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Immobilized Enzyme Electron Spin Resonance: A Method for Detecting Enzymatically Generated Transient Radicals

Bradley E. Sturgeon,* Yeong-Renn Chen, and Ronald P. Mason

Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

The study of enzymatically generated, transient radicals provides valuable information about radical reactivity as well as enzyme function. ESR methods to detect transient radicals are generally based on continuous flow and have the potential to consume large quantities of enzyme, substrate, and buffer. Experimental approaches have been pursued to minimize sample volumes, although none have made the continuous-flow ESR approach generally applicable for enzymes and substrates available in limited quantities. We have developed an alternative approach to the traditional continuous-flow ESR method that provides the same high-resolution ESR spectra, but does not consume large quantities of enzyme, substrate, or buffer. The method utilizes enzyme immobilized onto an inert substrate packed directly into an ESR flat cell. Flowing substrate solution over the immobilized enzyme generates in situ, transient radicals, which can then be observed on the submillisecond time scale. We have termed this method "immobilized enzyme ESR," abbreviated IE-ESR. In this paper, we have described the details of the IE-ESR technique and have presented data collected using the IE-ESR technique for transient radicals from limited quantity enzymes, limited quantity substrates, and D_2O buffers. An extension of this technique to ESR spin trapping has also been discussed.

The study of transient radicals produced from enzymatic catalysis provides valuable information about radical reactivity as well as enzyme function. (The term "transient radical" in the present discussion is defined as a radical with a half-life <1 s.) Because of their transient nature, these radicals are often difficult to observe. To detect these species, rapid-mixing techniques, originally described by Hartridge and Roughton,[1] were developed. The most convenient rapid-mixing technique to confirm the formation of a radical product uses a method referred to as "continuous-flow." In this experimental approach, two solutions, one containing enzyme and the other containing substrate(s), are continually flowed through a mixing chamber adjacent to an observation cell. This continuous-flow setup results in a steady-state concentration of reaction products, some of which may be transient radicals. The "age" of the mixed solution is dependent on the flow rate and the physical dimensions of the mixer/observation cell. The continuous-flow technique has been successfully applied to the detection of transient radicals using electron spin resonance (ESR) spectroscopy and is referred to as "continuous-flow ESR" (also referred to as high-velocity or fast-flow ESR; electron paramagnetic resonance (EPR) is often used in place of ESR).[2−8]

Yamazaki et al. were the first to report the continuous-flow ESR detection of an organic free radical from enzymatic oxidation.[2−4] In these seminal experiments, free radicals from ascorbic acid, dihydroxyfumaric acid, and hydroquinone were detected by peroxidase oxidation. Three key conclusions resulted from these experiments: (a) although it had been proposed that free radicals were products of the peroxidase oxidation reactions,[9,10] this was the first direct detection of a transient radical; (b) the radical concentration was 5−20 times the enzyme concentration, indicating the transient radical existed free in solution; and (c) the radical concentration depended on the concentration of the enzyme and substrate, thus allowing these investigators to determine kinetic parameters.

Whereas early continuous-flow ESR investigations required the local support of a master glassblower to manufacture delicate, high-precision continuous-flow ESR flat cells (the ESR sample holder), today this glassware is commercially available from the master glass blowers at Wilmad (Buena, NJ, part no. WG-804). The sample volume for this flat cell is ∼100 μL and the "dead volume" (the volume between the mixer and the entrance to the sensitive region of the ESR detection system) is ∼100 μL. Flat cells have been designed to have dead volumes as small as 5 μL.[8] To observe a reaction mixture that has an average age of 30 ms (also referred to as a 30-ms "observation time"), in the WG-804 flat cell, one must flow at a total volume of ∼5 mL/sec (300 mL/min). The total volume from the mixer to the center of the active

* To whom correspondence should be addressed. Current address: Department of Chemistry and Physics, Western Carolina University, Cullowhee, NC 28723. Phone: 828-227-3682. Fax: 828-227-7647. E-mail: bes@email.wcu.edu.

