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Biocompatible and Multifunctional Trityl Spin Probes for Electron Paramagnetic Resonance Spectroscopy

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ABSTRACT

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The primary objective of my thesis was to develop and utilize a biocompatible multifunctional trityl spin probe for concurrent measurement of pO_2, pH_e, and $[P_i]$ in vivo by electron paramagnetic resonance (EPR) spectroscopy (Chapter 2). My first goal was to synthesize the proposed probe we are terming HOPE71. Secondly, HOPE71 was characterized by X-band and L-band EPR spectroscopy. Next, the biocompatibility of HOPE71 was assessed through an albumin binding test, cytotoxicity assays, and in vivo intravenous tolerance. Then, the use of HOPE71 to measure the target parameters was demonstrated in a breast cancer mouse model. In tandem, I developed an application for user friendly fitting of the EPR spectrum of HOPE71 and the related monophosphated probe, pTAM (Chapter 3). Lastly, to demonstrate further skill in synthesis of trityl spin probes, I worked in collaboration with the Saxena lab at the University of Pittsburgh to develop a hydrophilic spin probe with a maleimide linker for structural biology studies using site directed spin labeling and dipolar EPR spectroscopy (Chapter 4).

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CHAPTER 1 – Multi-Parameter Assessment of the Tumor Microenvironment (Review)

1. Introduction/Abstract

The tumor microenvironment (TME) contains important elements for understanding tumorigenesis and therapy resistance. Three markers characteristic of the tumor microenvironment are hypoxia (low pO_2), acidosis (low pH_e), and elevated inorganic phosphate concentration $[P_i]$. These attributes are vital to study cancer as they relate to the mechanisms of tumor growth, the aggressiveness of the cancer, and the response to therapy. There are various methods for measuring each of these parameters individually, each with benefits and drawbacks. One notable method is electronic paramagnetic resonance (EPR) spectroscopy utilizing pTAM, a monophosphonated trityl radical, which allows for concurrent, non-invasive measurement of pO_2, pH, and $[P_i]$ for in vivo applications.

2. Tumor Microenvironment

With its complexity, prevalence, and burden on society, cancer is and will continue to be a significant and essential topic in research, and studying the tumor microenvironment (TME) is vital for understanding tumorigenesis and therapeutic response[1, 2]. The TME is a complex network of various immune and fibroblast cells, vasculature, and extracellular matrix[2]. Its characteristics are both affected by and affecters of the tumor story. Two physiological parameters of the TME, hypoxia and acidosis, are well-defined hallmarks of solid tumor development and play considerable roles in tumorigenesis and treatment resistance. More recently, high inorganic phosphate concentration was also identified as a tumor marker that may also indicate metastatic character[3].

2.1 Hypoxia

Oxygen is vital for normal metabolic function in the cell, and therefore, adequate oxygen supply to the tissue is essential for maintaining homeostasis. Tissue oxygenation (pO_2) is a measurement of the net supply and demand of oxygen. An inadequate amount of oxygen to the body or specific tissue leads to hypoxia. Normal tissue oxygenation can range from approximately 10 to 70 mmHg, while hypoxia is defined as oxygen levels below 10 mmHg[4-6]. Hypoxia is a well-established hallmark of solid tumors, with the initial observation made over 60 years ago[7], and the association has been verified across many types of cancer. The overall average oxygenation and the fraction of normoxic regions are considerably lower in tumors when compared to their corresponding tissue[5].

Tumor hypoxia is caused by an imbalance between oxygen delivery and consumption. The high rates of metabolism and proliferation in cancer cells create higher demands for oxygen, while tumor vasculature fails to deliver sufficient and consistent oxygen[8]. There are two primary types of oxygen deprivation: chronic or diffusion-limited hypoxia, which is due to an increasing distance between blood vessels and cells, and acute or perfusion-limited hypoxia, which is caused by a temporary drop in blood flow[9].

Tumor hypoxia has several consequences on tumorigenesis and therapeutic response. Evidence shows that a hypoxic environment may be present early in pre-malignant lesions due to the basal membrane limiting oxygen supply[10].This creates a selected pressure towards cells resistant to hypoxia-induced apoptosis[10]. Hypoxia has also been associated with a more metastatic character due to two main effects: (1) continued selective pressure that can lead to greater survival of more aggressive cells, (2) hypoxia-induced expression of genes that leads to metastases[8, 10, 11]. Also, hypoxia can lead to recruitment of inflammatory cells and reprogramming of the inflammatory pathways that promote the tumor's ability to escape immune patrolling, proliferate, remodel the extracellular matrix, and migrate[12, 13].

An additional major consequence of hypoxia is resistance to standard therapy, such as radiation and some chemotherapies. The radiosensitivity of a tumor is related to the oxygenation through the oxygen enhancement effect or oxygen enhancement ratio (OER)[14]. Radiation in the presence of oxygen creates oxygen-centered free radicals while damaged mitochondria leak more reactive oxygen species (ROS), both of which increase the DNA damage[15]. Consequently, hypoxic tissue requires up to three times more radiation dose than normoxic tissue for the same therapeutic effect[14, 16]. Hypoxic tumor cells can also be less sensitive to chemotherapy, which generally targets rapidly dividing cells, because of slowed proliferation from lack of nutrition[8]. The gene expression selected and induced by hypoxia can also protect cells from chemotherapy through loss of apoptosis[8].
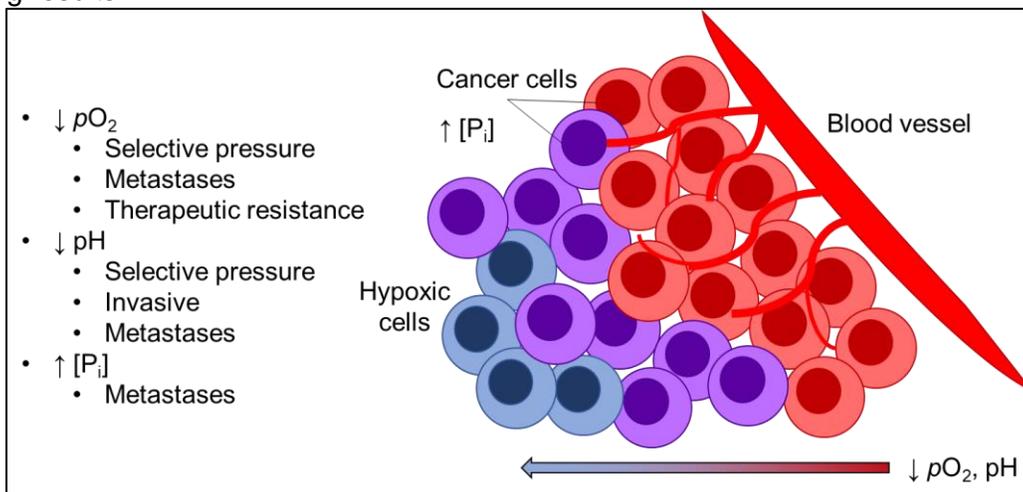
2.2 Acidosis

Another important chemical parameter in physiologic homeostasis is pH. Intracellular pH (pH_i) is around 7.2 and tightly regulated to preserve normal intracellular metabolism and function[17, 18]. The pH of the blood and the interstitial space (pH_e) of healthy tissue is generally around 7.4[19]. Systemically, the pH is maintained by the lungs and kidneys. Acidosis occurs when the tissue pH_e is more acidic, in the range of 6.5 to 6.9, and has been repeatedly associated with solid tumors for several decades[20]. In malignant tissue, there is an imbalance in increased proton secretion and poor proton removal. Cancer cells demonstrate a high rate of glycolysis to meet the high energy demands of a growing tumor[21]. An increased dependency on glycolysis can be a result of hypoxia, but glycolysis has been shown to persist even in well-oxygenated conditions, which suggests a high growth adaptation selected by intermittent hypoxia in the early stages of tumor development[10]. High rates of glycolysis create great amounts of lactate, which is exported from the cell along with protons[22]. As discussed earlier, the vasculature and perfusion of a tumor is often irregular and ineffective, and therefore, may not efficiently remove the buildup of protons.

Acidosis creates further selective pressure toward cells that are more resistant to acidic environments. It has also been associated with more aggressive and metastatic capabilities, possibly due to enhanced angiogenesis and degradation of the cellular matrix[10]. The acidic pH_e can have consequences on therapeutic response, especially when using chemotherapeutics with pK_a values around 6-8 because the increased protonation can alter the absorption, metabolism, and mechanism of action.

2.3 Inorganic Phosphate

Inorganic phosphate (P_i) is a major building block in nucleotides of DNA and RNA and phospholipids in cellular membranes. Phosphates are also an essential component of metabolism, with ADP and ATP acting as the main energy exchange source for cellular processes. Additionally, phosphorylation and dephosphorylation, or esterification and hydrolysis of phosphate groups on organic macromolecules, play vital roles in many enzymatic reactions and signaling mechanisms. More recently, an increased concentration of inorganic phosphate in the extracellular space was reported as a tumor marker in mice, differing from healthy tissue even more dramatically than pO_2 and pH_e[3]. Also, serum from patients with cancer had more than twice the amount of phosphorus from P_i than healthy patients[23, 24]. A major theory for this observation is related to the growth rate hypothesis (GRH), indicating that the tumor requires greater amounts of phosphorus to make components for excess proliferation[25]. P_i also may play a role in metastatic mechanisms as the concentration of interstitial phosphate has demonstrated a direct correlation with the metastatic potential of the cancer[3]. One aspect of the metastatic potential may be explained by the increased uptake of P_i which may enhance the ability of malignant cells to migrate and adhere[26]. The effects of phosphate on therapeutic response are still unclear, with studies showing differing results.

Figure 1. Summarized diagram of pO_2, pH, and [P_i] in the tumor microenvironment.

2.4 Importance for measurement and correlation

Hypoxia, acidosis, and elevated [P_i] are all strong indicators of malignancy, and they appear to have both cause-and-effect roles in tumor growth and aggression. Measurement of these parameters can be an instrumental tool in cancer research and therapy—studying the many mechanisms in tumorigenesis and metastasis, predicting outcomes, and optimizing therapeutic interventions. The ability to measure all three, pO_2, pH_e, and [P_i], unlocks even more potential investigations regarding the correlation between the variables.

Because these parameters can have significant temporal and special variance, more accurate correlation analysis would require the measurements to be made simultaneously and from the same location.

3 Measurement Techniques

There are currently methods for measuring each of these parameters in tissue independently. All these methods have their strengths and weaknesses. To the best of our knowledge, the only technique that allows for the simultaneous measurement of all three parameters of interest in vivo is Electron Paramagnetic Resonance (EPR) paired with a multifunctional radical probe. The following paragraphs aim at providing a short overview of the main techniques to measure pO_2, pH, and $[P_i]$ in vivo.

3.1 Oxygen Measurement

There are quite a few methods for measuring tissue oxygenation in vivo. One of the most common methods for measuring pO_2 is the Eppendorf polarographic electrode. This method can be used for in vivo tissue measurement using a microelectrode. However, this technique is invasive and consumes oxygen, which does not allow for repeated measurements in the same location[8]. Currently, there is no oxygen electrode available and suitable for clinical measurement of tumors[5].

Another common oxygen sensing technique involves the use of a fiber-optic probe that measures oxygen-dependent fluorescence quenching. This method has the advantage of high sensitivity at low oxygen partial pressures and does not require regular calibration but is also invasive as it requires the insertion of the probe into the area of interest.[8] A related optical method utilizes oxygen-dependent phosphorescence quenching of a phosphorescent agent that can be injected in vivo. This method allows for tissue oxygenation measurement to only a few millimeters depth because of the light penetration ability, but some methods and agents have been developed to utilize deeper penetrating X-rays[5].

Some drugs or markers selectively respond to hypoxic tissue. Bioreductive markers, such as those containing a 2-nitroimidazole moiety, are reduced selectively by hypoxic cells[8]. The reduced form covalently binds to thiols or amines of cellular components and therefore accumulates in hypoxic tissues. Pimonidazole (Hypoxyprobe) is a popular biomedical tool to image hypoxic tissue ex-vivo. However, this method requires hours of consistent hypoxia to viable cells[5]. PET tracers containing a nitroimidazole (e.g., [^{18}F]FAZA) are used clinically to track hypoxic regions non-invasively[27].

One way to indirectly measure tissue oxygenation is to measure oxygen in the blood. There is a strong relationship between the pO_2 in the blood and the oxygen saturation of hemoglobin (Hb), so techniques that can detect differences between oxyhemoglobin (HbO_2) and deoxyhemoglobin (dHb) can provide vascular pO_2[8]. However, vascular pO_2 does not translate well to tissue pO_2 because of the variable nature of perfusion and diffusion, especially in tumors, and the presence of myoglobin in some tissue[5]. The most clinically available method currently is pulse oximetry, which uses near-infrared range spectroscopy (NIRS) to measure the concentration of HbO_2 to total hemoglobin. It does have the major advantages of being inexpensive, real-time, and non-invasive.

Another technique that targets vascular oxygen is blood oxygen level-dependent (BOLD) MRI, where dHb acts as an endogenous contrast agent because of the paramagnetic electrons in Fe^{2+} [8]. Because BOLD contrast is only a function on the amount of deoxyhemoglobin, quantitative measurement of blood pO_2 is very challenging, but BOLD MRI may provide valuable insight into changes in blood oxygen or volume and is clinically available and non-invasive[5].

A more direct method for measuring tissue oxygenation uses ^{19}F-NMR probes. Molecular oxygen affects the NMR relaxation times of these probes. Therefore, biocompatible perfluorocarbons (PFOB, HFB, etc.) can be injected in vivo, and the relaxation times measured to derive the concentration of oxygen[8]. While MRI is readily available in most clinical settings, the use of ^{19}F-NMR spectroscopy and imaging is not yet available for clinical practice[5]. NMR-based methods are also limited by low resolution and very low sensitivity which is a major hurdle for exogeneous perfluorocarbons probes [28, 29].

Finally, electron paramagnetic resonance (EPR) oximetry operates on a similar principle as ^{19}F-NMR oximetry—molecular oxygen shortens the spin relaxation times of a paramagnetic probe. It results in a broadening of the EPR line of the probe. For soluble EPR probes, there is a linear relationship between the EPR linewidth of the probe and the oxygen concentration.[8] EPR oximetry is non-invasive and highly sensitive but does require the introduction of paramagnetic material or probes. Fortunately, the high sensitivity (≈1000 times more sensitive than ^{1}H-NMR) allows for smaller amounts of spin probe than would be required for NMR. EPR oximetry is available for clinical use with particulate paramagnetic material, but clinical EPR instruments are not widely accessible today, and no soluble probes have been approved yet for use in humans[5].

3.2 pH Measurement

One of the most common and oldest methods for measuring pH is with electrodes, and the glass electrode is generally used as the gold standard to calibrate and evaluate new methods[30, 31]. Most pH electrodes work by measuring the difference in potential across a membrane which depends on the concentration of H^+ compared to a reference electrode. In general, pH electrodes have high sensitivity, accuracy, and pH response range between 2 and 9[32]. However, these electrodes are temperature-dependent and often suffer from stability issues and drift, requiring frequent calibrations[32, 33]. Microelectrodes have been made with various materials (glass, polymers, silicon, metal) in attempt to optimize biostability, biocompatibility, reliability, and accuracy for in vivo use[32, 34]. Still, these microelectrodes are limited to superficial readings and are invasive.

