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Imaging of Enzyme Activity by Electron Paramagnetic Resonance: Concept and Experiment Using a Paramagnetic Substrate of Alkaline Phosphatase

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Abstract

Enzyme activities are well established biomarkers of many pathologies. Imaging enzyme activity directly *in vivo* may help to gain insight into the pathogenesis of various diseases but remains extremely challenging. In this communication, we report the use of EPR imaging (EPRI) in combination with a specially designed paramagnetic enzymatic substrate to map alkaline phosphatase activity with a high selectivity, thereby demonstrating the potential of EPRI to map enzyme activity.

Keywords

Enzymes; EPR; Imaging; Nitroxide; Phosphatase

Enzyme activities are important biomarkers of many pathologies, such as cancers, Alzheimer's disease and diabetes.^[1] While reliable methods have been developed to measure enzyme concentration, expression, and activity *in vitro* and *ex vivo*, the direct measurement of enzyme activity *in vivo* remains extremely challenging.^[2]

Magnetic resonance techniques such as magnetic resonance imaging (MRI) and electron paramagnetic resonance imaging (EPRI) are ideal for *in vivo* applications due to the high penetration depth of the radiofrequency into the tissue. A number of enzyme responsive MRI contrast agents have been developed to assess enzymatic activities.^[2–3] However, the

Conflict of interest

None.

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low sensitivity of MRI often limits its applications in spite of recent developments of advanced modalities such as chemical exchange saturation transfer (CEST) and hyperpolarization. EPRI has advantages over MRI in higher intrinsic sensitivity due to a larger magnetic moment of the electron and higher specificity due to the absence of endogenous background signal. The limited number and/or the low concentration of endogenous EPR active enzymes^[4] and enzymatically generated radicals^[5] make it necessary to use a specially designed exogenous paramagnetic substrate to assess enzymatic activity by EPR.

A few approaches for assessing enzyme activity via EPR were based on detecting time dependent spectral changes upon enzymatic hydrolysis of the paramagnetic substrate. These have relied on three major mechanisms: (1) the change in rotational correlation time resulting in line broadening or in alteration of the spectral peaks intensity ratio; (2) the elimination of spin exchange and/or dipole-dipole interaction between two radical fragments after bond cleavage resulting in spectral simplification; and (3) the modification of magnetic resonance parameters (hyperfine coupling, g-factor) between the substrate and product. Nakaie, *et al.* proposed several paramagnetic substrates of angiotensin I-converting enzyme (ACE), namely angiotensin I^[6] and bradykinin^[7] containing the stable nitroxide radical (NR) amino acid TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid). The authors measured ACE activity by detecting a change in the EPR spectrum following enzymatic hydrolysis of the NR-substrate peptide bond. The shorter rotational correlation time of the NR-product decreased the line broadening of the high-field component of the triplet EPR spectrum. An even more pronounced modulation of correlation times for NR-labeled proteins following enzymatic cleavage^[8] provided a basis for *in vitro*^[8b] and *in vivo*^[9] assessment of proteolytic activity using Overhauser-enhanced MRI (OMRI).

Hacker, *et al.*^[10] proposed the use of biradical substrates with two NRs connected via an enzyme cleavable motif. As a proof of concept, they synthesized an adenosine triphosphate (ATP) analogue, NR-ATP-NR, and monitored phosphoanhydride bond cleavage by Phosphodiesterase I via EPR.

Recent work demonstrated the detection of proteases using a β -phosphorylated NR-substrate prototype^[11] and an NR-labeled peptide specific to chymotrypsin and cathepsin G.^[12] Upon enzymatic hydrolysis of the peptide, the enol ester moiety of the NR is converted into a ketone moiety accompanied by a large 4 to 5 G change of the phosphorus hyperfine splitting (hfs) constant. Note that the measurement of hfs changes provides higher specificity than changes in correlation time or spin exchange, as the latter are sensitive to the local environment.

In this work, we utilize EPRI with a new phosphorylated paramagnetic substrate to image enzymatic dephosphorylation, a key biological processes playing a critical role in physiology and pathophysiology. We synthesized a phosphorylated NR whose nitrogen hfs is directly modified by the action of alkaline phosphatase (ALP) with high selectivity.

