Real-time Monitoring of Photoinduced pH-jumps by In Situ Rapid-scan EPR Spectroscopy

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Abstract

This work represents the first demonstration of a light-induced pH-switchable process monitored by *in situ* rapid-scan (RS) electron paramagnetic resonance (EPR) spectroscopy on the millisecond timescale. Here, we investigate the protonation state of an imidazoline type raical as pH sensor under visible light irradiation of a merocyanine photoacid in bulk solution. The results highlight the utility of photoacids in combination with pH-sensitive spin probes as an effective tool for real-time investigation of biochemical mechanisms regulated by changes of the pH value.

Introduction

One of the key physiological parameters in life science is the pH value. The pH regulates enzyme activity, transport of substances, ensures the stability of biomolecules and significantly influences the folding and functionality and of proteins. Typical spectroscopic methods employed for sensing pH dependent mechanisms on the subsecond timescale are fluorescence spectroscopy,[1] Fourier-transform infrared spectroscopy[2] or time resolved nuclear magnetic resonance (NMR) spectroscopy.[3] Electron paramagnetic resonance (EPR) spectroscopy, however, is more sensitive than NMR and provides complementary insights into biological systems and their interactions that are not readily accessible though other spectroscopic methods. A fundamental problem of using EPR for real-time pH determination is the restricted excitation bandwidth of pulsed measurements and the limited time resolution of traditional continuous wave (CW) experiments. Acquisition of a CW EPR spectrum often takes several minutes but can also take longer depending on the system under study, the spin concentration, or the required signal-to-noise ratio. Rapid-scan (RS) EPR, on the other hand, substantially improves the signal-to-noise ratio and time resolution. More specifically, it allows much higher microwave powers to be applied before saturating the spin system while being largely unaffected by bandwidth related constraints.[4] Rapid-scan is thus well suited to resolve spectral changes of the EPR resonance line accompanied by switching the pH with photoacids.

Photoacids are molecules that release protons upon illumination, lowering the pH value of aqueous solutions.[5] Light as an external trigger offers properties that are important to perform successful time-resolved RS experiments for biological studies. Light can be used with high spatial and temporal control, is not harmful to proteins at wavelengths above 300 nm, and unlike titration (with acids and bases) does not alter the sample volume or resonator tuning.

A metastable photoacid that switches with visible light is protonated merocyanine. Merocyanine and its derivatives are among the most widely studied photoacids allowing reversible and pronounced pH jumps from basic to acidic environments. They exhibit fast response times with proton transfer reactions on the subsecond timescale making them ideal for studies of fast biochemical dynamics.[6]

Monitoring of the pH jump upon proton release is accomplished by addition of pH-sensitive nitroxides. In particular, nitroxides of the imidazoline type have been extensively studied for pH determination.[7] Imidazoline nitroxides are stable,[8] both chemically and thermally, highly sensitive and cover an exceptionally wide pH range.[9]

Taken together, photoacid, pH-sensitive nitroxide and rapid-scan EPR spectroscopy form an excellent toolbox for studying various biochemical processes regulated by changes of the pH value.

This paper describes the most important characteristics of the pH-sensitive nitroxide and photoacid used in this work; provides details about the experimental setup, the synthesis of the required chemicals as well as data processing. We present first measurements of *in situ* RS-EPR pH monitoring and demonstrate how the pH of the solution can be controlled by changes of the sample composition or laser emission.

Characterization of the pH-sensitive nitroxide and photoacid

The structure of the imidazoline type nitroxide used for pH monitoring in this study is shown in Figure 1a. Its functionality as a probe for pH values is well understood[10] and we will therefore only briefly discuss its most important characteristics.

Imidazoline type nitroxides have a second nitrogen atom in the imidazole ring that can be

protonated depending on the pH of the solution. Protonation of the nitrogen at lower pH leads to a shift of the spin density towards the oxygen atom of the NO group which consequently lowers the isotropic hyperfine splitting (Figure 1c).