Optical-based methods are another common class of techniques for measuring pH in vivo. Generally, colorimetric or fluorescent pH indicator dyes are suspended in a solid matrix on an optical fiber-based sensor[33]. The optical fiber sensors can provide real-time blood or interstitial pH but can still experience drift from photobleaching[33]. These methods are also invasive, requiring implantation of the fiber into the area of interest. Therefore, the biocompatibility of the fiber, dye, and matrix is essential.

The first non-invasive method of measuring pH is with positron emission tomography (PET). Radiotracers will distribute between the extracellular and intracellular space depending on the pH and ion trapping of the cell membrane. Because the basis of the measurement is based on the affinity of the radio-labeled compound to cross the membrane, it can be imprecise and inaccurate.[35]

Magnetic Resonance Imaging (MRI) and spectroscopy (MRS) provide more valuable methods for measuring pH non-invasively. Specific gadolinium contrast agents have been developed with relaxivities modulated by the pH, providing pH-weighted MRI images[36] . Also, contrast agents for the chemical exchange saturation transfer (CEST) MRI modality have also been utilized for pH mapping in vivo[20, 35]. The major limitations to CEST-based MRI are that it requires relatively high concentrations (>10 mM) of contrast agent and a very stable magnetic field[35]. A clinically available way of measuring pH is by ^{31}P-NMR spectroscopy. The chemical shift between endogenous P_i and creatine phosphate on the MR spectrum can be used to calculate intracellular pH (pH_i)[37]. However, this method depends on the concentration of Pi, which is variable, lacks resolution, and is affected by ionic strength[38, 39]. In order to measure extracellular pH (pH_e), a cell impermeable ^{31}P-NMR probe such as 3-aminopropylphosphonate (3-APP) needs to be injected[40]. 3-APP is highly biocompatible but has not been approved for use in humans[40-42]. More recently, 2-imidazole-1-yl-3-ethoxycarbonyl (IEPA) was developed as a pH-sensitive probe for ^1H-NMR[35]. The poor sensitivity of NMR techniques can be enhanced through dynamic nuclear polarization (DNP), which has been used to enhance the NMR signal of ^{13}C-labeled bicarbonate for pH measurement[35]. However, DNP suffers from the fast decay of hyperpolarization (in only a few minutes) and high instrumental, operation costs, and complexity of the technique.

EPR has an advantage over NMR by having a much larger analytical sensitivity and the absence of background signal. Similar to NMR, changes in pH caused shifts in peaks field positions and hyperfine splittings[38]. Early non-invasive EPR measurements of pH in vivo utilized pH-sensitive nitroxide radicals[43, 44], but most nitroxides are quickly reduced in vivo to EPR-silent hydroxylamines[45]. Various trityl radicals, which have higher redox stability in vivo, have been shown to have an EPR spectrum sensitivity to both pH and oxygen[38, 39, 46-48]. Neither nitroxides nor trityl radicals are available for clinical use to date.

3.3 Phosphate measurement

Inorganic phosphate and phosphate-containing metabolites have been often measured in tumors using ^{31}P-NMR spectroscopy to differentiate from healthy tissue and to study growth mechanisms[41, 49-51]. The ^{31}P-NMR peaks are assigned to the different phosphates on NTP and NDP, phosphocreatine, phosphodiester, phosphomonoester, and inorganic phosphate, and the integrals of the peaks inform on the relative amount of each phosphorus-containing compound[50, 51]. However, this method using endogenous phosphorus does not allow for discrimination between extracellular and intracellular ^{31}P signals, and results in tumor tissue have been highly variable and unclear[3].

In clinical practice, P_i is often measured in serum, plasma, blood, and urine samples using a colorimetric assay based on a complex between P_i and molybdate with a recording of the absorbance at 340 nm[24]. While phosphate is also generally elevated in the blood of patients with cancer[23], it is not an accurate representation of the local $[P_i]$ in the tumor. Microdialysis and colorimetric quantification of the extracellular $[P_i]$ can be performed in pre-clinical settings, but this technique is invasive and difficult to implement[52].

Within the past decade, a monophosphonated trityl radical EPR probe, pTAM, was developed for concurrent measurement of pO_2 and pH_e, and it was also found to have spectral sensitivity to $[P_i]$[48, 53, 54]. With

increasing [P_i], the proton exchange rate between the ionic species of pTAM increases and causes the EPR lines to coalesce[48]. The exchange rate can be calibrated against [P_i][3, 53, 54]. Therefore, with pTAM and EPR spectroscopy, oxygen, pH, and [P_i] can be measured in vivo concurrently and non-invasively with high sensitivity[3]. Because all three parameters are derived from the same probe and not from multiple probes with different potential localization, pTAM allows for correlation analysis of these three parameters.

Table 1 summarizes the methods to measure pO_2, pH and [P_i] with their advantages and limitations.

Method	Advantages	Limitations	pO_2	pH	[P_i]	Concurrent
Microelectrode	- high accuracy - high sensitivity	- invasive - oxygen-consuming - frequent calibration	X	X		
Fiber-optic fluorescence	- high sensitivity	- invasive - photobleaching	X	X		
Fluorescence/ phosphorescent imaging	- High resolution - non-invasive	- low penetration depth	X			
18F-FAZA PET (nitroimidazole)	- clinically available - non-invasive	- requires use of radioactive material	X			
NRIS	- inexpensive - real-time - non-invasive - clinically available	- blood fraction only	X			
BOLD-MRI	- non-invasive - clinically available	- not direct pO_2 measurement - blood fraction only	X			
19F-MRI	-- non-invasive	- low sensitivity	X			
pH-sensitive radiotracer PET	- clinically available -non-invasive	- imprecise - low accuracy		X		
CEST-MRI	- clinically available - non-invasive	- low sensitivity		X		
31P-MRI	- no exogenous compound required (except for pH_e discrimination)	- low sensitivity - hard to discriminate between intracellular and extracellular P_i		X	X	X
Hyperpolarized 13C-MRI	- non-invasive	- limited measurement time - high operational cost and complexity		X		
Colorimetric Assay with Molybdate	- clinically available	- blood or urine fraction			X	
EPR	- high sensitivity - non-invasive - extracellular specific	- low resolution	X	X	X	X

Table 1. Comparison of methods for in vivo measurement of the pO_2, pH, and [P_i].

4 EPR

Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR) is based on the same principle as the most common nuclear magnetic resonance (NMR) technique, with a focus on the electron spins instead of the nuclear spins. Therefore, EPR applies to species with free electrons. A major difference between EPR and NMR is that the magnetic moment of the electron is 659 times greater than that of the proton, providing a much higher sensitivity[8].

The most common EPR spectrometers operate at X-band (around 9.5 GHz) frequency. At this frequency, the non-resonant absorption of aqueous samples limits the penetration depth of the electromagnetic radiation to about 1 mm, insufficient for in vivo applications. Low-frequency EPR spectrometers (<1 GHz) allows for higher penetration depth but comes at a decrease in sensitivity.[8]

Another significant difference between NMR and EPR is the need for paramagnetic materials. With the exception of melanin, there are no endogenous species present in sufficient quantities to be detected directly by EPR in vivo. Therefore, in vivo applications of EPR require the injection of exogenous paramagnetic compounds or probes. In contrast, MRI detects the protons of endogenous water molecules which are present in large concentrations (>50M), allowing this method to thrive for in vivo applications despite its much lower sensitivity. The most popular application of in vivo EPR is EPR oximetry. While oxygen is paramagnetic with two unpaired electrons, its fast relaxation makes it undetectable in solution by EPR (the lines are too broad)[8]. However, the collisions between oxygen and a paramagnetic compound, introduced as probe, result in a shortening of the probe's relaxation times and a broadening of the EPR line (Figure 2) of the probe[55]. After calibration in vitro, the EPR linewidth of a probe measured in vivo reports the oxygen concentration in the vicinity of the probe.

Interaction between molecular oxygen and spin probe ➡ Induces reversible changes in the EPR linewidth
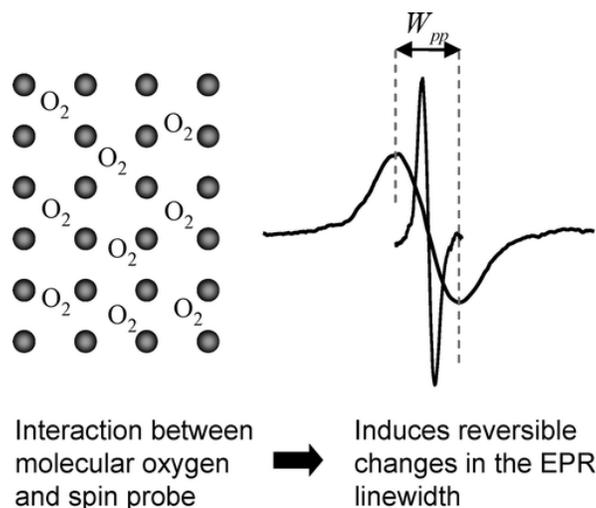
Figure 2. Effect of molecular oxygen on the EPR linewidth of a paramagnetic spin probe. Reproduced from Ahmad et. al.[55].

4.1 Paramagnetic Materials

There are two main categories of paramagnetic materials used for oxygen measurement, namely particulate and soluble probes.

Particulate paramagnetic substances, such as coals, chars, India ink, and lithium phthalocyanine, octa-n-butoxynaphthalocyanine (Figure 3A), have a high electron spin density and very high sensitivity to oxygen[8, 55]. They are also very stable and, once implanted in vivo, allow for repeated measurements at the same site over months. India ink and lithium octa-n-butoxynaphthalocyanine embedded in an FDA-approved biocompatible polymer (PDMS) are currently being used in clinics (Figure 3B)[5]. However, because of the variable nature of these materials, the EPR properties can vary between batches, and calibration of the oxygen sensitivity is required for each batch. Also, the high sensitivity of the probes to oxygen results in a significant loss in signal-to-noise ratio in normoxic conditions (as the intensity of the signal is inversely proportional to the square of the linewidth).[8]
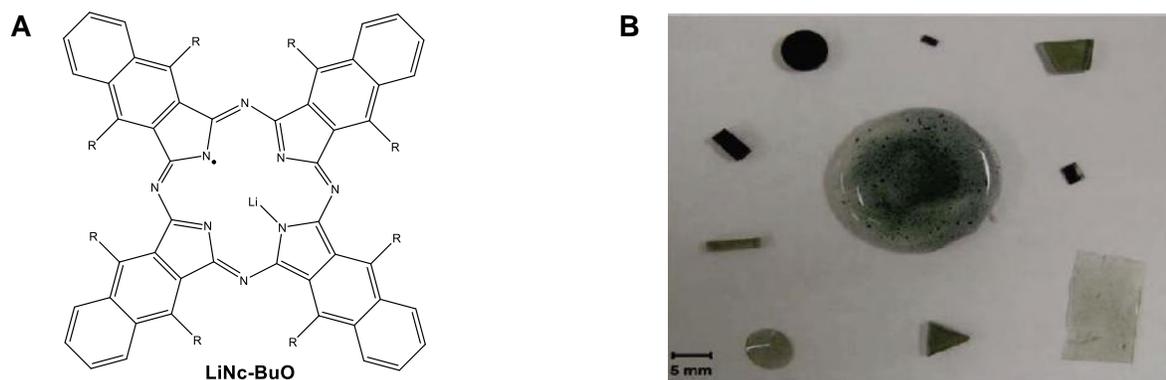
Figure 3. **A**. Structure of LiNc-BuO and **B**. LiNc-BuO emended into a gas permeable polymer. Reproduced from Ahmad et. al.[55].

Soluble probes include nitroxides and trityl radicals (Figure 4). Nitroxides radicals have been first developed for EPR applications. The well-developed chemistry of nitroxides allowed for the synthesis of a large variety of structures with functional sensitivity to important parameters such as oxygen, pH, enzyme activity, redox status, or reactive oxygen species or with higher resistance to reduction. The main limitation of nitroxides is their fast bioreduction in vivo (a few seconds to a few minutes)[45].
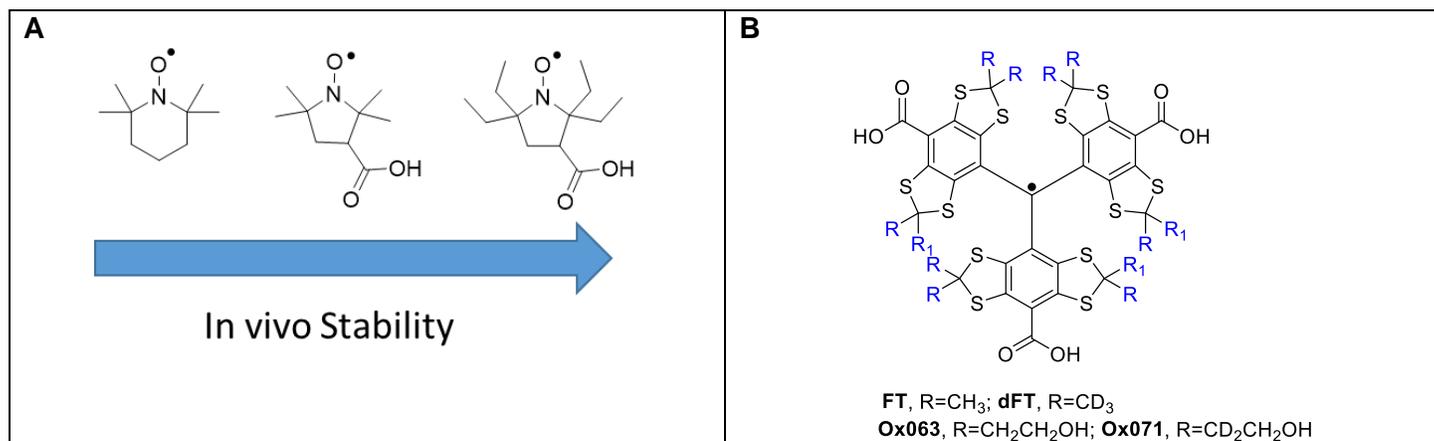
Figure 4. Structures of **A**. nitroxides and **B**. trityl radicals used for biomedical EPR.

On the other hand, triarylmethyl (TAM) or trityl radicals (Figure 4B) have extraordinary stability in vivo, with half-lives in blood in the range of hours to days and demonstrated resistance to most biological oxidoreducants[56]. Trityl radicals also have the advantages of longer relaxation time and narrower line widths, which allows for a higher signal-to-noise ratio, which is critical for in vivo application, and higher functional sensitivity[8]. The most popular structures are the Finland Trityl (FT), OX063, and their deuterated analogues dFT and OX071 (Figure 4B) developed in the late 90s by Nycomed Innovation (now subsidiary of GE Healthcare)[57]. Both structures exhibit extraordinary stability in biological media, high water solubility, and single-line EPR spectrum. Because of those features, trityl radicals are considered superior probes for biomedical EPR applications.