Alkaline phosphatase is an enzyme that catalyzes the dephosphorylation of a large number of substrates containing a phosphate monoester.^[1c, 13] In spite of the recognized importance

of ALP activity as a well-defined biomarker for the progression of many diseases^[1c], a robust imaging technique for ALP activity *in vivo* has yet to be developed. Recently, fluorescent probes have been reported to image ALP activity *in vivo*.^[14] However, these approaches suffer from light absorbance and scattering by living tissue, which limit imaging to superficial tissues and 2D resolution.

To create an EPR active substrate for ALP, we synthesized a phosphate enol ether of a nitroxide radical (**1**), assuming that the cleavage of the phosphate moiety by ALP would lead to the formation of an enol intermediate, which would then undergo keto-enol isomerization and result in the formation of the ketone **2** (TEMPONE-d₁₅, see Scheme 1).^[11] One can expect a significant change in the nitrogen hfs upon the change in ring conformation, therefore allowing for discrimination between NRs **1** and **2** that exhibit narrow EPR linewidths due to deuteration.

Compound **1** has been synthesized in a two-step sequence as depicted in Scheme 2. First, quenching of TEMPONE enolate by dimethylchlorophosphate produced phosphonated NR **3** with an excellent yield of 95%. Second, deprotection of the phosphonic ester using trimethylsilylbromine gave **1** with a moderate yield of 50% after purification. Note that the nitroxide fragment is reduced during the phosphate deprotection and is reoxidized by bubbling air into the solution (see SI for details).

The EPR spectrum of **1** exhibits a characteristic triplet pattern (Figure 1A) with a nitrogen hfs of 16.45 G between center and low-field components and peak-to-peak linewidth of 0.45 G for the low-field component. Incubation of **1** in the presence of ALP results in the appearance of a second triplet with an hfs of 15.78 G and peak-to-peak linewidth of 0.40 G, measured as previously (Figure 1B). The observed difference in hfs between substrate and product ($a_N=0.67$ G) allows for quantitative concentration measurements of both species during the enzymatic reaction.

In order to confirm that the enzymatic product was the ketone **2** (Scheme 1), the reaction was monitored by HPLC. Chromatograms B-D in Figure 2 show the conversion of **1**, upon incubation with ALP, into a single product with a retention time identical to TEMPONE (from commercial source, Chromatogram E), confirming that NR **2** (TEMPONE-d₁₅) is the product of the enzymatic reaction.

To determine the kinetic parameters of ALP-catalyzed dephosphorylation of substrate **1**, we performed EPR kinetic measurements over a range of substrate concentrations. Figure 3A shows the time evolution of the EPR spectrum's low-field component illustrating the enzymatic conversion of **1** into **2**. Figure 3B shows the corresponding kinetics for NR **2** formation given different substrate concentrations. A Hanes-Woolf plot (Figure 3C) yields the Michaelis-Menten parameters, $K_M = (24 \pm 6) \mu\text{M}$, $V_{\text{max}} = (0.031 \pm 0.001) \mu\text{M/s}$, and $k_{\text{cat}} = V_{\text{max}}/[\text{ALP}] = (193 \pm 6) \text{ s}^{-1}$. ALP catalytic efficiency for NR **1**, estimated by a $k_{\text{cat}}/K_M = 8.04 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, is significantly higher than that reported for commonly used fluorogenic ALP substrates ELF-97 and 4-MUP.^[15]

Figure 4 demonstrates the high stability of the NR **1** probe in aqueous solutions at various pH as well as the high selectivity of its dephosphorylation by ALP. The ALP inhibitor sodium orthovanadate, Na₃VO₄, was used to confirm the enzymatic nature of the reaction. Dephosphorylation of the probe was inhibited in a dose dependent manner with an IC₅₀ = 4 ± 2 μM, in good agreement with the value reported in literature (See SI).^[14c, 16] The incubation of **1** with other hydrolytic enzymes, such as acid phosphatase (ACP), protein phosphatase (PPase), esterase, phosphodiesterase (PDE), lysozyme, and trypsin did not result in significant probe conversion to **2**, confirming the high selectivity of the probe toward ALP.

Figure 5 shows the results of a cytotoxicity study of **1** performed using an MTT cell viability assay on MDA-MB-231 breast cancer cells (see SI for details). The probe does not show significant cytotoxicity in concentrations up to 1 mM, with cell viability above 80% after 24 hrs incubation. It is worth noting that the EPR enzymatic assay requires less than 10 μM of substrate, and EPRI can be performed with a local probe concentration of a few hundred micromolar.

