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Electron Spin Relaxation Times and Rapid Scan EPR Imaging of pH-sensitive Amino Substituted Trityl Radicals

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Abstract

Carboxy-substituted trityl (triarylmethyl) radicals are valuable in vivo probes because of their stability, narrow lines, and sensitivity of their spectroscopic properties to oxygen. Amino-substituted trityl radicals have the potential to monitor pH in vivo, and the suitability for this application depends on spectral properties. Electron spin relaxation times T_1 and T_2 were measured at X-band for the protonated and deprotonated forms of two amino-substituted triaryl methyl radicals. Comparison with relaxation times for carboxy-substituted triarylmethyl radicals show that T_1 exhibits little dependence on protonation or the nature of the substituent, which makes it useful for measuring O_2 concentration, independent of pH. Insensitivity of T_1 to changes in substituents is consistent with the assignment of the dominant contribution to spin lattice relaxation as a local mode that involves primarily atoms in the carbon and sulfur core. Values of T_2 vary substantially with pH and the nature of the aryl group substituent, reflecting a range of dynamic processes. The narrow spectral widths for the amino-substituted triaryl methyl radicals facilitate spectral-spatial rapid-scan EPR imaging, which was demonstrated with a phantom. The dependence of hyperfine splittings on pH, is revealed in spectral slices through the image.

Introduction

Localized measurement of pO_2 and pH is crucial to understand normal and diseased states.^[1] It would be particularly helpful to have probes that can monitor both properties. Water-soluble triarylmethyl (TAM) radicals have been shown to be useful probes for EPR oximetry.^[2, 3] Synthetic methods have been developed for larger scale preparations of TAMs to facilitate in vivo applications.^[4] The narrow EPR signals and long T_1 and T_2 values of carboxy-substituted TAM radicals make them sensitive probes of local oxygen concentration.^[5-7] Carboxylic groups on TAMs make the EPR signal sensitive to pH.^[1] However, the pK_a of carboxylic groups is too low to be appropriate for monitoring pH in the physiological range. Replacement of one of the carboxylic groups by an amino group to form aTAM₄ or its selectively deuterated analog aTAM₅ (Figure 1) has been shown to result in probes with continuous wave (CW) spectra that are oxygen responsive and pH sensitive in the range of pH = 6 to 8.^[8] The relatively long electron spin relaxation times for carboxy-substituted TAMs trityl-CH₃ and Oxo63 (Figure 1)^[9] are advantageous for pulsed oximetry.^[10-13] To assess the potential utility of aTAMs for pulsed oximetry and pH

measurement, the pH dependence of the relaxation times for aTAM₄ and aTAM₅ were studied at X-band (9.4 GHz). T_1 was measured by saturation recovery and inversion recovery and T_2 was measured by pulsed electron spin echo decay. To demonstrate the feasibility of using these probes for EPR imaging, rapid scan images at 250 MHz were obtained for a phantom consisting of tubes containing aTAM₄ at pH 6.5 and 7.1.

Experimental

Samples

Preparation of aTAM₄ and aTAM₅ was performed at The Ohio State University using literature procedures.^[8] Weighed samples of aTAM₄ were dissolved in absolute ethanol and diluted with potassium phosphate (1.0 M) or tris (1.0 or 0.5 M) buffer in an 20:80 percent ratio (ethanol:buffer) to yield a final radical concentration of 0.1 mM. Samples of TAM₅ were prepared analogously using 0.5 M tris buffer. For X-band experiments solutions in thin-wall 0.97 mm i.d. Teflon tubing were placed inside 4 mm o.d. quartz tubes. For the CW and pulsed EPR experiments oxygen was removed by passing N₂ gas over the sample using a thin Teflon tube inserted parallel to the Teflon tube that contained the sample.

The phantom for imaging was constructed from two quartz tubes, separated by a 2 mm foam spacer. A 7 mm O.D. tube contained 0.5 mM aTAM₄ at pH 7.1 and a 6.1 mm O.D. tube contained 0.5 mM aTAM₄ at pH 6.5. The solvent was 20:80 ethanol: phosphate buffer (1.0 mM). Oxygen was removed by bubbling the solutions with N₂ prior to flame sealing the tubes.

X-band Spectroscopy

CW spectra were obtained at 295 K on a Bruker EMX spectrometer with an SHQ resonator. Microwave power was selected to be in the regime where signal intensity increases linearly with the square root of power. CW spectra were simulated with locally written programs and with WINSIM.^[8] The nitrogen and proton hyperfine splittings obtained by the simulations are in good agreement with the literature values.^[8] Inclusion of 7% of trityl-CH₃ (Figure 1) was required to match the relative intensities of the peaks in the spectra of aTAM₄.