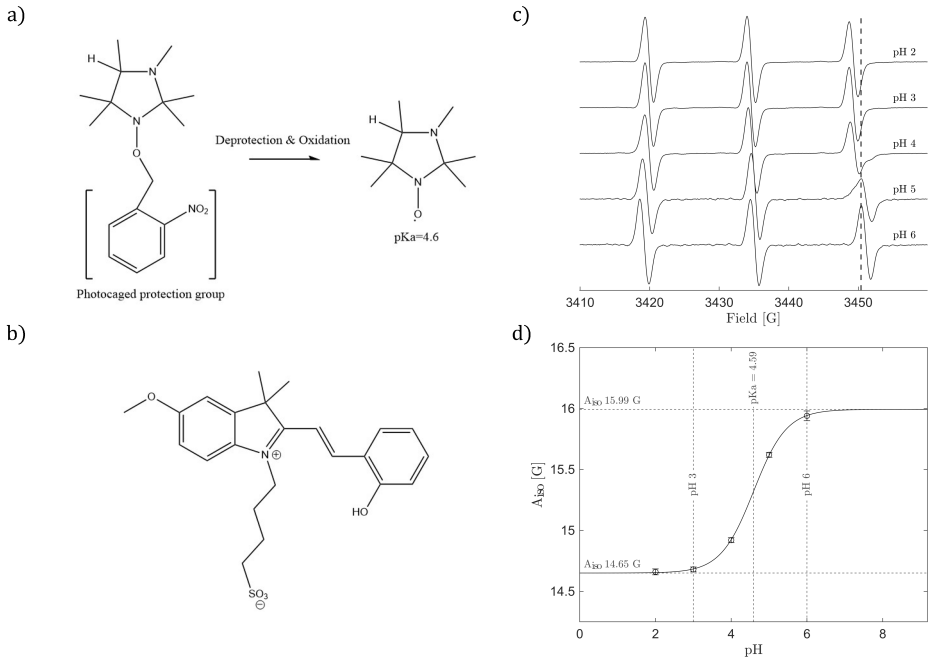

Figure 1: a) pH sensitive nitroxide before and after cleavage of the photoprotecting group. b) Photoacid. c) Titration experiment: CW EPR spectra taken at different pH values. Top to bottom: pH 2, 3, 4, 5, 6. d) pH dependence of the isotropic hyperfine splitting measured as the distance between the low and central-field components (circles). Fit to the Henderson-Hasselbalch titration curve (solid line).

Hence, these nitroxides have two spectroscopically distinguishable protonated and unprotonated forms. The two different hyperfine splittings are most prominent in the high field peak of X and L-Band spectra which prevails over shifts in their g-factors that would become apparent at higher microwave frequencies. Spectra at intermediate pH values are comprised of a superposition of the acidic and basic form of the nitroxide (Figure 1d) with splittings well described by the Henderson-Hasselbalch (Equation 1) equation

$$A_{iso}(pH) = \frac{A(R)10^{(pH-pK_a)} + A(RH^+)}{1 + 10^{(pH-pK_a)}} \tag{1}$$

where $A(R)$ and $A(RH^+)$ are the isotropic hyperfine coupling constants for the non-protonated basic and protonated acidic form of the nitroxide. The isotropic hyperfine splitting determined for the nitroxide used in this work is approximately 1.3 G making the spin probe suitable as a pH indicator over the range of about three pH units centered at a pK_a of 4.6. Figure 1b depicts the structure of the merocyanine photoacid (MCH) in its protonated from. MCH is a metastable photoacid with exceptional bulk pH switching properties allowing large tunable, reversible but long-lived pH jumps up to 3.5 units.[11]

Materials and methods

MCH was synthesized as previously described.[11] The pH-sensitive nitroxide comprising a photocaged protecting group was purchased from Enamine Ltd. Cleavage of the ortho-nitrobenzyl group with subsequent oxidation of the EPR silent hydroxylamine to the desired radical is achieved after two minutes of irradiation under a 8 W UV hand lamp at 302 nm. CW EPR titration experiments (Figure 1) were carried out on an EMXnano benchtop X-Band spectrometer (Bruker) using a set of standard buffer solutions purchased from ROTICalipure. All samples contained 19 μl of the buffer solution and 1 μl of the pH-sensitive nitroxide taken from a 10 mM stock solution in DMSO giving a final nitroxide concentration of 500 μM. Five CW EPR X-Band spectra were collected for each pH value with a modulation amplitude of 1 G, a microwave power of 1 mW, 100 G sweep width and 60 s acquisition time. Averaging of the recorded spectra is used to minimize deviations in the sample composition during titration.

Rapid-scan experiments were conducted with the Bruker rapid-scan accessory on an Elexsys spectrometer at X-Band, ambient temperatures and a microwave power of 20 mW. A 100 G sinusoidal scan modulated with 10 kHz is used to acquire the transient spin response with either 100 ms, 1 s or 5 s time resolution. Activation of the photoacid is carried out with a diode pumped Nd:YAG laser (EKSPLA) operating at 450 nm and a fixed repetition rate of

50 Hz. Samples (composition see below) are irradiated with the optical fiber directly coupled into the resonator from above.

Spectra are background corrected with the Xepr-software provided by Bruker or with the algorithm (based on non-quadratic cost functions) recently developed.[12] Data post-processing further included the combination of both half-cycles of the rapid-scan period and the Kramer-Kronig relation to add the corresponding absorption spectra calculated from the in-phase component of the incident microwave.[13] Deconvolution is used to preserve accurate lineshapes although no passage effects were observed.[14] The signals are finally smoothed by convolution with a Gaussian function whose full width at half maximum was set to 0.5 G.