However, FTs and their derivatives have some major limitations for biological applications. Firstly, the lipophilic triarylmethyl core of the trityl is responsible for lipophilic interaction with biomacromolecules such as albumin[58]. This binding results in a broadening of the EPR line, which significantly reduces the signal intensity. FT and dFT both showed an 80% loss in signal intensity with equal molarity of trityl radical and bovine serum albumin[58, 59]. OX063 and OX071, on the other hand, are very hydrophilic with the addition of twelve hydroxyl groups, eliminating albumin binding and showing no loss in signal[59]. FTs and their derivatives are also toxic upon intravenous injection, with the doses required for spectroscopy being lethal, which prevent their systemic delivery[60]. However, in vivo measurements have been successfully made with small dose intratissue injections[3]. Again, OX063 and OX071 resolve this limitation with extremely low toxicity (LD_{50}=8mmol/kg in mice) that is well above the required systemic dose for in vivo studies[61-63]. While the synthesis of FT was reported 20 years ago[64], the synthesis of OX063/71 remained a trade secret for more than 25 years. For this reason, most of the synthetic efforts for the development of trityl radicals for biological EPR applications relied on the modification of the Finland trityl. Conjugating FT to dextran or polyethylene glycol (PEG) eliminated albumin binding but still lacked in biocompatibility and water solubtility[65]. Replacing the carboxylates with sulfonates did significantly improve water solubility and reduced the affinity for albumin[59]. However, OX063/OX071 are still unmatched for their high aqueous solubility, low toxicity, and absence of interaction with blood biomacromolecules[59, 61]. Fortunately, our lab reported an efficient synthesis for OX063 and the deuterated OX071 in 2019[66], making biocompatible trityl EPR probes more accessible for in vivo research and enabling the development of derivatives with enhanced functionality.

4.2 Trityl Derivatives

Over the last 20 years, several derivatives of trityl radicals have been developed with extended sensitivity to important parameters such as pH[38, 46-48], redox status[67], thiol concentration[68], viscosity[69], and inorganic phosphate concentration[53]. The concept of dual-function trityl pO_2/pH probes was introduced by the group of Dr. Khramtsov in 2007[38]. Since then, creative synthetic efforts and optimizations lead to a mono-phosphonated

triarylmethyl radical (pTAM or HOPE) as a multifunctional EPR probe to measure pO$_2$, pH, and [P$_i$] concurrently (Figure 5).

pTAM is a simplified doublet with good water solubility and a pH-sensitive shift in hyperfine splitting that is independent of the frequency[48]. This phosphonated trityl also showed a sensitivity to [P$_i$] because the rate of proton exchange is affected by the amount of phosphate, and when the exchange rate increases, the peaks of the two ionic states coalesce[47, 48, 53]. Importantly, pTAM has been used successfully to measure pO$_2$, pH, and [P$_i$] simultaneously in mouse models of breast cancer after intratissue injection[3]. This led to the identification of inorganic phosphate as a possibly powerful marker of tumors and metastatic character[3]. Also, the concurrent measurement allowed for true correlation analysis because it eliminates the temporal and location variances that would arise from using multiple methods or multiple probes. However, because pTAM is derived from FT, it has the biological limitations of binding to albumin and intravenous toxicity, which limits delivery to intratissue injection. Therefore, a major and clear improvement that can be made to the monophosphated trityl radical is better biocompatibility.
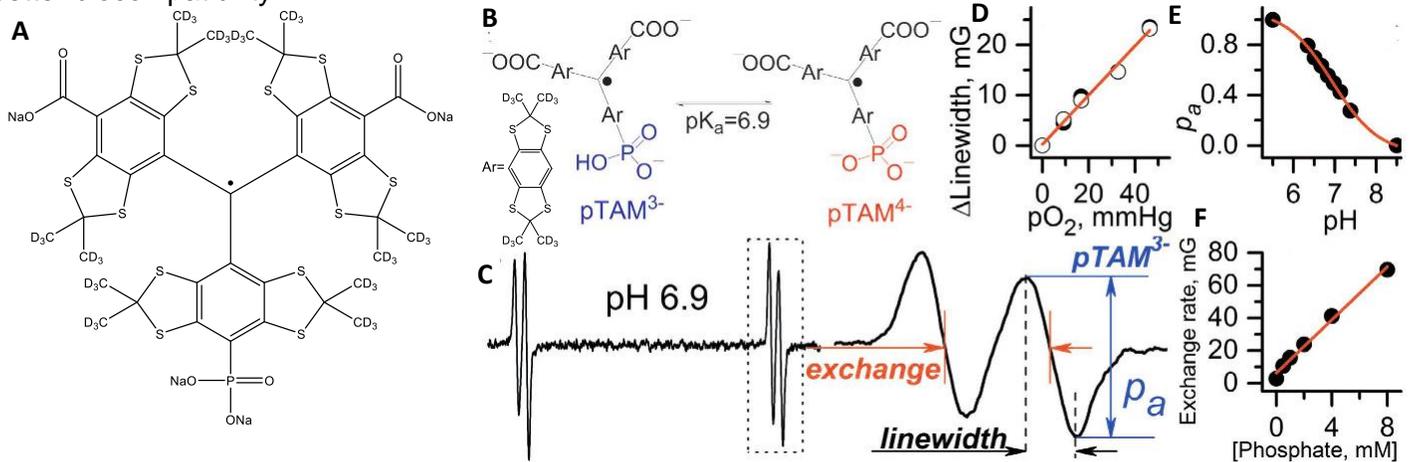
Figure 5. **A**. Structure of pTAM. **B.** Equilibrium of two ionic states of pTAM. **C**. Full EPR spectrum of pTAM and a zoom in on the high-field component with summary of effects of pO$_2$, pH, and [P$_i$] on spectral shape. **D**. Relationship between pO$_2$ and EPR linewidth. **E**. Fraction of the acidic state (pTAM^{3-}) against pH. **F**. The H$^+$ exchange rate depends on the concentration of P$_i$. **B-F** Reproduced from Bobko et. al.[3]

5 Summary

Oxygenation (pO$_2$), pH, and inorganic phosphate [P$_i$] are all important parameters of the tumor microenvironment that can inform on tumorigenesis, metastatic character, and therapeutic response. The ability to measure all three variables can help to better understand the tumor story, and simultaneous measurement with the same probe allows for deeper investigation with correlation analysis. EPR spectroscopy and imaging with the multifunctional probe, pTAM, provides a non-invasive way to concurrently measure pO$_2$, pH, and [P$_i$]. However, pTAM has major drawbacks in biological applications, and biocompatibility is a significant area for improvement.

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CHAPTER 2 – Biocompatible Monophosphonated Trityl Spin Probe, HOPE71, for In Vivo Measurement of pO₂, pH, and [P$_i$] in Mouse Tumors by Electron Paramagnetic Resonance Spectroscopy

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Biocompatible Monophosphonated Trityl Spin Probe, HOPE71, for In Vivo Measurement of pO₂, pH, and [P$_i$] in Mouse Tumors by Electron Paramagnetic Resonance Spectroscopy

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ABSTRACT

Hypoxia, acidosis, and elevated inorganic phosphate concentration are characteristic of the tumor microenvironment in solid tumors. There are a number of methods for measuring each parameter individually in vivo, but the only method to date for non-invasive measurement of all three variable simultaneously in vivo is electron paramagnetic spectroscopy paired with a monophosphonated trityl radical, pTAM. While pTAM has been successfully used for in vivo studies upon intratissue injection, it cannot be delivered intravenously due to systemic toxicity and albumin binding, which causes significant signal loss. Therefore, we present HOPE71, a monophosphonated trityl radical derived from the very biocompatible trityl probe, OX071. Here we present a straightforward synthesis of HOPE71 starting with OX071, and report its EPR sensitivities to pO₂, pH, and [P$_i$] with X-band and L-band EPR spectroscopy. We also confirm that HOPE71 lacks albumin binding, shows low cytotoxicity, and has systemic tolerance. Finally, we demonstrate its ability to profile tumors in vivo with a longitudinal tumor growth study.

Introduction

In recent years, the tumor microenvironment (TME) has gained focus in cancer research because it can hold key information about into tumorigenesis, immune escape, and therapeutic response. Several key physiological parameters of the TME that are of interest include tissue oxygenation (pO_2), extracellular pH (pH$_e$), and interstitial inorganic phosphate concentration [P$_i$]. Hypoxia ($pO_2 <10mmHg$) and acidosis (pH$_e <7$) have been recognized as hallmarks of solid tumors for quite some time, and elevated [P$_i$] was more recently identified as a biomarker of the TME[1]. Hypoxia occurs in tumors because of increased oxygen consumption along with decreased oxygen delivery. Cancer cells demand higher amounts of oxygen to support their fast metabolism and proliferation rates[2]. Furthermore, irregular tumor vasculature fails to delivery sufficient oxygen because of increased distance between cells and blood vessels (diffusion-limited) and temporary drops blood flow (perfusion-limited)[3]. Hypoxia can lead to aggressive disease with more metastatic character[4-6] and pathways for immune escape[7, 8]. Tumor hypoxia is also associated with therapeutic resistance, especially in radiation therapy. The presence of oxygen improves radiosensitivity, as described by the oxygen enhancement effect, and, as a result, hypoxic cells require up to three times the therapeutic radiation dose as normoxic cells[9, 10]. Cancer cells also demonstrate a high rate of glycolysis, even in well-oxygenated conditions, which leads to high amounts of lactate and protons being excreted from the cells, which may not be efficiently removed by tumor vasculature[5, 11, 12]. Acidosis has also been linked to more aggressive disease and metastatic potential[5], and acidic extracellular pH may also affect the absorption and effectiveness of chemotherapy drugs. Finally, inorganic phosphate may be elevated in the TME due to high growth rate demands for nucleotides, phospholipids, and cell-signaling[13]. Also, phosphate concentration demonstrated the ability to discriminate between metastatic and non-metastatic disease[1].

Therefore, measurement of pO_2, pH$_e$, and [P$_i$] is crucial for better understanding tumorigenesis, aggressive disease mechanisms, and therapy resistance. They also have the potential to play a role in optimizing therapy strategies clinically. There are various methods for measuring each parameter individually, and they all have their benefits and limitations, with the most notable drawbacks being invasiveness and low sensitivity. To to our knowledge, Electron Paramagnetic Resonance (EPR) Spectroscopy with a monophosphonated trityl probe, pTAM (Fig. 1), is the only method for non-invasive measurement of all three parameters concurrently. The EPR spectrum of pTAM is a doublet with a linewidth sensitive to oxygen and hyperfine splitting is sensitive to pH. The signal is also sensitive to [P$_i$] because the concentration of phosphate buffer affects the proton exchange rate on pTAM, which causes the different ionic species peaks to coalesce[4, 14, 15]. However, pTAM is a derivative of deuterated Finland trityl (dFT, Fig. 1), which has major limitations for biological applications. FTs bind to albumin which drastically reduces the signal intensity and may increase the line width[16, 17]. FTs are also toxic upon intravenous injection[18], which limits in vivo work to intratissue delivery.

Alternatively, OX063 and the deuterated OX071 (Fig. 1), trityl probes with twelve hydroxylethyl groups, are very hydrophilic and does not bind to albumin[17]. They also have a remarkably low toxicity (LD$_{50}$=8mmol/kg) in mice and has been used broadly for in vivo OMRI and EPR oximetry studies[19-22]. Therefore, we propose HOPE71 (Fig.1), a monophosphonated derivative of OX071 as a more biocompatible multifunctional trityl probe for concurrent EPR measurement of pO_2, pH$_e$, and [P$_i$]. First, we report a straightforward synthesis of HOPE71 with a 2-step process starting from OX071. Then, we characterize the EPR sensitives to pO_2, pH$_e$, and [P$_i$] with X-band and L-band spectroscopy. Next, we present biocompatibility improvements by showing the difference in albumin binding, performing cell viability assays, and testing intravenous tolerance in mice. Finally, we demonstrate the ability of HOPE71 to profile mammary gland tumors in mice models and report a longitudinal study indicating that TME pH increasing deviates from healthy tissue with tumor growth.

Figure 1. Structure of Finland trityl (FT), deuterated FT (dFT), pTAM, OX063, OX071 and HOPE71.

FT : $R_1=CH_3$, $R_2 = COOH$
dFT : $R_1=CD_3$, $R_2 = COOH$
pTAM/HOPE : $R_1=CD_3$, $R_2 = PO_3H_2$
OX063 : $R_1=CH_2CH_2OH$, $R_2 = COOH$
OX063d24/OX071 : $R_1=CD_2CH_2OH$, $R_2 = COOH$
HOPE71 : $R_1=CD_2CH_2OH$, $R_2 = PO_3H_2$
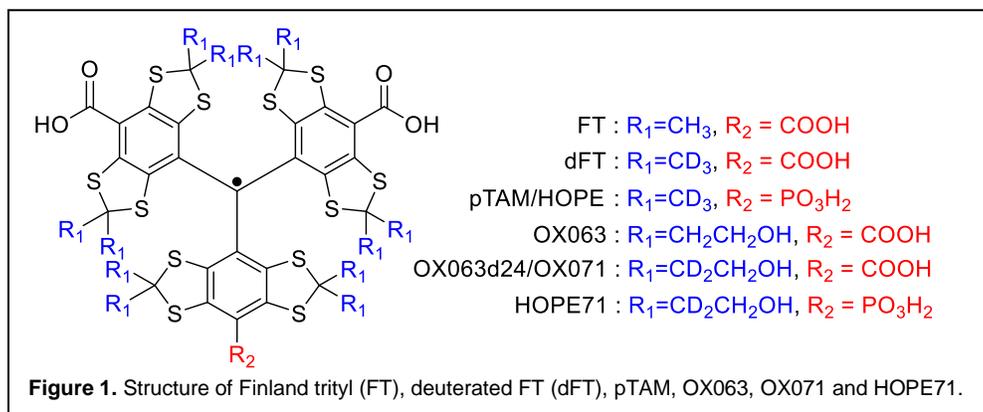
Materials and Methods
General
All reactions were carried out under argon in flame-dried glassware using anhydrous grade solvents, except for the phosphorylation of OX071 (1st step), which was performed in an aerated aqueous solution. All commercially available reagents were purchased from Sigma-Aldrich or Fisher Scientific and used as received without further purification. OX071 was synthesized according to a published protocol[23]. X-Band and L-Band EPR spectra were recorded using a Bruker Elexsys E580 (9.5 GHz) and a Magnettech (1.2 GHz) spectrometers, respectively. For X-band spectra, 50 µL of the solution was filled into a gas-permeable Teflon tube (1.14 mm diameter and 60 µm wall thickness) from Zeus, Inc. and the temperature and the nitrogen/oxygen gas mixture were controlled inside the resonator using a Noxygen temperature and gas controller. Gas was flushed for at least 10 min before the measurement or until the spectrum becomes time independent. For L-Band spectra, 800 µL of the solution was filled into a 1.5 mL conical tube. The temperature was controlled using a circulation thermostat. Nitrogen/oxygen gas mixture form the gas controller was bubbled into the solution for 25-30 min before the measurement. HRMS spectra were recorded on a Thermofisher Scientific Q Exactive Mass Spectrometer with an Electron Spray Ionization (ESI) source. HPLC-MS analyses were carried out using a Water Alliance e2695 separation module, a Water 2998 PDA detector, and a Water SQD2 mass detector. Purifications on C18 column were carried out using a CombiFlash Rf+ purification system using water (containing 0.1% TFA) and acetonitrile (containing 0.1% TFA). Freeze drying was carried out using a MartinChrist Alpha 2-4 LSCbasic. A Thermo Scientific Orion Star A111 Benchtop pH meter with a Fisherbrand accumet Micro Glass Combination Electrode was used and was calibrated with 4.00 and 9.00 buffer solutions from Fisher Chemical before the experiments.