Measurements of electron spin relaxation times at room temperature were performed on a locally-designed and built pulsed spectrometer^[14] equipped with a Bruker ER4118-X-MS5 split ring resonator and a 1 KW microwave amplifier. The resonator was over-coupled to $Q \sim 100$. Multifit, a locally written program based on the algorithms of Provencher,^[15] was used to determine T_1 or T_2 from the decay curves. T_2 was determined by two-pulse spin echo. For a sample of aTAM₄ in tris buffer, variation of the length of the 90° pulse from 40 ns to 160 ns caused no discernible change in T_2 . The values reported in Tables 1 to 3 were obtained with a 90° pulse length of 40 ns, which corresponds to a B_1 of 2.2 G. Longer pulses are more selective but result in poorer signal-to-noise. Field-swept echo-detected spectra were recorded with 90° pulse lengths of 160 ns, which corresponds to a microwave B_1 of 0.55 G, and a boxcar integration window of 0.5 μ s, to be field selective. T_1 was measured by inversion recovery. For aTAM₄ in tris buffer the value of T_1 decreased as the length of the 90° pulse was increased from 40 to 320 ns, which is attributed to spectral diffusion. Data reported in Tables 1 - 3 were obtained with a 90° pulse length of 40 ns. T_1 also was

measured by long-pulse saturation recovery on a locally-built spectrometer^[16] equipped with a Varian E231 resonator. The pump power was 12 dB below 600 mW, which is a B_1 of about 0.2 G and the observe power was 18 dB below 10 mW, which is a B_1 of about 10 mG. Recovery curves obtained with a 50 μ s saturating pulse could be fit well with a single exponential. Values of T_1 obtained by saturation recovery were systematically longer by about 1 μ s than values obtained by inversion recovery with a 90° pulse length of 40 ns. For these rapidly tumbling radicals it is unlikely that the difference between values measured by spin echo and saturation recovery is due to spectral diffusion. The difference is comparable to the estimated uncertainties in the values. The poorer signal-to-noise for the saturation recovery experiments may contribute to systematically longer values of T_1 .

Imaging parameters—The projections for the 2D spectra-spatial image were collected at 295 K on a spectrometer with an operating frequency of 251 MHz that includes a modified Bruker E540 console and gradient coils.^[17-19] The sinusoidal scans were generated with a driver similar to the one described previously.^[20] The cross loop resonator and scan coils are similar to the ones described in Ref. ^[21]. 18 projections were collected with 10.8 kHz sinusoidal scans and B_1 of 36 mG. The maximum gradient was 10 G/cm. The sweep widths ranged from 12 G at zero-gradient to 32 G at the highest gradient, which was sufficient to encompass the full gradient-broadened spectra. Signals were digitized with a Bruker Specjet II using 64k points per gradient and a 10 ns sampling interval which therefore encompassed 7.07 sinusoidal scan cycles within the digitizer time window. Each projection was averaged 49152 times which required 43s. The corresponding absorption signals were obtained by background correction,^[22] sinusoidal deconvolution,^[23] and combination of up-field and down-field scans. The image was reconstructed by filtered backprojection. To permit image reconstruction by filtered backprojection (FBP) the high-gradient spectra were ‘padded’ with zeros at the low- and high-field ends to generate projections with the widths required for spectral-spatial imaging.^[24]

Result and Discussion

CW spectra of aTAM₄ at pH 6.5, 7.6, and 8.5 (Figure 2A-C) and of aTAM₅ at pH 6.5, 8.2, and 9 (Figure 3) are pH dependent and in good agreement with spectra reported previously.^[8] Field-swept spin echo detection of the same samples provides the absorption spectra (Figure 2D-F), which are in good agreement with the first integrals of the CW spectra. Protonation of the amino group of aTAM₄ increases the coupling to the amino nitrogen from 0.69 G to 1.01 G and decreases the coupling to one of the protons on the CH₂ group from 2.04 to 1.05 G (Figure 1). The sample of aTAM₄ contained about 7% of the parent compound trityl-CH₃ which puts a sharp single line in the center of the spectrum (Figure 2). This signal provided an internal comparison between the properties of aTAM₄ and of trityl-CH₃. Because of the changes in the hyperfine coupling constants the contributions from the protonated and deprotonated species are well resolved in the high-field and low-field regions of the spectrum (Figure 2). In aTAM₅ the two protons of the methylene group that link the amino substituent to the aryl ring are deuterated, which reduces the corresponding hyperfine coupling constants to the methylene nuclei by about a factor of 6.^[8] The spectral width is therefore reduced, but more lines are resolved in the spectrum so deuteration does not simplify the spectrum. As discussed previously,^[8] the EPR

spectra at intermediate pH are the sum of contributions from the spectra of protonated and deprotonated radicals. Simulations can be used to find the coefficients of the two contributions, which can then be used to calculate the pH of the solution.^[8]

