[9,10] The quantum efficiency of the protonated species is low in water, and hence, it does not influence the observed lifetime. However, our two-photon excitation scheme ensures that the detected lifetime indeed belongs to fluorescein molecules inside the low-pH region (a species that exists only for 30 μs as verified by the FCS measurements). The quantum efficiency of the protonated species is higher in other solvents or in the presence of buffers, and the lifetime does change as a function of pH.[9,10] We can indeed observe these changes in a 50% glycerol−water mixture, as shown later.

We then show that the time scale of observation can be tuned over a large range, without substantially affecting the amplitude of the pH jump, by adjusting the solution viscosity and/or the focus parameters. Under strong illumination, the pH is sensitive only to the ratio of the τ_D of protons and the τ_D of *o*-NBA in the probe volume and is thus expected to remain approximately the same (see the Supporting Information). This feature allows us to follow slower kinetic events. To show this independence, we repeat the pH jump experiment in a 50% glycerol−water solution (viscosity of 6 cP), using a 20× objective lens for photoexcitation. The experimental probe volume in this case is very different from the earlier experiment, and FCS measurements show that the residence time of fluorescein in the probe volume is now 50.0 ± 3.3 ms (Supporting Information Figure 2,

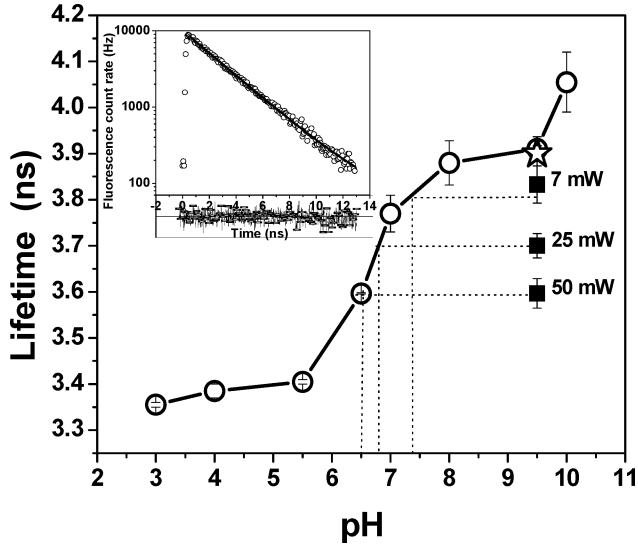

Figure 2. Fluorescence lifetime measurements. Fluorescence lifetime of 100 μM fluorescein as a function of various pHs ranging from 3 to 10 (open circles). Similar measurement from the pH jump experiment using 12 mM of *o*-NBA (as a proton donor) is plotted at different excitation power (as marked in the figure), ranging from 7 to 50 mW (filled squares). The initial pH of fluorescein solution is 9.5. The pen star marks the lifetime of fluorescein at pH 9.5 in the absence of *o*-NBA. Inset: Fluorescence lifetime decay curve obtained using TCSPC. Fluorescence lifetime decay of 100 μM fluorescein at pH 7.0 (open circles), fitted to a single exponential decay (solid line). The residuals are shown at the bottom.

open circles). The fractional difference of fluorescence between the specimen containing *o*-NBA and a control (without *o*-NBA) is calculated, as the excitation power is varied from 7.5 to 35 mW. The fractional changes (Supporting Information Figure 3, open circles) are compared to that with the data obtained previously without glycerol (Supporting Information Figure 3, filled circles), and confirms our expectation.

This glycerol−water solution also demonstrates that we can measure the changes of fluorescein lifetime upon protonation. The lifetime in the absence of *o*-NBA is 3.9 ± 0.02 ns (Figure 2, open star). The presence of *o*-NBA, if it is not excited, does not affect the lifetime. This is observed in a separate experiment using single-photon excitation of fluorescein at 488 nm, where *o*-NBA is not excited (Figure 2, open circle at pH 9.5). Also, the addition of *o*-NBA changes the steady-state fluorescence intensity only by 8 ± 2.5%, indicating the absence of substantial quenching of fluorescein by *o*-NBA in glycerol solution. However, the lifetime of fluorescein in this solution is sensitive to pH change, as summarized in Figure 2 (open circles). At pH 9.5, the lifetime is 3.9 ± 0.1 ns, as expected, and does not change substantially until pH 8. However, there is a sharp change in lifetime between pH 7 and pH 5.5. Below this pH, the lifetime value does not change significantly and remains 3.4 ± 0.1 ns. This observation is also consistent with the steady-state fluorimetry data (Supporting Information Figure 3, open circles).

We expect the spatial pH jump experiment to report this protonation-dependent change in the fluorescein lifetime. The lifetime of this glycerol−water solution (with a starting pH of 9.5) is recorded under several different powers of two-photon excitation, as shown in Figure 2 (filled squares). Indeed, we observe a significant drop in the fluorescence lifetime of fluorescein. The lifetime is about 3.83 ± 0.04 ns at 7 mW of excitation power, which further reduces to 3.59 ± 0.03 ns at 50 mW. In specimens which do not contain *o*-NBA, the fluorescence lifetime remains close to 3.92 ± 0.03 ns over this range of excitation powers (data not shown). We compare these values with those obtained with single-photon excitation (at 488 nm) at different bulk pH values (Figure 2, open circles). The calibration curve shows that we can successfully change the pH of the reaction volume from pH 9.5 to 6.3 using 50 mW of excitation light. Similar experiments are also repeated using a starting pH of 7. The lifetime changes from 3.8 ± 0.06 to 3.56 ± 0.08 ns, suggesting a pH jump from pH 7 to 6.1 (data not shown), which is consistent with our steady-state fluorescence data (Supporting Information Figure 2, open circles). We conclude that our scheme faithfully reports the protonation induced changes in the fluorescence lifetime of the molecules which enter the reaction volume.

Here, we have demonstrated a spatial pH jump, but we can in principle use other photoinducible changes in such experiments (such as a temperature jump or a change in the concentration of any photouncageable reagent). Our scheme has a time resolution comparable to the best fluid mixing experiments (stopped flow or continuous flow[11,12]) and allows indefinite data averaging times in a steady-state setup. In an elegant series of hydrodynamic focusing experiments using semiconductor nanostructures, Austin and co-workers demonstrated a mixing time resolution of less than 10 μs.[13] However, our scheme effectively creates such nanostructures using light, and therefore avoids chemical contact with nearby surfaces and does not require any flow apparatus. The laser induced pH-jump and T-jump experiments can easily achieve sub-μs time resolutions,[14−16] but for probing techniques with long averaging times (such as TCSPC), the effective time resolution becomes much longer. Also, our scheme can have a sub-fL reaction volume, and thus uses much less material. Our scheme bears a certain analogy to the FCS method, which can also determine the chemical kinetics in a similar steady-state experiment.[2,4] However, unlike FCS, the signal amplitude does not have to depend on the rare spontaneous chemical fluctuations coming from a restricted number of molecules in the probe volume. We believe these features present unique advantages for following the fast kinetics of pH or temperature induced reactions such as protein folding.

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Supporting Information Available: Supporting Information about the material used, experimental techniques, and supplementary results. This material is available free of charge via the Internet at http://pubs.acs.org.

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