Synthesis
Step 1: Monophosphorylation of OX071. The starting material, OX071 tri-sodium salt (959 mg, 0.661 mmol, 1 eq.), was dissolved to a concentration of 1 mM in Na-phosphate buffer (10 mM, pH=7.4, 660 mL). Then, a solution of potassium hexachloroiridate(IV), K_2IrCl_6, (940 mg, 1.946 mmol, 3 eq.) in deionized water (70 mL) was added to generate the triarylmethyl cation; the green solution turned deep green-blue. The reaction was stirred 10 seconds, and triethyl phosphite, $P(OEt)_3$, (2.195 g, 13.210 mmol, 2.27 mL, 20 eq.) was added. The reaction mixture was stirred for 10 minutes, and the conversion checked by HPLC-MS. The mixture was acidified to pH ≤ 2 using trifluoroacetic acid and loaded into a C18 loading cartridge (25 g). The crude product was purified by reverse-phase chromatography using a C18 column (86 g) with a gradient of acidic water/acetonitrile (95/5 to 85/15) and freeze-dried. 138 mg of **2** was recovered as a green solid (14% yield). 431 mg (47%) of OX071 was also recovered.

Step 2: Deprotection of the phosphonic acid. Diethyl phosphonate HOPE71 **2** (138 mg, 0.094 mmol, 1 eq.) was dissolved in anhydrous dimethylformamide (15 mL) under argon. The solution was cooled to 0°C, and bromotrimethylsilane, TMSBr, in excess (1mL) was slowly added. After stirring for 10 minutes at 0°C, the reaction was heated at 50°C for 5 hours, and the deprotection was monitored by HPLC-MS. The TMSBr was removed under reduced pressure. Methanol (10 mL) was added, stirred 1 min, and removed under reduced pressure. The DMF solution was diluted 20 times with deionized water and freeze-dried. The final product was purified using a C18 column (26 g) with a gradient from acidic water to acidic water/acetonitrile (80/20) and freeze-dried. The residue was dissolved in water and titrated to pH=7 with NaOH (0.1M) and freeze-dried again to afford 121 mg (88%) of HOPE71 as a green powder.

Spectral sensitivity to oxygen, pH, and inorganic phosphate.
X-band Acquisition parameters. For each condition, a focus on the low-field part of the spectrum was recorded six times. For certain conditions (identified below), a full spectrum was recorded three times. Settings were as follows: power; 0.04743 mW, mod. freq.; 30 kHz, mod. Ampl.; 50 mG, conv. time; 30.01 ms, number of points; 2054 for the full spectrum and 1024 for the low-field spectrum, sweep width; 6.0 G for the full spectrum and 2.0 G for the low-field spectrum. All spectra were measured at 37°C.

L-band Acquisition parameters. For each condition, a focus on the low-field part of the spectrum was recorded four times. Parameter settings for the spectrometer were as follows: mod. freq.; 100 kHz, mod. Ampl.; 0.005 mT,

scan time; 30s, number of points; 4096, sweep width; 0.0960 mT and non-saturating power. All spectra were measured at 37°C.

Spectral fitting. A MATLAB-based application developed in house with graphical user interface for a non-linear fitting function (lsqcurvefit) was used to fit each EPR spectrum[24](Fig. S2). Fitting was performed on the low-field focused spectra. The following values were determined as constants for the spectral modelling: Gaussian linewidth, individual intrinsic Lorentzian linewidth of the acidic and basic peaks, and the distant between the acidic and basic peaks. The Gaussian linewidth and intrinsic Lorentzian linewidths were determined by sextuplicate EPR spectra measured with no oxygen, and with acidic (pH < 5) or basic (pH > 10) conditions so that only the acid or basic peak was present. The acidic to basic peak distance was determined by sextuplicate EPR spectra measured with no oxygen and no phosphate buffer at pH≈7. The variable parameters that also contributed to or were dependent on the curve-fitting include oxygen-induced Lorentzian linewidth broadening, proton exchange rate, and acidic fraction of the population.

X-band Oxygen calibration. To determine the linewidth sensitivity to oxygen for both ionic forms. 200 μM solutions of HOPE71 in 1 mM phosphate buffer and 137 mM NaCl at pH=4.8 and 10 were prepared, and spectra were recorded for pO_2 = 0 mmHg, 19 mmHg, 38 mmHg, 76 mmHg, 114 mmHg, and 159 mmHg. Full spectra were recorded for both pH=4.8 and pH=10 with pO_2 = 0 mmHg. The spectral fitting described above was used to determine the oxygen-induced line broadening for each pO_2. Linear fit using OriginLab allow for determination of the oxygen sensitivity for both ionic forms combined.

X-band pH calibration. To determine the pKa of the probe, a 200 μM solution of HOPE71 1 mM phosphate buffer and 137 mM NaCl was titrated by addition of small amounts of HCl and NaOH. For each titrated pH, spectra were recorded with pO_2 = 0 mmHg. Full spectra were recorded of pH=7.1. The spectral fitting described above was used to determine the acidic fraction at each measured pH. A plot of pH versus acidic fraction (P_a) was fitted to the equation $P_a = \frac{1}{1+10^{pH-pKa}}$ using OriginLab to determine the pKa.

X-band Inorganic phosphate calibration. To determine the exchange rate dependence to phosphate concentration, 200 μM solutions of HOPE71 at pH=7.2 and pO_2 = 0 mmHg. were recorded for phosphate concentration of 0 mM, 1 mM, 4 mM, 7 mM, and 10 mM. The spectral fitting described above was used to determine the exchange rate for each phosphate concentration. The proton exchange between phosphate and HOPE71 is express by the following equation:

$$HOPE^{3-} + HPO_4^{2-} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} HOPE^{4-} + H_2PO_4^-$$
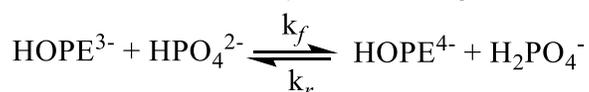
The rate of proton loss of HOPE[3-] to HPO₄[2-] is $k_a = k_f$ [HPO₄[2-]]. The rate constant k_f can be determine by linear approximation of the dependence of ka on [HPO₄[2-]] or the total phosphate concentration [P$_i$] using [HPO₄[2-]] = [P$_i$] (H^{+} + K$_a$[B])/(2πK$_a$[B]). Where K_a^B is the dissociation constant of phosphate buffer (K_a^B=10[-6.66])[25]. Linear fit of the equation $\frac{k_a(H^+ + K_a^B)}{2\pi K_a^B} = k_f[P_i]$, using OriginLab allows determination of the phosphate sensitivity.

Validation of the calibration with blind sample. (X-band). Three solutions with 200 μM of HOPE71 and 137 mM NaCl were prepared with various concentrations of inorganic phosphate (1-10 mM), pH and pO_2 that were that were blinded to the researcher acquiring the spectra. The spectral fitting described above was used to extract the oxygen-induced Lorentzian linewidth broadening, proton exchange rate, and acidic fraction. The calibrations above were used to determine the EPR measured pO_2, pH, and [P$_i$].

L-band calibrations and validation. The pO_2, pH, and [P$_i$] calibrations and validation were repeated for L-band EPR with 500 μM solution of HOPE71. Oxygen calibration spectra were recorded for pO_2 = 0 mmHg, 17 mmHg, 34 mmHg, 68 mmHg, 103 mmHg, and 159 mmHg. Phosphate calibration spectra were recorded on solutions with 0 mM, 1 mM, 4 mM, 7 mM, and 10 mM of inorganic phosphate.

Biological Application Characterizations.

Albumin binding. To study the interaction of HOPE71 with albumin, 200 µM solutions of HOPE71 at pH=7 were prepared, one with no bovine serum albumin (BSA) as control and one with 200 µM BSA (1 equiv.). For comparison, 200 µM solutions of pTAM at pH=7 were also prepared with 0 µM, 50 µM, 100 µM, and 200 µM BSA. Spectra were recorded for each solution with X-band EPR. Spectra of pTAM were acquired with mod. Ampl. = 25 mG.

MTT assay for cell toxicity. MDA-MB-231 (triple-negative breast cancer) cells were plated on a 96-well flat-bottom plate and allowed to grow over night to 60-70% confluency. The medium used was DMEM with 10% fetal bovine serum. The cells were then incubated with increasing concentrations of pTAM or HOPE71 in medium for 24 hours. DMSO (2% and 5% in medium) was used as a cytotoxic control. Medium with corresponding concentrations of pTAM or HOPE71 were used as a background control. All conditions were performed in quadruplicate. The MTT assay was performed using ThermoFisher Vybrant MTT Cell Proliferation Assay Kit according to the manufacturer's protocol. Absorbance was measured at 570 nm minus 630 nm to correct for cell debris. Statistically analysis was performed using one-way ANOVA. This process was repeated with HUVECs (Human umbilical vein endothelial cells) using vascular cell basal medium plus ATCC Endothelial Cell Growth Kit-VEGF.

In vivo Applications.

Mouse model. Female MMTV-PyMT (PyMT+/−) mice with spontaneous mammary tumors and their wildtype littermates (PyMT−/−), 10-15 weeks in age, were used for HOPE71 in vivo EPR studies. Mice were anesthetized by inhalation of isoflurane in air prior to injection and during acquisition of spectra.

In vivo L-band Acquisition parameters. For each in vivo EPR measurement of HOPE71, the resonator coil was placed on mammary gland 4 (MG4) or 9 (MG9). Focus on the low-field part of the spectrum was recorded six times. Parameter settings for the spectrometer were as follows: mod. freq.; 100 kHz, mod. Ampl.; 0.005 mT, scan time; 30s or 60s, number of points; 4096, sweep width; 0.0960 mT and non-saturating power.

Intravenous Tumor Profiling. Six female MMTV-PyMT mice, 10-15 weeks in age, with MG9 tumors approximately 1 cm in diameter (370-580 mm^3), were administered a bolus dose of HOPE71 in saline (90 µL 75mM, 10mg, 0.17-0.31 mmol/kg, pH=7) by retroorbital injection. EPR spectra focused on MG9 tumors were recorded immediately after injection.

Systemic Toxicity. Six mice from the intravenous tumor profiling were observed for apparent signs of toxicity for 7-10 days after injection. The mass of five mice was tracked during this time.

Longitudinal Tumor profiling. Eight female MMTV-PyMT mice were watched starting at 9 weeks old for tumor growth on mammary gland 9 (MG9). Once palpable, the tumors on MG9 were profiled by HOPE71 EPR once to twice per week (once for early stage; twice for late stage). Four female wildtype (PyMT−/−) littermates were also profiled on the same days. The mass of the mice and the tumor volume (V=L x W x D x 3.14/6 or V= L x W^2 x 3.14/6) was recorded along with each EPR measurement. HOPE71 in saline (15-50 µL 2mM, pH=7) was injected intratissually into the tumor or the fat pad mammary gland. EPR spectra were recorded immediately after injection. Correlation analysis was done using OriginLab software. T-tests were performed to detect differences in group means and slopes.

Intratissue versus intravenous injection. To detect if there are significant differences in the tumor profiling based on delivery method, the pO_2, pH, and [P_i] of tumors profiled with intravenous injection were compared to those of sized matched tumors profiled by intratissue injection. Two-tailed T-test for independent means was used test for statistically significant difference.

Results and Discussion.

Synthesis of HOPE71. In order to make pTAM more biocompatible, we planned to substitute a carboxylic acid on OX071 with a phosphonate group. The strategy was to use the *ipso* aromatic nucleophilic substitution reaction for trityl radicals reported by DeCroos et. al.[26]. The one electron oxidation of OX071 using potassium hexachloroiridate(IV) in phosphate buffer leads to the trityl cation, which was delocalized across the aromatic groups. Then, the nucleophilic attack of triethyl phosphite at the para-position triggers an oxidative decarboxylation leading to the mono phosphonated trityl radical. The recovery of OX071 indicates that the oxidant of the intermediate **1** is the trityl cation. Therefore, for this mechanism, the theoretical yield of substituted product is capped at 50%. Also, the trityl cation was found to be very short lived in aqueous solution, and the best yield came from adding the phosphite within 10 seconds of adding the hexachloroiridate. Adding the triethyl phosphite first degraded the phosphite before forming the trityl cation. The first step afforded 14% of **2**, the protected target compound (HOPE71). Bromotrimethylsilane efficiently deprotected the phosphonate to yield 88% of HOPE71 after purification. (Sch. 1)

Scheme 1. Synthesis of HOPE71 starting with OX071. Our strategy was based on the *ipso* aromatic nucleophilic substitution for TAM radicals reported by DeCroos, et al.[26].
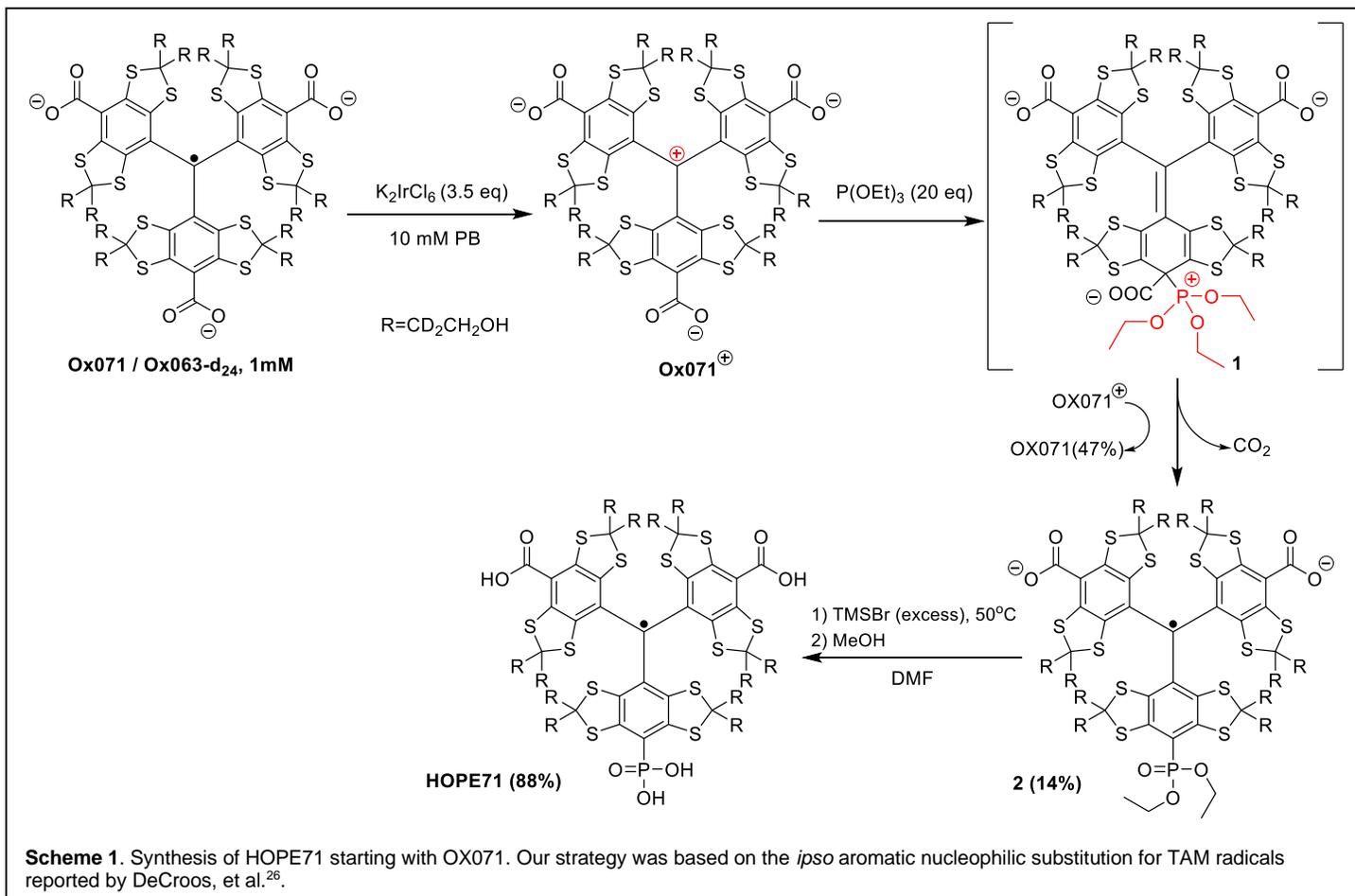
EPR Characterization of HOPE71. EPR characterization was first performed at X-band EPR. Fig. 2A shows the full spectrum doublet of HOPE71 at pH=5, 7, and 10. The phosphorus (I=1/2) causes hyperfine splitting that is dependent on the protonation state. HOPE71^{3-} has a greater hyperfine splitting constant (a_p = 3.54 G, pH=5) than HOPE71^{4-} (a_p = 3.26 G, pH=10) (Fig. 2).