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region of the detection system is ~150 μL. To have an observation time of 30 ms, one must flow at 150 μL/30 ms = 5 mL/s; sample age distribution is ±10 ms. The time needed to acquire an ESR spectrum is highly dependent on the radical concentration and typically varies from 20 to 1200 s, using 100−6000 mL of reaction solution. Two recently published continuous-flow ESR spectra of the tyrosyl radical from enzymatic oxidation used quite different flow conditions, but recorded similar spectra using ~20 mg of horseradish peroxidase (~20 000 units based on ABTS assay).[11,12] The highly desirable feature of continuous-flow ESR technique is that it provides a high-resolution ESR spectrum of the transient radical. From this spectrum, hyperfine coupling constants are derived which, in turn, give detailed electron spin density information as it relates to molecular structure. This high-resolution spectrum, however, comes at the expense of relatively large quantities of enzyme, substrate, and buffer. Because of the large quantity of enzyme needed to perform a continuous-flow ESR experiment, readily available enzymes, such as hemoglobin,[13−17] myoglobin,[18,19] and horseradish peroxidase,[4,11,14,20−28] are commonly used. Continuous-flow ESR experiments using lactoperoxidase,[20,25] lignin peroxidase,[29,30] lipoxygenase,[31] hematin,[24] ascorbate oxidase,[4] cytochrome b_5 reductase,[32] and cytochrome *c* reductase[32] have been reported in the literature. Experimental approaches have been pursued to minimize sample volumes[8,33] (see also Bruker BioSpin Corp., Billerica, MA; AquaX and dielectric mixing resonator), although none have made the traditional continuous-flow ESR approach feasible for enzymes available in limited quantities.

We have developed an alternative approach to the traditional continuous-flow ESR method that provides the same high-resolution ESR spectra, but does not consume large quantities of enzyme, substrate, or buffer. This method takes full advantage of the enzyme's catalytic nature. The method utilizes enzyme immobilized onto an inert substrate that is packed directly into the ESR flat cell (Figure 1). Whereas the traditional continuous-flow method flows two solutions (at relatively high flow rates, >20 mL/min), the method described here flows only a substrate solution (at relatively low flow rates, 0.5−3.0 mL/min) over the immobilized enzyme, generating the transient radicals in situ with essentially a zero dead volume and, hence, a submillisecond observation time. This technique is still considered a continuous-flow method, although the method of enzyme/substrate mixing is a variation on traditional methods. We have termed this method "immobilized enzyme ESR," abbreviated IE-ESR, and we have used a subscript "i" to refer to the immobilized enzyme (ex: HRP_i for immobilized HRP). Data using the IE-ESR technique have been recently published.[34,35] In the present paper, we describe the details of the IE-ESR technique and present examples of IE-ESR detection of transient radicals from limited quantity enzymes,