To demonstrate the potential of EPRI to map ALP enzymatic activity, we performed *in vitro* imaging of enzymatic dephosphorylation of **1** using a pre-clinical 800 MHz rapid scan EPR imager. 4D images (3D-spatial, 1D-spectral) were acquired every 15 minutes of a phantom composed of three tubes, one containing **1** with ALP, one containing only **1**, and one containing only TEMPONE-d₁₆ (see Figure 6C and SI for the details). A slice through the three tubes was selected and spectra from each tube were extracted for each voxel to produce images of substrate and product. The series of images clearly demonstrates a decrease of the substrate (Figure 6A) and formation of the product (Figure 6B) in the tube containing ALP. Figure 6D shows the reaction kinetics calculated from the imaging data by integration of the corresponding voxel intensities for substrate and product. The sum of the substrate and product signals did not change over the course of the experiment, validating the stability of the radicals and the accuracy of the image reconstruction.

In summary, this work demonstrates the feasibility of mapping enzyme activity using EPRI in combination with a paramagnetic substrate for the first time. *In vitro* 4D spectral-spatial EPR imaging of an ALP-catalyzed dephosphorylation reaction has been performed using a nitroxide radical substrate. Recently, *in vivo* EPR of a pH-sensitive trityl probe helped to identify inorganic phosphate (Pi) as a new microenvironment factor for tumor progression.^[17] Therefore, imaging of ALP activity *in vivo* could lead to new insights into phosphorous metabolism in tumors. One of the potential problems with nitroxide substrates is their limited stability in biological media. Trityl probes have advantages over nitroxides including higher biostability and very narrow linewidths, which are particularly important features for imaging technologies such as EPRI^[18] and OMRI.^[19] Therefore, the synthesis of enzyme responsive trityl-based substrates may provide an interesting future direction for the biomedical imaging of enzyme activity *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

X-band EPR spectra of 200 μM **1** measured at 37°C in 10 mM Tris, pH=7.5, containing 1 mM MgCl_2 and 0.1mM ZnCl_2 , (A) before and (B) 50 min after incubation with 0.08 U/mL ALP.

Figure 2.

Reverse phase HPLC chromatograms of 300 μM **1** (A) alone and (B-D) after incubation with 0.8 U/mL ALP at 37°C. Chromatogram E shows TEMPONE injected as a reference.

Figure 3.

Enzymatic conversion of NR 1 into NR 2. (A) Time evolution of the low-field EPR component of NR 1 in the presence of 0.08 U/ml ALP measured under conditions indicated in Figure 1. (B) The kinetics of ALP-catalyzed NR 2 formation measured by EPR for various concentrations of NR 1. (C) Hanes-Woolf plot with linear fit yielding $K_M = 24 \pm 6 \mu\text{M}$ and $V_{\text{max}} = 0.031 \pm 0.001 \mu\text{M/s}$.

Figure 4.

Selectivity of NR **1** dephosphorylation by the ALP enzyme. The probe (10 μ M) was incubated for 30 min in the presence of ALP alone, with Na₃VO₄, and in the presence of other hydrolytic enzymes: acid phosphatase, protein phosphatase, esterase, phosphodiesterase, lysozyme, and trypsin. To verify the stability of NR **1** in acidic and alkaline conditions, the probe was incubated for 30 min in aqueous solutions at pH 1 or pH 13.

Figure 5.
MTT cell viability assay performed on MDA-MB-231 breast cancer cells.

Figure 6.

In vitro 4D spectral-spatial EPR imaging of ALP activity. The images were acquired using an 800 MHz rapid scan EPRI instrument at room temperature. Acquisition time was 10 minutes, number of projections was 2520, field of view 18 mm, and matrix size 64×64×64. Slices (xz-plane) of the images for (A) the substrate and (B) the product are shown. (C) Schematic of the phantom, which consisted of 3 tubes with 3 mm diameter filled with either 1 mM **1** (S), 1 mM TEMPONE-d₁₆ (P), or 2 mM **1** with 0.5 U/mL ALP (S->P). All contained 100 mM Tris buffer at pH=7.4, 1 mM MgCl₂, and 0.1 mM ZnCl₂. Note that commercially available TEMPONE-d₁₆ and product NR **2** (TEMPONE-d₁₅) exhibit similar EPR spectra. (D) Time course of integrated substrate and product signal intensities.

Scheme 1.

Schematic representation of alkaline phosphatase catalyzed dephosphorylation of the NR substrate.

Scheme 2.
Synthesis of **1**.