Figure 2. EPR Spectrum and pKa of HOPE71. **A**. Full X-band EPR spectra for HOPE71 measured under nitrogen atmosphere and 37°C at pH 5, 7, and 10. The protonated species HOPE71^{3-} has a greater phosphorus hyperfine splitting than the deprotonated species HOPE71^{4-}, forming resolvable peaks for the different ionized species. **B**. The primary ionized species HOPE71^{3-} and HOPE71^{4-} are in equilibrium around physiological pH.

To determine the sensitivity to oxygen, low field spectra were recorded with increasing oxygen partial pressure on solutions of HOPE71 at pH=5 and 10. Fig. 3A demonstrates the effect of oxygen on the linewidth and signal intensity at pH=10. The extent of line-broadening was determined by fitting each spectrum, allowing the oxygen-induced Lorentzian linewidth to vary. The line-broadening has a strong linear correlation with oxygen partial pressure (0.51 mG/mmHg) (Fig. 3B).

Figure 3. X-band EPR Sensitivity to Oxygen. **A**. The low field component spectra measured at 37°C and pH 10 with 0, 19, 38, 76, or 114 mmHg partial pressure of oxygen (pO_2). **B**. The linear relationship between oxygen partial pressure (mmHg) and oxygen-induced line-broadening (mG) for pH=5 and pH=10 is 0.51±0.05 mG/mmHg (R^2=0.998).
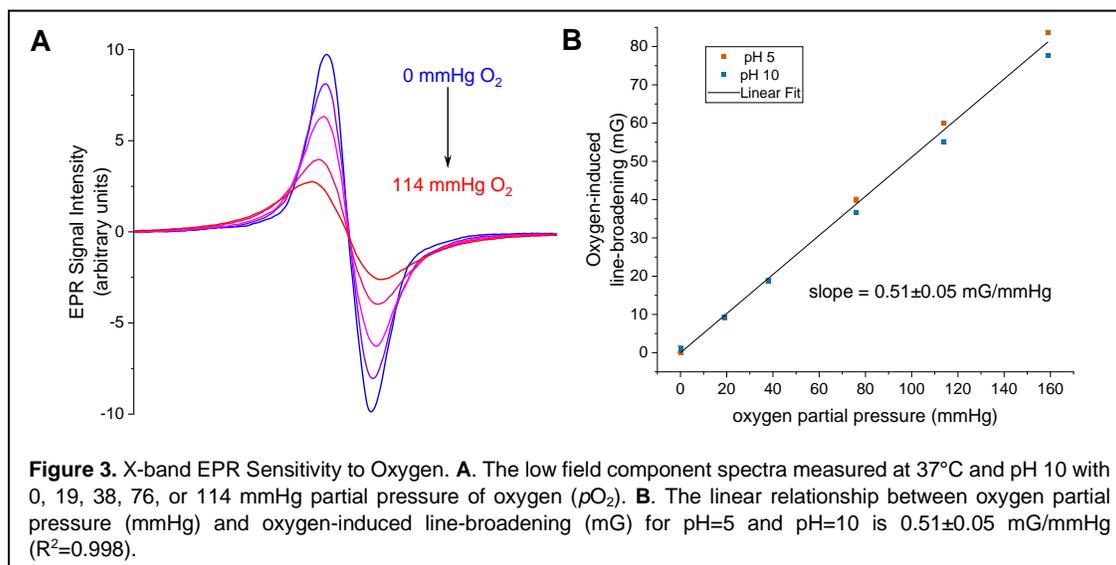
In order to determine the pKa of HOPE71, a solution of HOPE71 was titrated with small amounts of acid or base, and X-band EPR spectra were recorded for six pH points between 6 and 8 in nitrogen atmosphere. The ratio of the peaks is directly correlated to the fraction of acidic and basic states (Fig. 4A). Spectral line fitting was used to determine the acidic fraction (HOPE71^{3-}) for each pH point. By plotting the acidic and basic fractions versus pH, the titration curves show the pKa to be 7.1±0.1 (Fig. 4B).

Figure 4. X-band EPR Sensitivity to pH. **A**. The low field component spectra measured under nitrogen atmosphere and 37°C at various pH values between 6 and 8. The ratio of the peaks corresponds to the ratio of acidic and basic species. **B**. Titration curves for the fraction of $HOPE71^{3-}$ and $HOPE71^{4-}$ verses pH show a pKa=7.1±0.1 (R^2=0.999).
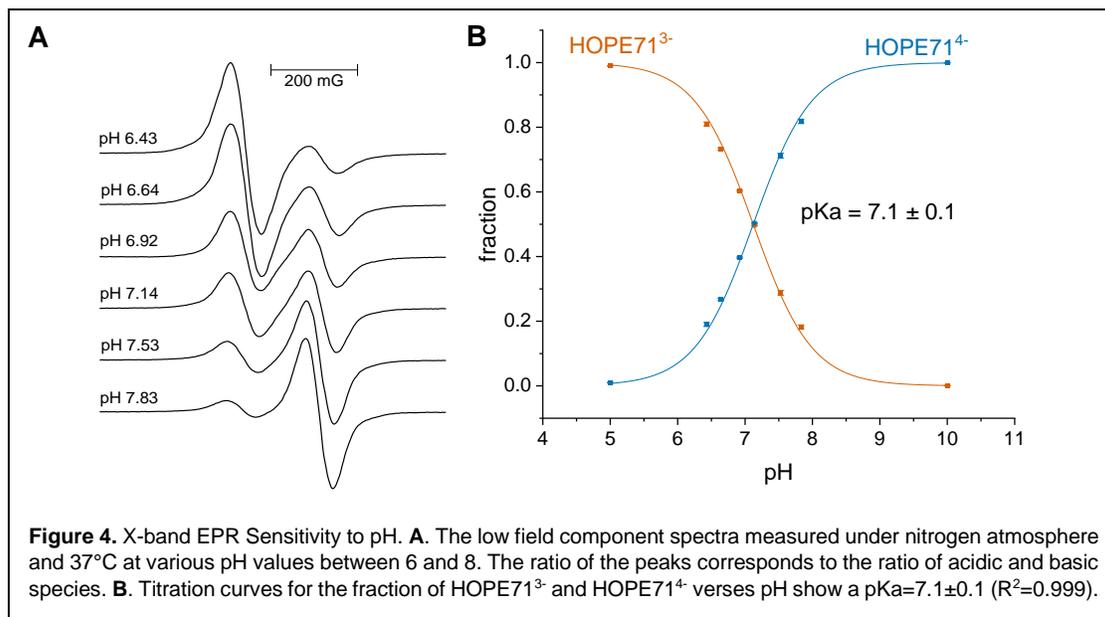
For the calibration of phosphate, solutions of HOPE71 with increasing concentrations of P_i were placed under nitrogen atmosphere in the X-band EPR to record spectra of the low field peaks. The presence of phosphate buffer [HPO_4^{2-}] increases the proton exchange rate on the phosphate group of HOPE71, which causes the acidic and basic peaks to coalesce (Fig. 5A). The proton exchange rate (k_a) can be derived from spectral fitting. The exchange rate adjusted for pH has a linear relationship against [P_i] and is equal to $2.36±0.05×10^4 s^{-1}mM^{-1}$ (Fig. 5B)[25].

Figure 5. X-band EPR Sensitivity to [P_i]. **A**. The low field component spectra measured under nitrogen atmosphere at 37°C and pH 7 with 0, 1, 4, 7, or 10 mM of inorganic phosphate [P_i]. The distance between the peaks relates to the proton exchange rate and decreases as the [P_i] increases. **B**. The proton exchange rate k_a, adjusted for pH, has a linear relationship versus inorganic phosphate concentration (k_f=2.36±0.05×10^{4}s^{-1}mM^{-1}) (R^2=0.999).

To validate the X-band sensitivities, EPR spectra were recorded for HOPE71 solutions with random pO_2, pH, and [P_i] within range of the calibrations that were blinded at time of measurement. The spectra were fitted allowing oxygen linewidth, acidic fraction, and exchange rate to vary. The values for random pO_2, pH, and [P_i] were extracted using the calibrations in Figs. 3-5. When comparing the prepared values to the EPR-derived values, the accuracy was within 2 mmHg of oxygen, 0.1 units of pH, and 0.3 mM of [P_i] (Tbl. 1).

	Gas Controller Set (mmHg)	EPR measured pO_2 (mmHg)	pH meter	EPR measured pH	[Pi] Prepared (mM)	EPR measured [Pi] (mM)
Sample 1	47.9	47.0 (±4.7)	7.28	7.26 (±0.1)	1.88	2.14 (±0.10)
Sample 2	34.9	32.2 (±3.2)	7.09	7.08 (±0.1)	6.88	7.00 (±0.10)
Sample 3	19.0	17.3 (±1.7)	6.94	6.97 (±0.1)	8.25	8.22 (±0.10)

Table 1. Validation of the X-band EPR Calibrations. Low field component spectra were recorded for 3 samples with blinded values of pH, pO_2, and [P_i]. The spectra were fitted, and the corresponding values were derived using the calibrations in Figs. 2-4 and compared to the prepared values.

With the EPR sensitivities characterized at X-band, the calibrations were repeated for L-band for in vivo measurements. The oxygen and P_i calibrations needed to be repeated to adjust for small differences in line shape and width due to inst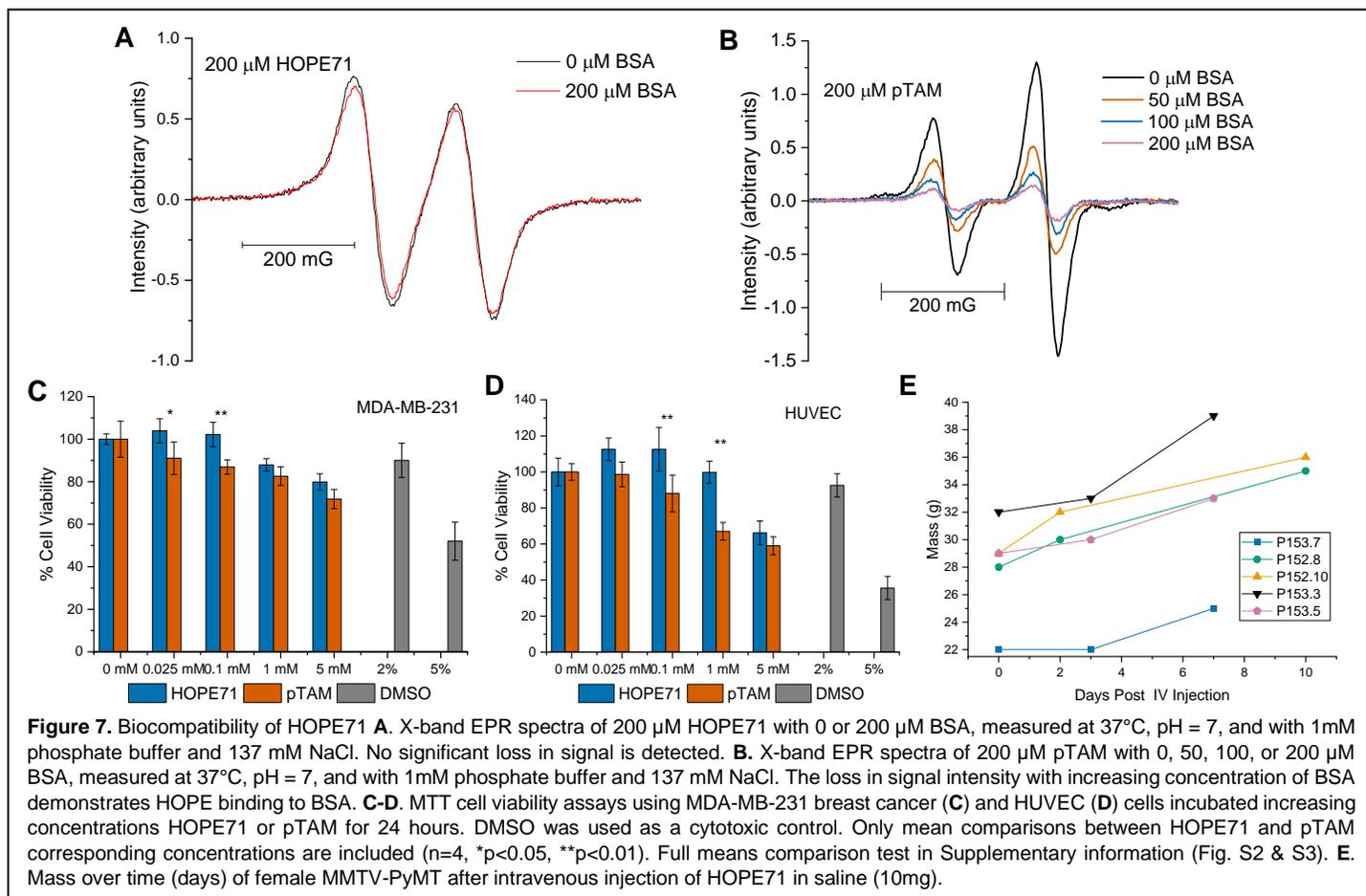rument variances. The pH calibration was repeated for rigor. The L-band slope for oxygen sensitivity was determined to be 0.41±0.05 mG/mmHg (Fig. 6A). The pKa measured by L-band EPR agrees with the X-band measurement of 7.1±0.1 (Fig. 6B, Fig. 4B). For the calibration of inorganic phosphate, the L-band slope, or kf, was found to be 1.86±0.05x10^{4}s^{-1}mM^{-1} (Fig. 6C). Blind sample testing was repeated for the L-band calibrations and the accuracy is estimated to be within 4 mmHg of oxygen, 0.1 units of pH, and 0.3 mM of [P_i] (Fig. 6D).