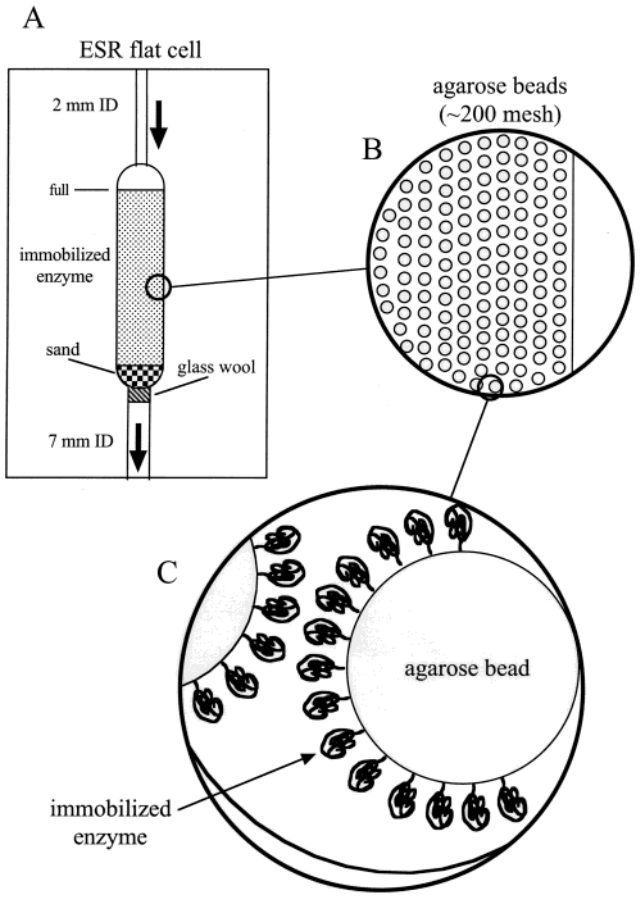

Figure 1. A, an overview of ESR flat cell packed with immobilized enzyme. The immobilized enzyme is prevented from exiting the flat cell by a glass-wool plug at the exit port on the flat cell. B, an expanded view of the flat cell showing the agarose beads (~50 μm). C, a further expanded view diagrams the coupled enzyme to the agarose support material. Enzyme and support bead dimensions are not meant to be factual.

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Table 1. Parameters for Immobilized Enzymes

enzyme	enzyme mol wt (kD)	isoelectric point	[enzyme] before coupling (mg/mL, μM)	[enzyme] immobilized (mg/mL beads, μM)	coupling efficiency (%)	ϵ ($mM^{-1}\ cm^{-1}$)
horseradish peroxidase	44	7.2	20.0, 455	9.6, 218	32	102 @403 nm
lactoperoxidase	78	9.2	20.0, 256	26.67, 342	89	114 @412 nm
myeloperoxidase	130	9.2	1.0, 8	~1.0, ~8	>95	178 @429 nm
cytochrome c	12	9.3	13.3, 1108	16.7, 1392	86	79 @409 nm
myoglobin	17	6.8 − 7.8	13.3, 782	18.2, 1071	91	121 @435 nm[a]
microperoxidase-11	1.861	8.0	0.93, 500	1.2, 645	84	18.5 @550 nm
xanthine oxidase	290	5.3	15.0, 52	14.8, 51	66	66 @450 nm
catalase	230	5.7	20.0, 87	28.0, 122	93	92 @405 nm
glucose oxidase	160	4.3	20.0, 125	23.2, 145	77	15.2 @450 nm

[a] Based on the deoxy form.

limited quantity substrates, and D_2O buffers. An extension of this technique to ESR spin trapping is also discussed.

EXPERIMENTAL SECTION

Reagents. L-Tyrosine, *N*-acetyl-L-tyrosine, xanthine, uric acid, gly-gly-tyr-arg peptide, 3-[*N*-morpholino]propanesulfonic acid (MOPS), diethylenetriaminepentaacetic acid (DTPA), and glycine ethyl ester were purchased from Sigma (St. Louis, MO) and were used as received. Horseradish peroxidase (EC 1.11.1.7) type-VI-A, xanthine oxidase (EC 1.1.3.22), myoglobin, and microperoxidase-11 were purchased from Sigma. Lactoperoxidase (EC 1.11.1.7) and glucose oxidase (EC 1.1.3.4) were purchased from Boehringer Mannheim (Indianapolis, IN). Myeloperoxidase (EC 1.11.1.7) was purchased from Planta Naturstoffe (Vienna, Austria). Hydrogen peroxide (H_2O_2) was purchased from Fischer Scientific (Pittsburgh, PA), and its concentration was verified using UV absorbance at 240 nm ($\epsilon = 43.6\ cm^{-1}\ M^{-1}$). Tyrosine isotopes and deuterium oxide were purchased from Isotec, Inc. (Miamisburg, OH) and used as received. Affi-Gel 10, Affi-Gel 15, and Chelex-100 were purchased from BioRad Laboratories, Inc. (Hercules, CA). Enzyme dialysis was performed using Slide-A-Lyzer cassettes, 10 000 MW cutoff, purchased from Pierce Inc. (Rockford, IL). Phosphate buffer salts, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic heptahydrate were purchased from Mallinckrodt (Paris, KY). All buffers were made fresh daily from a 10-fold concentrate that underwent a 24-hour Chelex-100 treatment; DTPA was added to the buffer concentrate after removal of Chelex-100. Final buffer concentrations (50 mM phosphate and 50 μM DTPA) were obtained by dilution with HPLC grade water followed by pH adjustment to 7.4.