The values of T_1 for aTAM₄ exhibit little dependence on pH or position in the spectrum. Pulsed imaging of T_1 for Oxo63 is a powerful method for in vivo oximetry.^[25] The pH invariance of T_1 for TAMs suggests that oxygen concentration can be measured independent of pH using T_1 . T_2 for aTAM₄ and aTAM₅ are strongly dependent on pH, varying between 0.76 and 3.7 μ s (Table 1-3). Values of T_2 are consistently shorter for the deprotonated form than for the protonated form. When the radicals were dissolved in D₂O instead of H₂O, the values of T_2 for the deprotonated form became similar to that for the protonated form. Since the magnetic moment of a deuteron is 1/6 that of a proton, the increase in T_2 in D₂O indicates that water dynamics that modulates electron coupling to the H(D) contribute to the decrease in T_2 for the deprotonated form of the radical. In a prior study^[8] the lineshapes of CW spectra of a deoxygenated solution of deprotonated aTAM₄ found that the Lorentzian component had a peak-to-peak linewidth of 96 mG. If the Lorentzian component is assumed to correspond to the spin packet linewidth, the T_2 would be 0.68 μ s, which is shorter than the 0.76 μ s found by spin echo at pH = 8.5.

Values of T_1 for aTAM₄ and aTAM₅ (Table 3) are similar to values reported for trityl-CH₃ and Oxo63.^[26] This similarity is consistent with the assignment that the dominant contribution to T_1 for TAM radicals in solution at ambient temperature is a local mode.^[26] Replacement of a substituent on one of the aryl rings does little to change the local mode, which presumably is centered in the hydrocarbon and sulfur core of the radical. Values of T_2 vary substantially as a function of pH and structure of the substituents, which presumably reflects a range of dynamic processes occurring in solution. The dependence of T_2 on both pH and on oxygen concentration would make it more complicated to determine pH or oxygen concentration independently from T_2 . The substantial pH dependence of aTAM hyperfine splittings makes these spectral features the preferred metric for determining pH.

Rapid scan imaging of pH dependence—Because of the spectral dimension, an EPR spectral-spatial image constitutes a mathematical pseudo-object.^[27] The equivalent of rotation in the spectral dimension is achieved by changing the gradient amplitude. The maximum angle for a particular set of experimental constraints can be calculated using Eq. (1):^[28]

$$\alpha_{\max} = \arctan(LG_{\max}/\Delta B) \quad (1)$$

where L is the spatial length of the image, B is the spectral length of the image, and $G_{\max}$ is the maximum gradient. If B is too large, Eq. (1) shows that either $G_{\max}$ must be extremely large (which may result in poor signal-to-noise), or the length L of the reconstructed image must be artificially increased relative to the sample size; otherwise $\alpha_{\max}$ becomes too small relative to 90°. Missing angle algorithms can compensate for small decreases in $\alpha_{\max}$.^[29] For aTAM₄ the spectral length is about 8 G, which is much smaller than the ~ 40 G for nitroxide radicals. The narrower width is advantageous for spectral-spatial imaging. Even

with this narrow width, if the maximum gradient is 10 G/cm and $L = 2$ cm, $\alpha_{\max}$ would be 68° which would require many missing projections for the reconstruction. Instead, the $\alpha_{\max}$ was set at 85° which corresponds to $L = 9$ cm. The full image was reconstructed, but only the region of interest is displayed.

For a spectral-spatial EPR image, rapid scan has been shown to provide substantially higher signal-to-noise than CW for the same amount of data acquisition time.^[21] A 2D spectral-spatial image of a phantom consisting of two tubes of aTAM₄ at pH = 6.5 and 7.1 was reconstructed from projections acquired by rapid scan (Figure 4). Rapid scan EPR produces the absorption spectrum, rather than the first-derivative that is produced by conventional CW EPR. The spectral slices through the image reveal the absorption spectrum at positions in the sample, which are similar to the absorption spectra obtained by field-swept echo detection. The spectral differences between the probe at the two different pHs are well defined. Rapid scan EPR of trityls holds substantial promise for imaging pH with aTAM radicals.