	Gas Controller Set (mmHg)	EPR measured pO_2 (mmHg)	pH meter	EPR measured pH	[Pi] Prepared (mM)	EPR measured [Pi] (mM)
Sample 1	36.7	34.7 (±4.2)	7.09	7.12 (±0.1)	3.75	3.82 (±0.10)
Sample 2	25.3	23.0 (±2.8)	6.94	6.96 (±0.1)	7.00	6.71 (±0.10)
Sample 3	15.8	16.3 (±4.1)	7.16	7.19 (±0.1)	1.66	1.77 (±0.25)

Figure 6. L-band EPR Calibrations for pH, pO_2, and [P_i]. **A.** The peak line broadening has a linear relationship versus the partial pressure of oxygen at pH 5 and 10 with slope=0.41±0.05 mG/mmHg (R^2=0.997). **B.** Titration curves for the fraction of HOPE71^{3-} and HOPE71^{4-} verses pH give a pKa=7.1±0.1 (R^2=0.999). **C.** The proton exchange rate k_a, adjusted for pH, has a linear relationship versus inorganic phosphate concentration with k_f=1.86±0.05x10^{4}s^{-1}mM^{-1} (R^2=0.997). **D**. Validation of the L-band EPR Calibrations.

Biocompatability of HOPE71. The biocompatibility improvements of HOPE71 over pTAM were evaluated in several ways. Firstly, X-band EPR spectroscopy was used to show the difference in albumin interaction. For HOPE71, there was no significant loss in in the spectrum signal intensity when BSA was included in equal concentration to the probe (Fig. 7A). In contrast, when equal concentration was included with pTAM, there was over 80% loss in signal intensity (Fig. 7B). This is likely due to the addition of twelve hydroxyethyl groups in HOPE71, increasing hydrophilicity and lessening protein interaction. Because albumin concentration is so high in blood, this lack of binding is imperative for intravenous dose efficiency. Next, the cytotoxicity of HOPE71 and pTAM were tested using MTT cell viability assays on rapidly dividing breast cancer cells (MDA-MB-231) and endothelial cells (HUVEC). On the breast cancer cells, both showed a minor loss in cell viability at higher concentrations (1-5mM), but cells treated with 25 μM and 100 μM HOPE71 had statistically more viability than those with the same concentrations of pTAM (Fig. 7C). Similarly, HUVECs had a significant loss in cell viability

for both at the highest concentration (5 mM), but with 100 µM and 1mM, cells treated HOPE71 did better than those treated with pTAM (Fig. 7D). Because the target local concentration for L-band EPR spectroscopy is several hundred micromolar, we conclude that HOPE71 has a small decrease in cell toxicity at the required concentrations. However, in vivo systemic toxicity is much more complex than cytotoxicity. pTAM has been used in vivo by intratissue injection without obvious negative effects[1], but whenever Finland based trityls are delivered intravenously, the mice quickly die[18]. Therefore, we observed the health of mice injected intravenously with a bolus dose of HOPE71 required for EPR spectroscopy. No mice died in the short time after injection or showed apparent signs of distress or weight loss for a week following the injection (Fig. 7E). While HOPE71 appears to have low toxicity in vivo, further evaluation by pharmacokinetics, histology, and acute toxicity (LD50) testing would help us better understand the systemic safety of HOPE71.

Figure 7. Biocompatibility of HOPE71 **A**. X-band EPR spectra of 200 µM HOPE71 with 0 or 200 µM BSA, measured at 37°C, pH = 7, and with 1mM phosphate buffer and 137 mM NaCl. No significant loss in signal is detected. **B.** X-band EPR spectra of 200 µM pTAM with 0, 50, 100, or 200 µM BSA, measured at 37°C, pH = 7, and with 1mM phosphate buffer and 137 mM NaCl. The loss in signal intensity with increasing concentration of BSA demonstrates HOPE binding to BSA. **C-D**. MTT cell viability assays using MDA-MB-231 breast cancer (**C**) and HUVEC (**D**) cells incubated increasing concentrations HOPE71 or pTAM for 24 hours. DMSO was used as a cytotoxic control. Only mean comparisons between HOPE71 and pTAM corresponding concentrations are included (n=4, *p<0.05, **p<0.01). Full means comparison test in Supplementary information (Fig. S2 & S3). **E**. Mass over time (days) of female MMTV-PyMT after intravenous injection of HOPE71 in saline (10mg).

Profiling tumors with HOPE71. Because the major advantage of HOPE71 over pTAM is the potential for systemic delivery, we demonstrated its ability to profile tumors in the mammary glands of mice with spontaneous breast cancer. We found that 10 mg (0.17-0.31 mmol/kg) of HOPE71 in saline, was a sufficient bolus dose by intravenous injection to have a reliable signal-to-noise ratio for L-band EPR spectroscopy for at least 30 minutes after injection. These tumors were all on mammary gland 4 or 9 and approximately 1 cm in diameter (average volume = 437±91 mm^3) and their pO_2, pH, and [P_i] were measured on average to be 23.6±18.3 mmHg, 7.09±0.14, and 4.68±0.45 mM, respectively (Fig. 8A).

To investigate how these variables deviate from healthy tissue with tumor growth, mammary glands of the breast cancer mouse models and their wildtype littermates were longitudinally profiled by EPR spectroscopy. For these measurements, HOPE71 was injected directly into the tumor or mammary gland. The intention for intratissue (IT) as opposed to intravenous (IV) injection was to consume less probe per measurement and target the core of the tumor, which may be poorly vascularized. To determine if there was a significant difference in the EPR-measured values of pO_2, pH, and [P_i] with IV and IT injection, volume-matched data points from the IT longitudinal study were compared to the measured values in IT (n=6). Our hypothesis was that pO_2 and pH measured with IV injection would be higher than those measure with IT injection because the probe would be delivered to the better vascularized regions of the tumor. The pO_2 values had large variance in both groups so

no statistical conclusion can be made for oxygen, but the pH for IV delivery was significantly higher than pH for IT delivery (Fig. 8A). This may indicate that there is a pH gradient from the core of the tumor and that the probe is not as well delivered to the core upon intravenous injection. There also was no significant difference between IV and IT in the EPR-measured values of $[P_i]$, but elevated levels of Pi have been observed both locally in the tumor[1] and systemically in patients with cancer[27].

Oxygenation, pH, and $[P_i]$ of growing mammary gland tumors were compared to those of healthy mammary gland tissue of the wildtype littermate, based on the days since the tumors were recorded to be palpable. The wildtype mice were assigned a palpable date that matched their tumor littermates for comparison purposed. Throughout the entire tumor growth period, oxygen measurements appeared to be generally lower in tumors, but did not show significant difference from the wild type (Fig. 8B & 8F). On the other hand, pH was not significantly different in early tumors, but a difference did begin around 20-29 days (Fig. 8C). Acidosis continued to deviate significantly in tumors from healthy tissue as time went on (Fig. 8C & 8G). Inorganic phosphate was found to be elevated throughout most of the tumorigenesis (Fig. 8D), but linear deviation from healthy tissue was not clear (Fig. 8H). No significant correlation was found between pO_2, pH, and $[P_i]$ (Fig. S5).
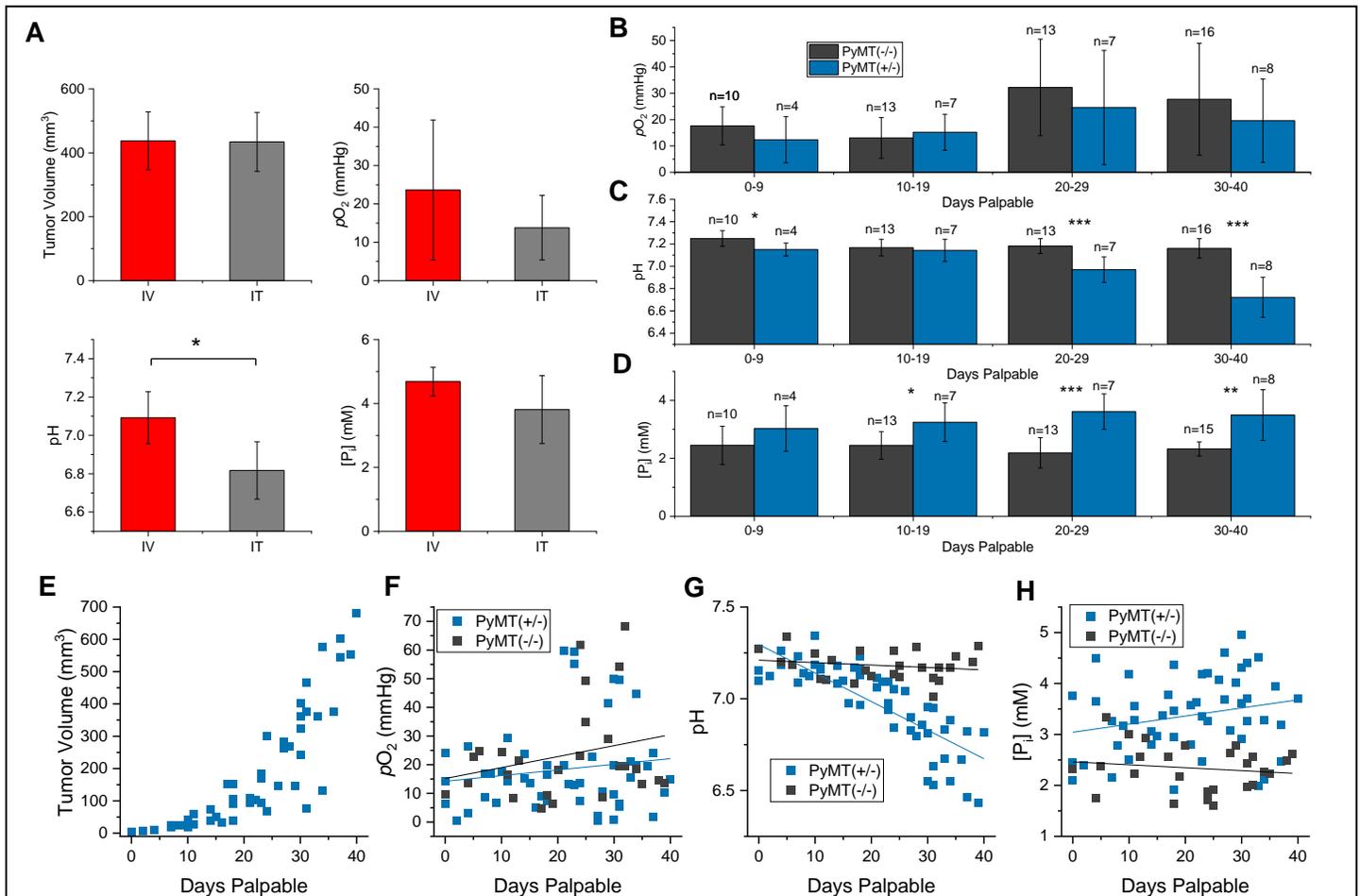
Figure 8. Profiling mouse breast tumors with HOPE71 **A.** The volumes, pO_2, pH, and $[P_i]$ of tumors profiled by EPR after either intravenous (IV) or intratissue (IT) injection of HOPE71 (n=6, *p<0.01). **B-D**. Average pO_2, pH, and $[P_i]$ for PyMT(-/-) (n=4) and PyMT(+/-) (n=8) for days palpable grouped by 10 days (*p<0.05, **p<0.01, ***p<0.001). **B**. No significant difference for pO_2. **C**. The difference in pH grew and was significant at 20-29 and 30-40 days. **D**. The difference in $[P_i]$ was significant at 10-19, 20-29 and 30-40 days. **E-H.** Data points by days since palpable (PyMT(+/-) mice=8, n=52; PyMT(-/-) mice=4, n=26) **E** Tumor volume of PyMT(+/-) show exponential growth versus days palpable. **F.** No significant correlation was found between pO_2 and days palpable for PyMT(+/-) (r=0.15, p=0.30) or PyMT(-/-) (r=0.25, p=0.21), and no significant difference was detected between PyMT(+/-) and PyMT(-/-) slopes (p=0.60). **G**. The slope between pH and days palpable for PyMT(+/-) was significantly different from zero, using the F-test (r=-0.80, p=1.5x10⁻¹²), but the slope for PyMT(-/-) was not (r=-0.19, p=0.35). Significant difference was detected between PyMT(+/-) and PyMT(-/-) slopes (p=1x10⁻⁸). **H.** No significant correlation was found between $[P_i]$ and days palpable for PyMT(+/-) (r=0.23, p=0.09) or PyMT(-/-) (r=-0.15, p=0.48), and no significant difference was detected between PyMT(+/-) and PyMT(-/-) slopes (p=0.08).

Conclusion

In conclusion, we propose HOPE71, a monophosphated hydroxyethyl trityl radical probe, as a biocompatible multifunctional EPR probe for in vivo measurement of pO_2, pH, and [P_i]. HOPE71 can be synthesized starting with OX071 by a 2-step process. The EPR-sensitivities of HOPE71 with X-band and L-band spectroscopy were verified and comparable to pTAM. Furthermore, HOPE71 has clear biocompatibility improvements over pTAM, including lack of albumin binding and systemic tolerance. The ability of HOPE71 to profile tumors in mouse models of breast cancer by EPR spectroscopy was demonstrated with both intravenous and intratissue injection. Finally, HOPE71 was used to longitudinally profile mammary gland tumors in mice, which found that pH strongly deviates with tumor growth and inorganic phosphate appears to be consistently elevated throughout most of the tumorigenesis.

Acknowledgment

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CHAPTER 3 – Large-Scale Synthesis of a Monophosphonated Tetrathiatriarylmethyl Spin Probe for concurrent *in vivo* measurement of pO$_2$, pH and inorganic phosphate by EPR

Contribution: This paper was written in co-authorship with labmate, Martin Poncelet. My contribution was in the development of the MATLAB-based fitting application and performing and analyzing the cytotoxicity assay. The manuscript was recently accepted in RSC Advances.

Large-Scale Synthesis of a Monophosphonated Tetrathiatriarylmethyl Spin Probe for concurrent *in vivo* measurement of pO$_2$, pH and inorganic phosphate by EPR

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ABSTRACT

Low-field electron paramagnetic resonance spectroscopy paired with pTAM, a mono-phosphonated triarylmethyl radical, is an unmatched technique for concurrent and non-invasive measurement of oxygen concentration, pH, and inorganic phosphate concentration for in vivo investigations. However, the prior reported synthesis is limited by its low yield and poor scalability, making wide-spread application of pTAM unfeasible. Here, we report a new strategy for the synthesis of pTAM with significantly greater yields demonstrated on a large scale. We also present a standalone application with user-friendly interface for automatic spectrum fitting and extraction of pO$_2$, pH, and [P$_i$] values. Finally, we confirm that pTAM remains in the extracellular space and has low cytotoxicity appropriate for local injection.