Immobilization of Enzymes. All enzymes were immobilized onto the commonly used Affi-Gel 10, with the exception of glucose oxidase (Affi-Gel 15), on the basis of the protein's isoelectric point, as outlined in the product literature (bulletin no. 1085).[36] Many immobilization techniques have been published.[37−39] All enzymes were immobilized using 0.1 M MOPS buffer (pH 7.5). Coupling was allowed to proceed for 4-hours at 4 °C, followed by a 1-hour glycine ethyl ester blocking step. The ratio of enzyme solution to Affi-Gel bead volume was 1.5 (in most cases, 3 mL of enzyme solution was mixed with 2 mL of Affi-Gel beads). The coupling efficiency was determined for the enzyme at a fixed enzyme concentration (mg/mL) by comparing the concentration of free enzyme before coupling and after coupling using UV−visible spectroscopy. The coupling parameters for the enzymes used for IE-ESR investigations are listed in Table 1.

ESR Experiments. There are three experimental aspects to the IE-ESR experiment: (1) loading the immobilized enzyme into the ESR flat cell, (2) mounting the flat cell and controlling substrate flow, and (3) acquiring the ESR data. Immobilized enzymes were loaded into a standard ESR flat cell as shown in Figure 1. The typical ESR flat cell has an upper and a lower tube connected to the flat cell region. One of the tubes is generally a larger bore (~7-mm i.d.), and the other has a smaller bore (~2-mm i.d.). A small glass wool plug was placed in the 7-mm-i.d. tubing and pushed all the way toward the flat cell region. This glass wool was washed (with water flow from the 7-mm-i.d. side to the 2-mm-i.d. side) with the help of a "tube washer" (Kimble-Kontes, Vineland, NJ). Tygon tubing (~5 cm on the 2-mm-i.d. side and ~15 cm on the 7-mm side) was connected to both ends of the flat cell. While the flat cell was held with the 7-mm-i.d. tubing down and the connected Tygon tubing up (the Tygon tubing will form a "J"), the entire system was filled with buffer. To minimize pump back pressure (and to minimize enzyme needed), a small amount of 50-mesh sand was placed on top of the glass wool to fill the glass tubing flat cell region (sand stuck in the flat cell region can be removed using a sonicating bath). Sand was added to the 2-mm-i.d. side and allowed to gravity-feed (allowing buffer to flow out of the 7-mm-i.d. end, while buffer was added to the 2-mm end). Once the sand was properly positioned in the flat cell, the buffer flow was stopped by putting a gloved finger over the 7-mm-i.d. end of the Tygon tubing. The container of immobilized enzyme was agitated to suspend the enzyme/support particles, and 50-uL aliquots were transferred into the 2-mm-i.d. tubing end of the flat cell using a large-bore pipet tip. The beads were allowed to gravity-feed into the flat cell region while the 2-mm-i.d. tubing side was continually filled with buffer to prevent air from entering the flat cell. Approximately 100 μL of immobilized enzyme was needed to fill the flat cell region. Care must be taken not to overfill the flat cell region (see Figure 1). Once the flat cell was loaded with enzyme, the Tygon tubing from the 2-mm-i.d. side was quickly

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removed, and a septum (Aldrich) inserted. Then the Tygon tubing from the 7-mm-i.d. side was removed, and a septum was inserted.