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Figure 1.
Structures of trityl (TAM and aTAM) radicals.

Figure 2.

On the left, X-band CW spectra of aTAM₄ obtained with 10 kHz modulation frequency, 0.05 G modulation amplitude, 10 G sweep with 2048 points, 20.48 ms time constant and conversion time, 41 s sweep time, 0.318 mW incident power. (A) at pH 8.5, (B) at pH 7.5, and (C) at pH 6.5. On the right, field-swept echo detected spectra (D) at pH 8.5, (E) at pH 7.5, and (F) at pH 6.5. The positions in the spectrum where relaxation times were measured are marked as 'a' and 'b'. The line marked with 'b' has a contribution from about 7% of trityl-CH₃.

Figure 3.

X-band CW spectra of aTAM₅ obtained with 30 kHz modulation frequency, 0.1 G modulation amplitude, 5 G sweep with 1024 points 163.84 ms time constant and conversion time, sweep time 167.77 s, 0.318 mW power. (A) at pH 9.0, (B) at pH 8.2, and (C) at pH 6.4. The positions in the spectrum where relaxation times were measured are marked as 'a', 'b', and 'c'.

Figure 4. 2D spectral-spatial image (A) of a phantom consisting of two tubes of 0.5 mM aTAM₄. (B) Spectral slices through the image at the locations of the centers of the tubes with pH=6.5 (green) and pH=7.1 (blue).

Table 1
pH dependence of T_1 and T_2 for aTAM₄ at X-band⁺

Line (Fig. 2) [*]	pH	$T_1(\mu\text{s})$	$T_2(\mu\text{s})$ in H ₂ O	$T_2(\mu\text{s})$ in D ₂ O
b	6.5	18±0.3	2.9±0.1	
b	7.6	18±0.6	3.7±0.1	
b	8.5	18±0.5	3.6±0.2	3.5±0.1
c	6.5	15±0.6	3.2±0.3	
c	7.6	17±0.2	1.3±0.1	
c	8.5	17±0.8	0.76±0.1	2.7±0.1

⁺ Values in tris buffer obtained with 90° pulse lengths of 40 ns.

^{*} The positions in the spectrum where the relaxation times were measured are marked in Fig. 2. The signal designed as line 'b' is an overlap of contributions from aTAM₄ and about 7% impurity of trityl-CH₃.

Table 2
pH dependence of T_1 and T_2 values for TAM₅ at X-band⁺

Line (Fig. 3) *	pH	T_1 (μ s) in H ₂ O	T_1 (μ s) in D ₂ O	T_2 (μ s) in H ₂ O	T_2 (μ s) in D ₂ O
a	6.5	20 $\pm$ 0.1		3.6	
	8.2	20 $\pm$ 0.7	20 $\pm$ 0.3	1.5 $\pm$ 0.1	3.2 $\pm$ 0.1
a	9			1.5 $\pm$ 0.1	
b	6.5	20 $\pm$ 0.7		3.7 $\pm$ 0.1	
	8.2	21 $\pm$ 0.4	21 $\pm$ 0.1	1.5 $\pm$ 0.1	3.1 $\pm$ 0.2
b	9	21 $\pm$ 0.1		1.5 $\pm$ 0.1	
c	6.5	20 $\pm$ 0.4		3.6	
	8.2	20 $\pm$ 1.0	20 $\pm$ 0.3	1.5 $\pm$ 0.1	3.1 $\pm$ 0.1
c	9			1.5 $\pm$ 0.1	

⁺ Values in tris buffer obtained with 90° pulse lengths of 40 ns.

* The positions in the spectrum where the relaxation times were measured are marked in Fig. 3.

Table 3
Comparison of T_1 and T_2 for Trityls at X-band

Compound	T_1 (μ s)	T_2 (μ s)
Trityl-CH ₃ [26]	16	9.1
Oxo63 [26]	15	6.4
aTAM ₄ (line b)	18	2.9 to 3.7
aTAM ₄ (line c)	15 to 17	0.76 to 3.2
aTAM ₅ (line a)	20	1.5 to 3.6
aTAM ₅ (line b)	20	1.5 to 3.7
aTAM ₅ (line c)	20	1.5 to 3.6