Low-field Electron Paramagnetic Resonance (EPR) with the use of a molecular spin probe is a powerful technique to non-invasively measure important physiological parameters in a living animal[1, 2]. EPR combines high sensitivity and good penetration depth. Stable tetrathiatriarylmethyl radicals (TAMs or trityls) are ideal spin probes for in vivo EPR applications. They exhibit unprecedented stability in vivo and ultra-narrow linewidths, which result in a high signal-to-noise ratio.[3] TAM structures with spectral sensitivity to oxygen[4], pH[5, 6], thiol concentration[7, 8], microviscosity[9], ROS[10-12], or redox[13, 14] have been developed. We recently reported on a mono-phosphonated tetrathiatriarylmethyl radical **pTAM** (Figure 1) whose EPR spectrum is sensitive to multiple parameters, namely oxygen concentration, pH, and inorganic phosphate concentration, $[P_i]$.[15-18] This multifunctional probe was utilized to profile the tumor microenvironment (TME) in various mouse models of cancer.[17] The unmatched capability to measure $[P_i]$ has resulted in the identification of this biomarker as a new TME marker for tumor progression.[17] Moreover, the ability to measure pO_2, pH, and $[P_i]$ concurrently using the same probe allows for the direct correlation of these important parameters independent of the probe distribution, providing insight into the biological processes occurring in the TME.
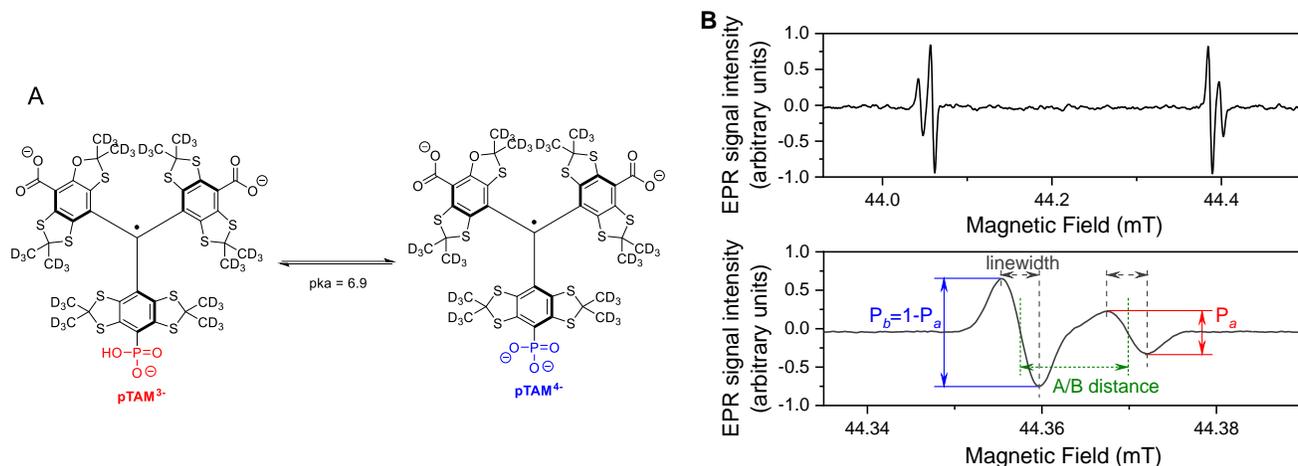

Fig. 1. A. Structure of **pTAM** spin probe and ionic forms at physiological pH. B. L-Band full spectrum (top) of **pTAM** at pH=7.13 showing both ionic forms present in the spectrum and zoom on the high field component (bottom). The molar fraction of the acidic form P_a versus basic form P_b is a function of the pH of the solution while the linewidths are functions of the oxygen concentration. Inorganic phosphate modulates the exchange rate between the two ionic forms and the A/B distance. Spectral simulation allows the three parameters to be extracted from the spectrum.

While this spin probe has proven to be of great importance for the study of tissue microenvironment in vivo, its current synthesis suffers from a very low yield. Indeed, the published synthesis[15] (Scheme 1) uses a lithiation of tetrathiatriarylmethanol **1** and subsequent reaction with a (2:1) mixture of diethyl carbonate and diethyl chlorophosphate. This reaction leads to a statistical mixture of mono-, di- and tri-phosphonated tetrathiatriarylmethanol **2n** that requires tedious purification and drastically decreases the yield of the desired **2b**. After hydrolysis of the ethyl esters using sodium hydroxide and deprotection of the phosphonic acid by TMSBr, the final **pTAM** probe was isolated with a yield of less than 5% from **1**. The published procedure allowed for isolating milligram quantity of the probe for limited in vivo studies.[17] However, more extensive utilization of this probe would require a synthetic method that enables gram-scale synthesis of **pTAM**.
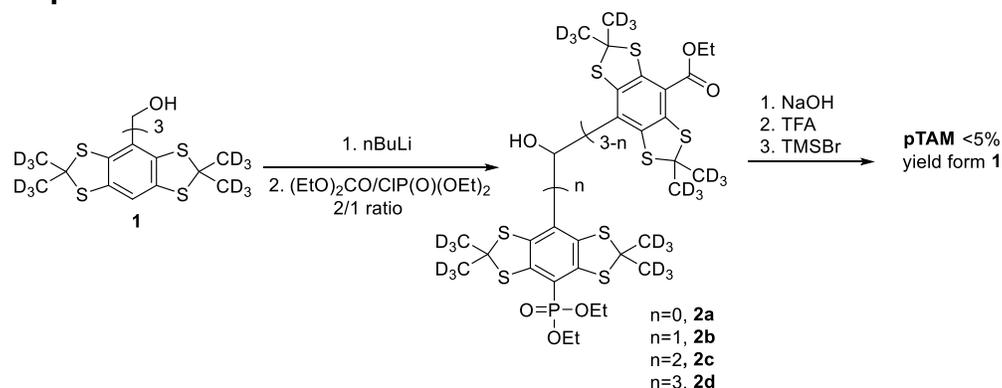

Scheme 1. The first reported synthesis of **pTAM** from **1**.[15]

Hereby we report an efficient protocol for the large-scale production of the **pTAM** probe as well as a MATLAB application for the automatic fitting of the EPR spectra and determination of the physiological parameters, namely pH, pO_2, and $[P_i]$. Our new strategy takes advantage of a reaction of *ipso* nucleophilic substitution of an aromatic hydrogen or a carboxyl group on tetrathiatriarylmethyl radicals reported previously.[19, 20] Our synthesis starts with the deuterated Finland trityl (**dFT**) which can be synthesized at large scale without chromatography (Figure 2A).[21, 22] The one-electron oxidation of **dFT** with one equivalent of potassium hexachloroiridate(IV), K_2IrCl_6, in water leads to the trityl carbocation **dFT⁺**, which is immediately treated with ten equivalents of trimethyl phosphite. The nucleophilic addition of the phosphite in the *para*-position of the aryl ring triggers an oxidative decarboxylation, leading to the mono-phosphonic ester **pTAM-(OMe)₂** in 35% conversion, as determined by HPLC/MS (Figure 2B and S6). Importantly, the HPLC/MS chromatogram shows that **dFT** radical was also generated back from the trityl carbocation **dFT⁺** in 65% yield, consistent with preferential oxidation of intermediate **3** by **dFT⁺** in line with previous reports. [19, 20] **dFT** can therefore be recycled for future reactions. In addition, <5% of quinone methide (**QM**) was also generated from the nucleophilic addition of water on the trityl cation (See ESI for mechanism). The use of additional equivalents of K_2IrCl_6 did not increase the yield of **pTAM-(OMe)₂** but did lead to higher conversion to the **QM**, TAMs with multiple phosphonates, and unidentified products. The preferential oxidation of **3** by **dFT⁺** explains 50% of the back conversion of the trityl radical from the cation. The slightly higher formation of **dFT** observed (65%) could be the result of the direct reduction of the trityl cation by the trimethyl phosphite.
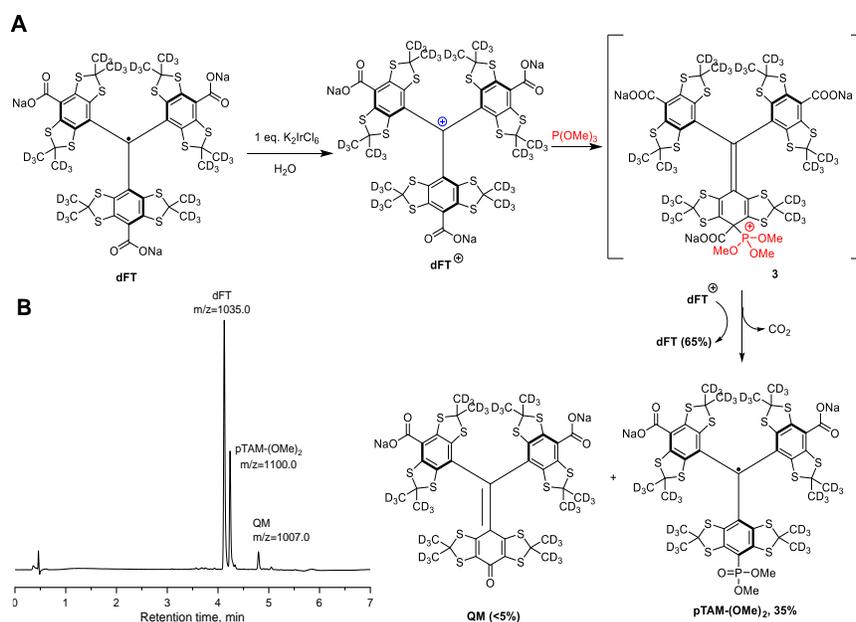
Fig. 2. A. Synthesis of **pTAM-(OMe)₂** from **dFT** and B. HPLC/MS chromatogram and m/z ratio of the products after addition of P(OMe)₃.

The mono-phosphonated derivatives **pTAM-(OMe)₂**, and **dFT** can be separated using a C18 column in 30% and 60% yield, respectively. Finally, the phosphonic acid was deprotected by treatment of **pTAM-(OMe)₂** with TMSBr in 95% yield (Scheme 2).
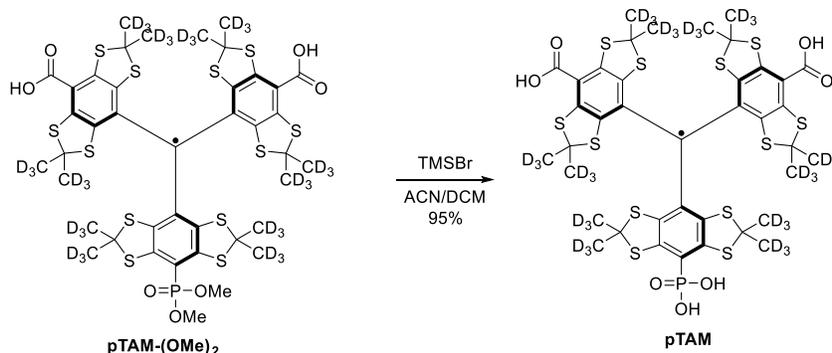
Scheme 2. Deprotection of the phosphonic acid leading to **pTAM**.

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However, for a multigram scale, we found the separation of **dFT** and **pTAM-(OMe)₂** to be more challenging. The use of other phosphites with longer alkyl chains (triethyl-, triallyl- or tributyl phosphite), allowed for easier purification but led to smaller conversion (15-25%). On a large scale (tens of grams), the quantitative esterification of the carboxylic acids using methyl iodide and sodium carbonate in DMF directly on the **dFT/pTAM-(OMe)₂** mixture (Scheme 3) allowed for easy purification by flash chromatography on silica gel. The esterified **pTAM-(OMe)₄** was isolated in 35% yield from **dFT** starting material alongside with **dFT-(OMe)₃** (63%). Then, the phosphonic acid was deprotected by TMSBr in DCM, and the methyl esters hydrolyzed using lithium hydroxide in 1,4-dioxane/water leading to **pTAM** in 95% yield after purification on a C18 column. **dFT-(OMe)₃** was also hydrolyzed, leading to dFT in 99% yield with no purification needed. The relatively low conversion of **dFT** to the monophoshonated ester is compensated by the recovery of the starting material. The calculated yield based on the recovery of the starting material reaches 92%. Our large scale synthesis allowed for the selective mono-phosphorylation of **dFT** in 4 steps and two purifications. The key step is the nucleophilic quenching of the trityl cation by trimethyl phosphite leading to the mono-phosphonated derivative.
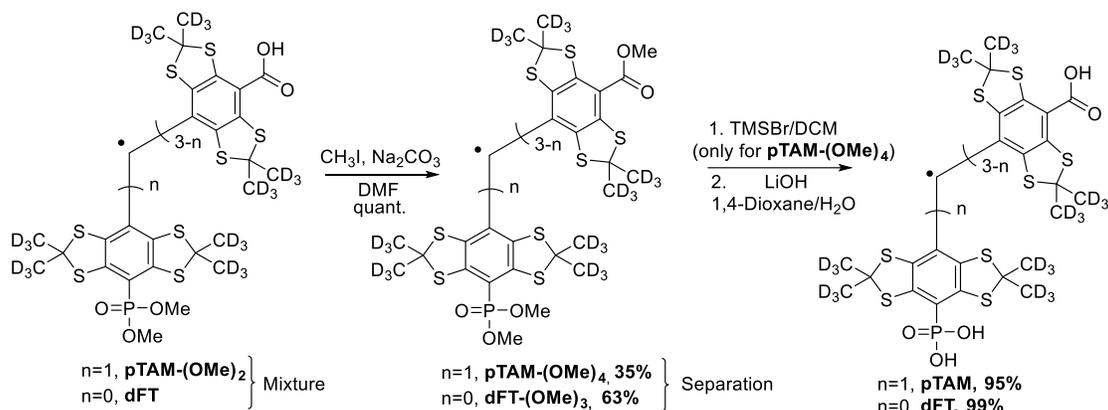
Scheme 3. Esterification of the carboxyl groups to allow for large-scale separation of **pTAM-(OMe)₂** and **dFT-(OMe)₃**. Then the carboxyl and phosphonic acids are deprotected, leading to **pTAM** and **dFT**.

The extraction of pO_2, pH, and $[P_i]$ from the spectrum can be achieved using spectral fitting of the whole spectrum (see Fig. 1B, top) or only the high or low field EPR lines (Fig. 1B, bottom) using a homemade MATLAB algorithm as reported previously.[16, 17] However, to provide a user-friendly interface for those unfamiliar with MATLAB, we developed a graphical user interface for fitting the spectra and deriving the values for pO_2, pH, and $[P_i]$. Figure 3 demonstrates the use of the standalone application to fit a spectrum of **pTAM** administered into the mammary gland of a MMTV-PyMT mouse (See SI for calibration and use of the App).
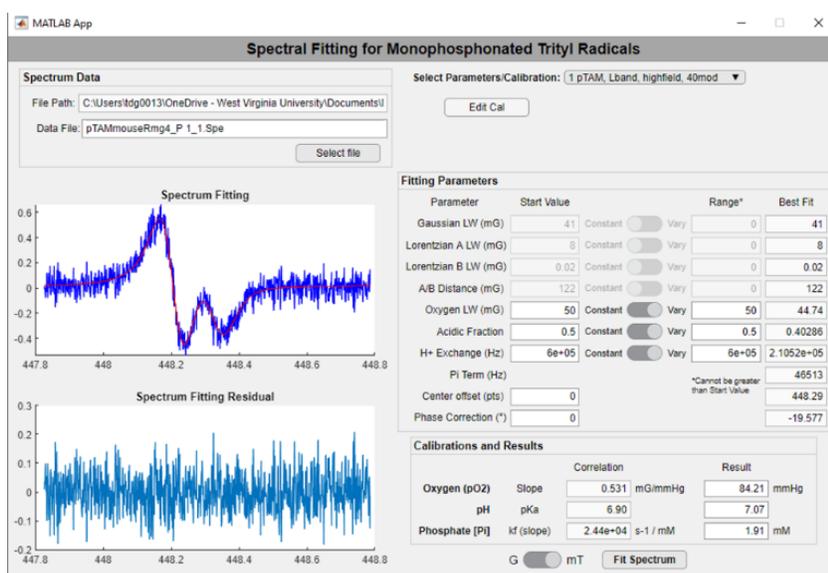
Fig. 3. Screenshot of the **pTAM** spectrum fitting App developed in-house with a spectrum measured of **pTAM** injected directly in the mammary gland of a MMTV-PyMT mouse. Values of pO_2=84.21 mmHg, pH=7.07 and $[P_i]$=1.91 mM are automatically calculated from the experimental spectrum.