The loaded flat cell was mounted into the ESR cavity from the bottom with the 2-mm-i.d. side up ("upside-down") and secured using compression fittings. The flow of the substrate solution was controlled using a syringe pump (Harvard Apparatus, Holliston, MA). Connections from the top of the flat cell (2-mm-i.d. end) to the syringe mounted in the syringe pump were conveniently made using a 19-gauge needle (with tubing) from a blood collection set (Becton Dickinson). Rubber tubing was connected to the bottom 7-mm-i.d. outlet and directed to a waste container. Substrate flow rates were generally 0.5−3.0 mL/min. Radical concentration was dependent on substrate flow rates and enzyme and substrate concentrations; conditions must be optimized for the radical of interest. Although the radical generation occurs inside the active region of the flat cell, the "age" of the radical will vary over the active region of the flat cell (from 0 to 100 μL/flow rate). For example, the age of a sample using a 2.0 mL/min flow rate ranges from 0 to 3 s, depending on where in the flat cell the radical was formed. Very slow flow rates (0.3 mL/min) have been used to increase the sample age (20 s).

ESR data acquisition parameters are listed in the figure captions.

Enzyme Stability. The IE-ESR experiment relies on the stability of the immobilized enzyme. The mechanism of peroxidase inactivation for peroxidases used in protein engineering has been recently reviewed.[40] The stability of the immobilized enzyme used for an IE-ESR experiment can be evaluated by monitoring the intensity of an ESR transition over time. For IE-ESR experiments in which radical concentrations are of interest, care must be taken to continually verify consistent enzyme activity. In our previously published IE-ESR experiments with HRP_i/H_2O_2/*N*-acetyltyrosine/NO/ascorbate, control spectra were collected before and after each set of conditions.[34] In the case of HRP (immobilized at 7.5 mg/mL), this enzyme was very stable under standard conditions (1 mM H_2O_2, 0−4 mM substrate). Stability was dependent on relative enzyme/substrate concentrations. Under these same standard conditions, myeloperoxidase (immobilized at <0.5 mg/mL) enzyme activity (based on tyrosyl radical concentration) decreased by 50% over 20 min. Enzyme stability needs to be evaluated under specific radical generating conditions.

RESULTS AND DISCUSSION

The main disadvantage of the traditional continuous-flow ESR method is the consumption of large quantities of enzyme, substrate, and buffer. Figure 2A shows our published continuous-flow tyrosyl radical spectrum generated by HRP/H_2O_2 oxidation.[11] The tyrosyl radical collected using IE-ESR with immobilized HRP is presented in Figure 2B. The higher signal-to-noise (S/N) (~3×) in Figure 2B was a result of many experimental parameters, including improvements in instrumentation (~20% better S/N for a 10-mm flat cell in a SHQ cavity used for IE-ESR versus a 17-mm flat cell in a TM_{110} cavity used for continuous-flow ESR), increased enzyme concentration as a result of immobilization (~200 μM in IE-ESR versus ~300 nM in continuous-flow ESR), and a shortened observation time (~0 s in situ in IE-ESR versus ~0.1 s in continuous-flow ESR). Although the increase in S/N is highly desirable, it should not be considered *the* significant point in the context of the current discussion; the more important point was the overall amount of materials used. Whereas the continuous flow ESR experiment used ~20 mg of HRP, the IE-ESR experiment used only 0.75 mg; in addition, the HRP_i was not consumed and was available to be used again. From the perspective of substrate and buffer, the IE-ESR technique was quite frugal, using only 14 mg of tyrosine (~500 mg in continuous-flow), 5 μL of 30% H_2O_2 (~500 μL in continuous-flow), and 40 mL of buffer (~1400 mL in