Results and discussion

Activation of the photoacid (MCH) leads to a sudden proton release and subsequent ionization of the pH-sensitive nitroxide. CW EPR experiments allow to determine the shift in the isotropic hyperfine splitting at constant pH values (Figure 1d), but cannot cover the overall process of ionization in a time resolved manner. The recorded titration curve, however, can be used as a calibration for time-resolved rapid-scan measurements. This allows the pH value to be determined from the splitting of the nitroxide peaks in reverse.

Protonation of the nitroxide is well resolved by acquiring RS spectra with 1 s time resolution (Figure 2a). As a first demonstration, we aimed to induce the pH jump right at the turning point of the titration curve, where sensitivity is highest. For this, the initial pH value was adjusted to 5.4 by adding a small amount of NaOH before irradiation.

The light-induced proton transfer reaction to the nitroxide radicals is clearly evident. Extraction of slices along the magnetic field axis allows accurate determination of the point in time where the protonated and deprotonated forms are present at equal concentrations. This is readily apparent from the high-field peak that after the onset of laser irradiation, separates into two distinct slopes (Figure 2b) revealing the precise hyperfine splitting of the

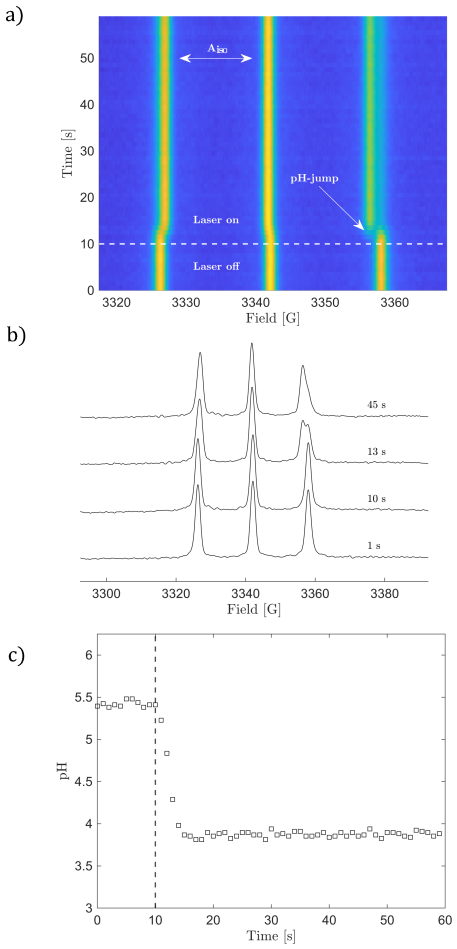

Figure 2: Time-resolved rapid-scan experiment. a) pH jump and corresponding decrease in the hyperfine splitting of the pH-sensitive nitroxide radical. Signal intensities are color-coded (view from above). A 100 G sinusoidal scan modulated at 10 kHz is used to acquire spectra with 1 s time resolution. The laser was switched on after 10 s measuring time (energy per pulse 5.8 mJ). b) Slices along the magnetic field axis. Bottom to top: 1 s, 10s, 13 s, 45 s. c) pH Readout. Sample composition: 12.5 μl MilliQ-water, 1 μl NaOH (10 μM), 1.5 μl nitroxide (10 mM in DMSO), 5 μl merocyanine (10 mM in DMSO).

two forms of the radical. The distances between the central peak of the triplet and the two observable slopes agree very well with the two splittings of 14.6 G and 16 G already known from the CW titration curve. The corresponding pH values are read out from the splitting between the low and the center field peak (Figure 2c).

The excellent time resolution of RS EPR spectroscopy allows to capture the process of ionization also on the millisecond time scale (Figure 3). Here, the decreasing isotropic hyperfine splitting is observed as a smooth shift rather than a sudden transition.

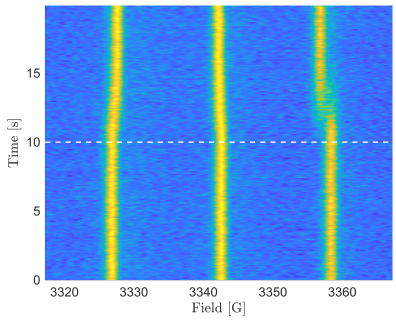

Figure 3: pH jump monitored by recording spectra with 100 ms time resolution. The on switching of the laser is indicated as horizontal dashed line. Sample composition and laser energy: 13.5 μl MilliQ-water, 1.5 μl nitroxide (10 mM in DMSO), 5 μl merocyanine (10 mM in DMSO), 5.8 mJ per pulse.