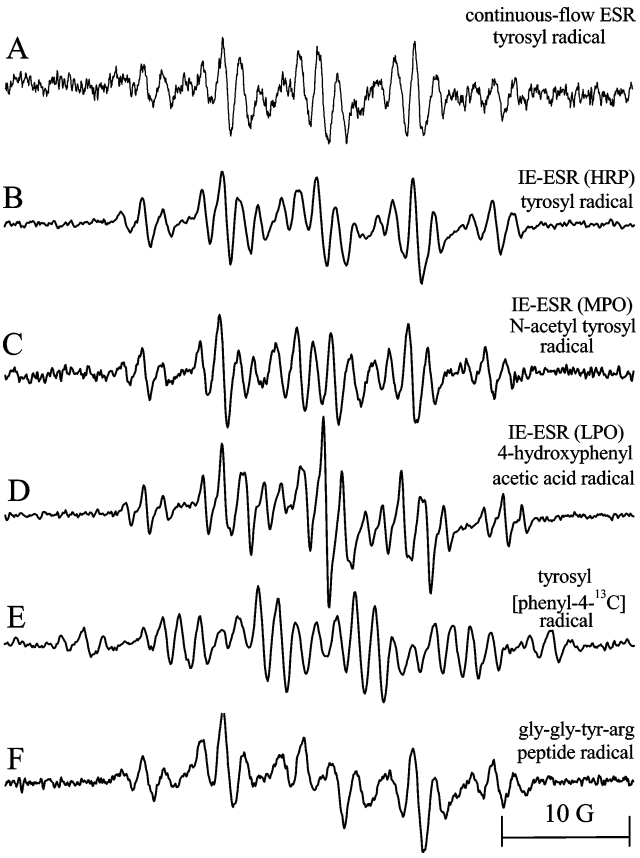

Figure 2. ESR detection of the tyrosyl radicals using traditional continuous-flow ESR (A) and immobilized enzymes (B−F). Spectrum in A was collected using 13.7 μg/mL HRP (1400 mL total), 2.1 mM tyrosine, 1.9 mM H_2O_2, 50 mM phosphate buffer at pH 7.4, 50 μM DTPA, 60 mL/min flow rate on a Bruker ER200 spectrometer with a TM_{110} resonator (17-mm flat cell), 9.7-GHz microwave frequency, 20-mW microwave power, 45-G sweep width, 0.5-G_{pp} modulation amplitude, 5-s time constant, and sweep rate of 0.0333 G/s. Spectrum B was collected using HRP_i (0.75 mg/flat cell), 2 mM tyrosine, 1 mM H_2O_2, 50 mM phosphate buffer at pH 7.4, 50 μM DTPA, 2 mL/min flow rate on a Bruker EMX with a SHQ resonator (10-mm flat cell), 9.8-GHz microwave frequency, 10 mW microwave power, 80-G sweep width (45 G shown), 0.5-G_{pp} modulation amplitude (100 kHz), 164-ms time constant, 84-s scan time, and an average of 14 scans. Spectrum C was collected using MPO_i (0.1 mg/flat cell) and *N*-acetyl tyrosine (2 mM). Spectrum D was collected using LPO_i (2.7 mg/flat cell) and 4-hydroxyphenyl acetic acid (2 mM). Spectrum E was collected using HRP_i (0.75 mg/flat cell) and tyrosine[phenol-4-^{13}C] (2 mM). Spectrum F was collected using HRP_i (0.75 mg/flat cell) and glycine-glycine-tyrosine-arginine peptide (2 mM). Spectra C, E, and F were collected under the same conditions as spectrum B. Spectrum D was collected under the same conditions as B, except an average of five scans and 1 G_{pp} modulation amplitude.

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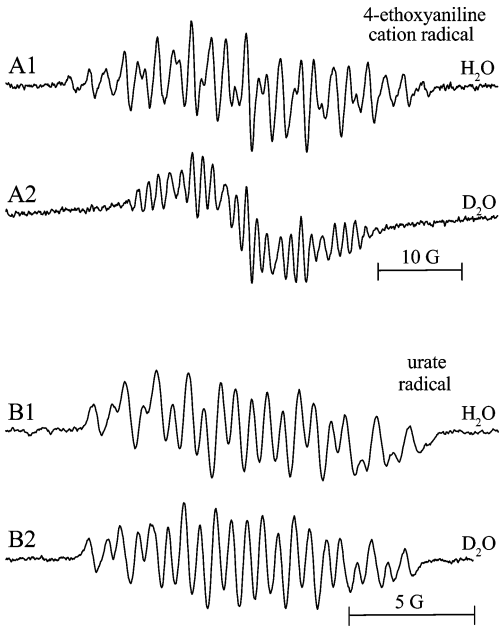

Figure 3. IE-ESR detection of the 4-ethoxyaniline cation radical in H_2O (A1) and D_2O (A2). ESR spectra were collected using 0.75 mg/flat cell HRP_i, 4 mM 4-ethoxyaniline, 1 mM H_2O_2, 50 mM phosphate buffer at pH 7.4, 50 μM DTPA, 3 mL/min flow rate on a Bruker EMX spectrometer with a SHQ resonator (10-mm flat cell), 9.8 GHz, 20-mW microwave power, 60-G sweep width, 0.6-G_{pp} modulation amplitude (100 kHz), 328-ms time constant, 168-s scan time, and four averaged scans. IE-ESR detection of urate radical in H_2O (B1) and D_2O (B2). ESR spectra were collected using 0.75 mg/flat cell HRP_i, 2 mM urate, 1 mM H_2O_2, 50 mM phosphate buffer at pH 7.4, 50 μM DTPA, 1 mL/min flow rate on a Bruker EMX spectrometer with a SHQ resonator (10-mm flat cell), 9.8 GHz, 10-mW microwave power, 30-G sweep width, 0.21-G_{pp} modulation amplitude (100 kHz), 328-ms time constant, 84-s scan time, and 13 (B1) and 26 (B2) averaged scans.

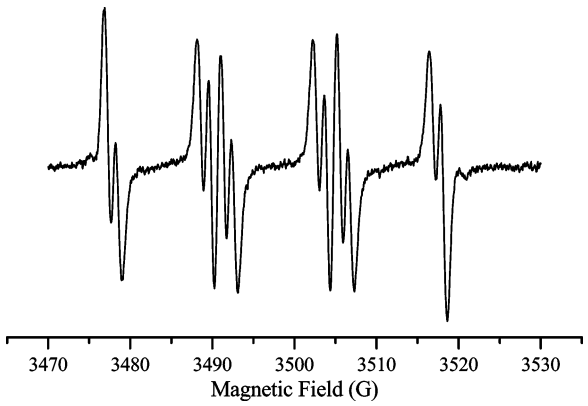

Figure 4. IE-ESR detection of the DMPO/•OOH spin adduct using immobilized xanthine oxidase. Data were collected with 100 μM xanthine, xanthine oxidase (1.5 mg/flat cell), and 30 mM DMPO on a Bruker EMX with a SHQ resonator (10-mm flat cell), 9.8-GHz microwave frequency, 10-mW microwave power, 2 mL/min flow rate, 0.5-Gpp modulation amplitude (100 kHz), 163-ms time constant, 167-s sweep time, and an average of two scans.

continuous-flow). The frugal nature of the IE-ESR technique allows for investigation of enzymes and substrates available in limited supply.

Enzymes. Table 1 lists the enzymes immobilized and used for IE-ESR studies. In most cases, the peroxidase activity of these enzymes has been investigated. Of particular interest is the enzyme myeloperoxidase (MPO). A continuous-flow ESR experiment using $MPO/H_2O_2/$tyrosine would be cost-prohibitive. The ability of MPO to oxidize tyrosine to the tyrosyl radical has been inferred by ESR spin-trapping studies.[12] The IE-ESR detection of the tyrosyl radical from the MPO_i/H_2O_2 system has now been confirmed (Figure 2C). IE-ESR experiments can be done routinely using lactoperoxidase (LPO) to detect free radical metabolites (Figure 2D), although this enzyme chemistry is mildly complicated by the formation of a protein radical.[41] Immobilized glucose oxidase, as a precolumn, has been used frequently in conjunction with these peroxidase studies to convert molecular oxygen into the required hydrogen peroxide.