We now demonstrate that the pH jump is reversible with thermal recovery on the timescale of minutes and that the solution's pH can be maintained under constant irradiation. Precise regulation of the proton transfer reaction is accomplished either by the sample's composition or the energy of the laser.

Activation of the photoacid followed by thermal recovery is depicted in Figure 4a. Switching off the laser after short term exposure of the sample results in the photoacid slowly returning to its stable isomer. Since protonation of the nitroxide is reversible as well, this process is coupled with a gradual increase of the hyperfine splitting. In contrast, splitting of the triplet remains constant under continuous irradiation (Figure 4b), i.e. the solution's pH stays unchanged.

The time evolution of the isotropic hyperfine splitting is well captured by a logistic decay which allows to determine the rate of the reaction kinetics under variable experimental conditions (Figure 4c). Lowering the energy of the laser visibly decelerates the pH jump but does not delay the onset of proton release. Reducing the concentration of the photoacid

ultimately leads to fewer protons being released into the solution, i.e. a smaller pH jump. The energy of the laser and the sample's composition therefore allow the pH value of the solution to be precisely controlled.

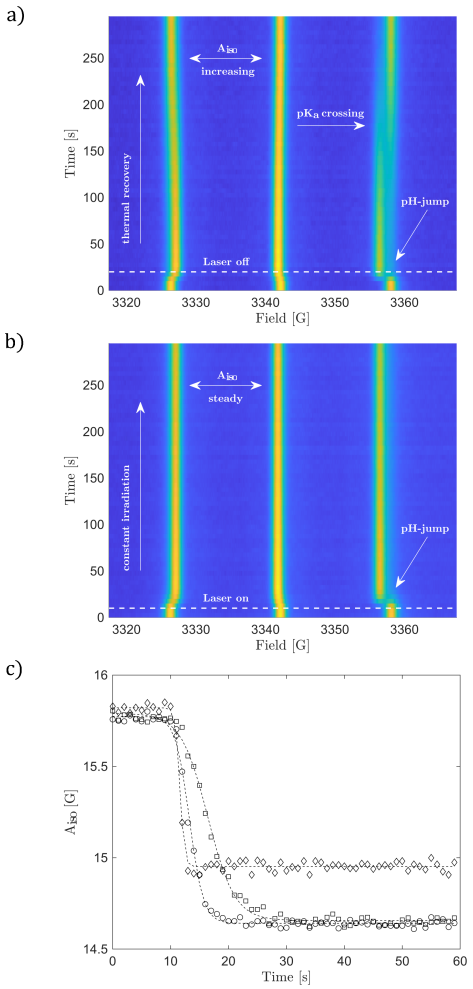

Figure 4: a) pH jump and thermal recovery. Only the switching off of the laser (after 10 s of exposure) is indicated for illustration purposes. b) Constant irradiation after 10 s of measuring time. Sample composition and laser energy: 13.5 μl MilliQ-water, 1.5 μl nitroxide (10 mM in DMSO), 5 μl merocyanine (10 mM in DMSO), 1.4 mJ per pulse. c) Influence of the laser's energy and photoacid concentration 3.2 mJ (circles), 1.4 mJ (squares), 5.8 mJ with only 2.5 μl of the photoacid (diamonds). Determined time constants from fitting the data to a logistic decay: 0.4 s (circles); 0.8 s (squares); 2.7 s (diamonds).

Concluding remarks

Photoacids offer spatial and temporal control of the pH, making them extremely attractive for non-invasive fast and well synchronized time-resolved biology studies. Activation of the photoacid accompanied with proton release and subsequent ionization of the pH-sensitive nitroxide can be monitored in real-time by *in situ* RS EPR spectroscopy. The pH of the solution can be determined without delay from the hyperfine splitting of the nitroxide.

Constant laser irradiation maintains the pH of the solution, with the possibility to precisely control its value by adjusting the energy of the laser or photoacid concentration.

The kinetics studied here are well captured with a time resolution of seconds. RS EPR however, offers the opportunity of recording the proton transfer reaction on the millisecond timescale. The available time resolution will surely prove crucial for investigating biochemical processes through photo-induced pH jumps in the near future.

It is also worth mentioning that imidazolidine-type nitroxides exhibit tunable pK_a values covering a wide pH range by selection of various substituents on the nitroxide moiety. Notably, there also exist metastable photobases that function similar to photoacids: They donate OH^- ions and thus increase the solution's pH upon irradiation.[15]

In conclusion, the experiments presented open up entirely new perspectives to use spin probes for kinetic measurements. This work represents a novel area of research that offers great potential for studying biological systems with RS EPR spectroscopy.

Acknowledgement

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