Substrate. Spectra E and F in Figure 2 show the IE-ESR spectra from relatively expensive substrates. The ESR spectra of these substrate radicals have not been previously published. The HRP_i/H_2O_2-generated radical originating from phenyl-4-^{13}C-tyrosine (Figure 2E) has a ^{13}C isotropic hyperfine coupling constant of 9.1 G, similar to the value reported.[42] The HRP_i/H_2O_2-generated radical originating from the peptide gly-gly-tyr-arg (Figure 2F) clearly shows a characteristic tyrosyl radical spectrum, although this radical was significantly more hindered in its rotation, resulting in clear alternating line widths.[43] Although these radicals were collected on different days, a single ESR flat cell packed with immobilized HRP can be used for multiple substrates. IE-ESR spectra of selectively deuterated tyrosyl radicals have been published.[34]

Buffer. D_2O buffers are commonly used in ESR spectroscopy to verify exchangeable protons. The gyromagnetic ratio of deuterium is 1/6 that of a proton; hence, the deuterium couplings are 1/6 that of a proton and are typically within the intrinsic ESR line width; the overall result is that the ESR spectrum is simplified. Because of the minimal quantities of buffer needed to collect an IE-ESR spectrum, D_2O can be used routinely. In Figure 3, the IE-ESR spectrum is presented for HRP_i/H_2O_2-generated 4-ethoxyaniline cation radical in H_2O (Figure 3A1) and D_2O (Figure 3A2).[25] This radical has two solvent-exchangeable amine protons, and hence, the ESR spectral assignment can be verified. Figure 3A2 points out one of the disadvantages of using immobilized enzymes. Underlying this spectrum was a broad "g = 2" background signal resulting from the buildup of polymeric radical material that adhered to the support surface. This background signal has *not* been subtracted, although it is relatively simple to subtract, since once the flow has stopped, the solution-phase radical decays quickly, and then the longer-lived "background" spectrum can be collected. In some cases, such as the detection of the acetaminophen radical,[44] the polymeric radical material dominates the ESR spectrum, making spectral subtractions difficult. The IE-ESR

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spectra for the HRP_I/H_2O_2-generated urate radical[24] in H_2O (Figure 3B1) and D_2O (Figure 3B2) are shown. Although there are no solvent-exchangeable protons in the urate radical, deuterium substitution of the weakly, dipolar-coupled solvent (H_2O) molecules generated a more symmetric D_2O ESR spectrum with narrower lines; this allows for a higher level of confidence in simulating this ESR spectrum.

Spin Trapping. The ESR technique of spin trapping uses a "spin trap" to react with (or trap) a short-lived radical to form a longer-lived "radical (or spin) adduct". In most cases, the ESR spectrum of the "radical adduct" is characteristic of the trapped radical and can lead to its identification. Under some circumstances, the lifetime of a radical adduct is not long enough to obtain an ESR spectrum with the desired signal-to-noise. The IE-ESR technique can be applied to the detection of short-lived "spin adducts". Figure 4 shows the ESR spectrum of the DMPO/superoxide radical adduct (DMPO/•OOH) collected using immobilized xanthine oxidase and flowing xanthine and DMPO in an oxygenated buffer. The DMPO/•OOH radical adduct is relatively short-lived ($t_{1/2}$ = 50 s at pH 7.4, 25 °C).[45] Using IE-ESR, a DMPO/•OOH ESR spectrum can be collected free of radical adduct decay or of secondary radical products (DMPO/•OH, because of the high signal-to-noise ratio, a very small amount (<3%) of the DMPO/•OH can be seen in Figure 4).

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CONCLUSION

The use of immobilized enzymes for the study of transient radicals appears to be a reasonable alternative to the traditional continuous-flow ESR method. Whereas our current application of IE-ESR was primarily to detect and identify radical metabolites, future investigations will extend the method to addressing mechanistic and kinetic parameters of interest. In addition, initial IE-ESR studies are underway to investigate systems that contain protein-bound radicals.

It is anticipated that immobilized enzymes can be used for a variety of other spectroscopic techniques that use continuous-flow methods. The immobilized enzymes presented here are all soluble enzymes. Membrane-bound enzymes, when immobilized, should generate similar results. Multiple enzymes (coimmobilization) used in single experiments should provide unique results.

NOTE ADDED IN PROOF

It was recently brought to our attention that the following used immobilized enzymes to observe ESR signals from transient radicals. See: Damerau, W.; Lassmann, G.; Flemming, C. *Stud. Biophys.* **1973**, *35*, 39−44.

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