When applied in vivo, the charged nature of the probe and its large size (MW = 1073 g/mol) is expected to prevent its diffusion through the cell membrane. In order to verify that **pTAM** cannot enter the cytosol, **pTAM** (200 μM) was incubated with 8.5×10^6 MDA-MB-231 cells (human triple negative breast cancer cells) with and without 10 mM Gd-DTPA, a paramagnetic extracellular broadening agent.[5] Figure 4 shows a large broadening of the EPR lines of **pTAM** upon addition of Gd-DTPA and no residual narrow component confirming the absence of **pTAM** spin probe in the intracellular compartment. In vivo, the physiological parameters reported by **pTAM** are therefore the extracellular ones.
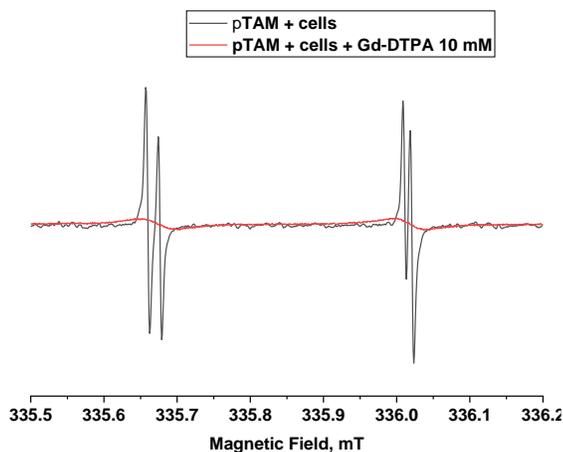
Fig. 4. X-Band EPR spectra of **pTAM** (200 μM, 100 μL) incubated with 8.5×10^6 MDA-MB-231 cells without (black) and with 10 mM of Gd-DTPA (red) as extracellular broadening agent.

Next we assessed **pTAM** cell toxicity using the MTT assay for cell viability and proliferation. MDA-MB-231 cells at 60-70% confluency were incubated with various concentration of **pTAM** for 24h. The result (Figure 5) shows that up to 1 mM, the probe is well tolerated with ~ 80% cell viability after 24h. It is worth noting that only a few hundred micromolar range is required for in vivo L-Band spectroscopy and the MTT results show no significant difference between 100 μM of probe and the control. Moreover, the **pTAM** was incubated 24h with cells while in vivo the probe is cleared from the tissue in less than 1h.[23] Therefore, the probe can be considered as non-toxic upon local injection, which is the mode of delivery for **pTAM**.[17]
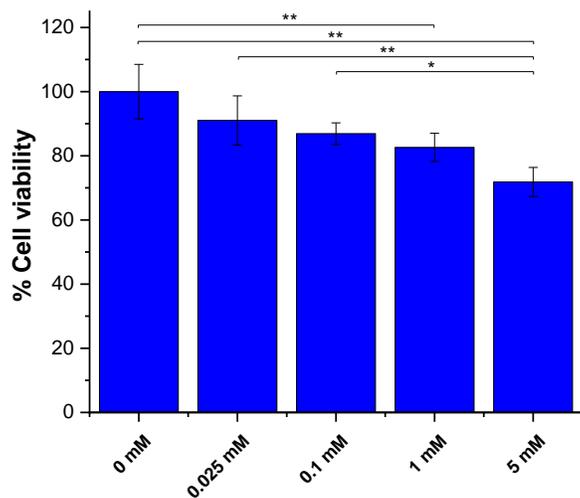
Fig. 5. MTT assays for **pTAM** at various concentration incubated with MDA-MB-231 cells for 24h. (n=3, *$p<0.05$, **$p<0.01$).

Conclusions

In conclusion, we have reported a procedure to synthesize gram quantities of **pTAM** and a MATLAB application for automatic extraction of pO_2, pH, and [P_i] from an experimental spectrum. Furthermore, we showed that **pTAM** does not cross the cell membrane and has a low cell toxicity for local delivery.

Author Contributions
B.D. conceived, supervised the project, performed the experiment and wrote the manuscript. T. D. G developed the App, performed the MTT assay and wrote the manuscript, M.P performed the synthesis and wrote the manuscript. S.D developed the fitting algorithm. M.G performed the cell and animal work, E.H.H performed the animal work. T.D.E supervised and discussed the cell and animal work. V.V.K. provided guidance on the fitting of pTAM radical and provided the initial fitting function. All authors have read and edited the manuscript.

Conflicts of interest
There are no conflicts to declare.

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CHAPTER 4 – Cleavage-resistant Protein Labeling with Hydrophilic Trityl Enables Distance Measurements *In-Cell*

Cleavage-resistant Protein Labeling with Hydrophilic Trityl Enables Distance Measurements *In-Cell*

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ABSTRACT

Sensitive *in-cell* distance measurements of proteins using pulsed-Electron Spin Resonance (ESR) requires spin-labels that are both reduction-resistant and cleavage-resistant. Among the reduction-resistant radical moieties, the hydrophilic trityl core known as OX063 is particularly promising due to its long phase-memory relaxation time (T_m). This property of OX063 allows for the detection of sufficiently intense ESR signal to reliably measure longer distances. Furthermore, the OX063's T_m remains sufficiently long at higher temperatures, which opens up the possibility for distance measurements to be done at temperatures above 50 K. In this work, we synthesized a deuterated OX063 with a maleimide linker (mOX063-d$_{24}$). The combination of deuterated OX063's hydrophilicity and the maleimide linker allows for highly efficient labeling of protein while also being uncleavable in cells. We show that the long T_m of mOX063-d$_{24}$ allow distance measurements at temperatures up to 150 K. Additionally, the distance measurement at 150 K is more sensitive than the measurement at 80 K. The sensitivity gain is due to the significantly short longitudinal relaxation time (T_1) at higher temperatures which allows for more data to be averaged given the same amount of time. In addition to *in vitro* experiments, we show that mOX063-d$_{24}$ allows distance measurements in *Xenopus laevis* oocytes. Interestingly, the T_m of mOX063-d$_{24}$ is still sufficiently long even in the crowded environment of the cell, which allows for distance measurements in-cells. Overall, mOX063-d$_{24}$ provides highly sensitive distance measurements both *in vitro* and *in-cells*.

Introduction

Understanding how proteins adapt in their cellular environments is of immense interest in structural biology. The crowded environment inside cells can affect protein folding and stability[1–7]. For example, phosphoglycerate kinase (PGK) is more stable in zebrafish tissues[8] and human osteosarcoma cells[5] than *in vitro*. The increase in stability due to molecular crowding has also been observed with other proteins such as frataxin[9], ubiquitin[10], hen egg white lysozyme[11], and calcineurin[12]. In contrast, the dimerization of baculoviral IAP repeat (BIR1) domain of X chromosome-linked inhibitor of apoptosis (XIAP) is destabilized *in vivo*[13]. The destabilization effect are also seen in other protein dimers that are not spherical in shape[14,15]. These experiments are indicators that the *in-cell* environment modulates protein structure and function, which vary case-by-case. Overall, *in-cell* experiments are required to understand the behavior of proteins in the context of cellular function.

Electron Spin Resonance (ESR) emerged as a widely applicable technique to measure dynamics and distance constraints *in vitro* and *in-cell*. For such ESR measurements, the normally diamagnetic proteins can be functionalized with a spin-label using site-directed spin labeling methodologies[16–19]. The combination of ESR and spin labeling enables the measurement of the dynamics at the labeled site[20,21] or measuring distances between the labeled sites of a protein[22–28]. Distance measurements have been particularly useful for shedding light on the changes in protein conformations[29–37], the assembly of large complexes[38–41], and the binding of substrates and metal ions[42–45]. Additionally, these distance measurements have been performed *in-cell* for proteins[46–48] and DNA[49,50]. The primary challenge for distance measurements *in-cell* is the reduction of spin-labels within the highly reducing cytosolic environment[51]. An intriguing new strategy for *in-cell* measurements is the use of genetically encoded non-canonical amino acids technology as an *in situ* labeling strategy[52–55]. In particular, a photo-caged radical amino acid can be incorporated into a protein during translation[56]. Only after the induction of light will the photo-cage is released to expose the nitroxide radical for ESR measurements. In addition to non-canonical amino acids, reduction resistant spin-labels such as sterically shielded nitroxides[57,58], Gd(III)-based spin-labels[59–61], and triarylmethyls (TAMs, trityls)[62–64] have been developed.

Trityls have a lot of potential as a class of spin-labels for several reasons. First, trityls are highly resistant to reduction *in-cell* due to the steric-shielding of its radical[65–67]. Second, trityls have appreciable relaxation times even at physiological temperatures[68]. Third, trityls have narrow spectral shape that leads to efficient excitation of the electrons and intense ESR signal[69]. Overall, trityl spin-labels have proved suitable for distance measurements at physiological temperatures or *in-cell*. The most explored trityl spin-labels are based on the Finland trityl radical (FT) shown in Figure 1A, which have successfully provided distance measurements at room temperature[70,71] and *in-cell*[72,73]. However, FT-based spin-labels usage is still challenging due to the complications in the labeling process.

The spin-labeling process typically entails a reaction between the spin-label and a cysteine residue to label the protein at a specific site. However, FT can bind non-specifically to membranes[74] or proteins[75]. Additionally, FT tends to self-aggregate[76,77]. As a result, efficient labeling of FT requires extensive washing of proteins that are immobilized on a solid support[63] or maintaining FT concentration to be less than 30 μM throughout the process to minimize aggregation[78].

Even after the labeling process, the phase-memory relaxation time (T_m) of FT is significantly reduced upon protein binding[63,78], which leads to a weaker signal. Additionally, the longitudinal relaxation time (T_1) is significantly long at temperatures that are typical for ESR distance measurements ($\leq 50K$)[78], which leads to longer experimental time. Overall, the short T_m and long T_1 of FT-based spin labels diminish the sensitivity gain from the efficient excitation of FT. As a result, FT's sensitivity for distance measurements is comparable to distance measurements using commercially available nitroxide spin-label[78].

As an alternative, a hydrophilic trityl spin-label,based on the OX063 radical shown in Figure 1A, has been recently developed that allows for straightforward labeling procedure without non-specific binding or aggregation[79]. Interestingly, deuterated OX063 (OX063-d_{24}) was reported to have the longest transversal relaxation time at 50K to date ($T_m = 6.3\ \mu s$)[79]. Additionally, OX063-d_{24} has been shown to also have a sufficiently long phase-memory relaxation time even at 200 K ($T_m = 3\ \mu s$)[46]. Because T_1 is generally shorter at higher temperatures, OX063-d_{24} has the potential for highly sensitive distance measurements at temperatures higher than 50K. Despite OX063-d_{24}'s improvement over FT, OX063-d_{24} only utilized a methanethiosulfonate linker so far, which is a limiting factor for *in-cell* experiments. This linker labels a protein by forming disulfide bonds with cysteines, which can be cleaved inside cells[48]. On the other hand, a maleimide linker reacts with a cysteine to form a thioether bond, which is uncleavable under normal physiological condition[47]. In response to the need of a hydrophilic trityl with an uncleavable linker, a hybrid of OX063 and short-linker maleimide (SLIM)[73] known as Ox-SLIM was recently developed (Figure 1A)[80]. Unlike OX063, the trityl core of Ox-SLIM has one of its bisthioketalaryl moieties remain unhydroxylated to bear the short maleimide linker. The hydrophilicity of Ox-SLIM

permitted the labeling efficiency of ~85%. These results motivate the development of hydrophilic trityl labels with high labeling efficiency for *in-cell* distance measurements.

To increase the viability of OX063-d$_{24}$-based spin-labels, we have developed a new OX063-d$_{24}$ spin label with a maleimide linker (mOX063-d$_{24}$) shown in Figure 1A. The maleimide linker allows for mOX063-d$_{24}$ to maintain its linkage with the protein *in-cell*[81]. We explored two aspects of mOX063-d$_{24}$ for distance measurements in proteins. First, we show how mOX063-d$_{24}$ provides highly sensitive distance measurements at temperatures higher than the typical $\leq 50K$ *in vitro*. Second, we showcase the usage of mOX063-d$_{24}$ for experiments *in-cell*, specifically in *Xenopus laevis* oocytes. When exploring these two aspects, spin-labeling and distance measurements were done on the immunoglobulin binding domain of protein G (GB1)[82], a 56-residue globular protein (Figure 1B).
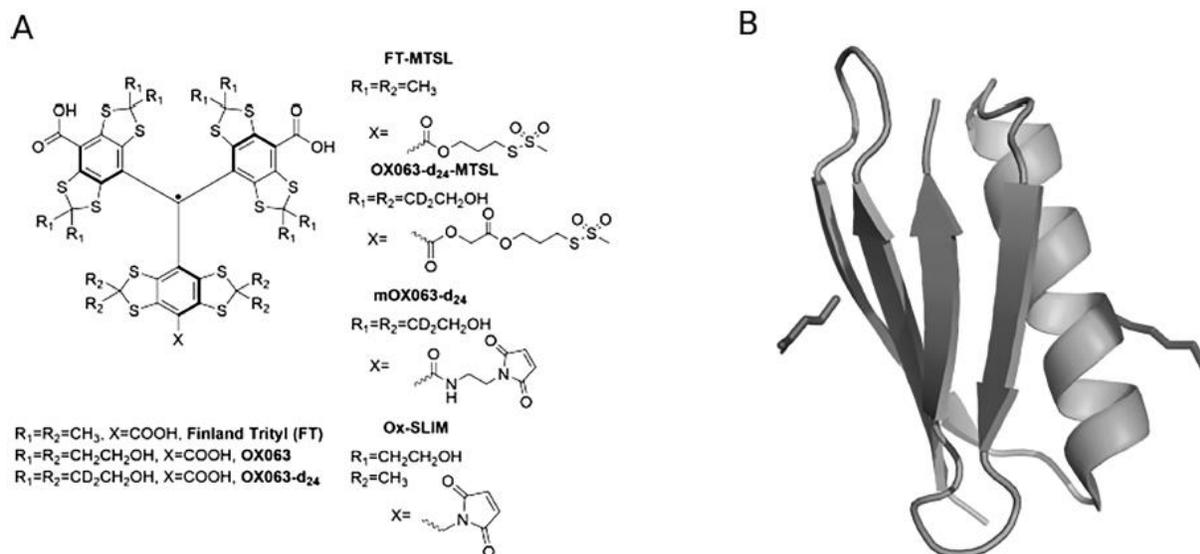
Figure 1. A) Representation of TAM-based spin labels, FT-MTSL, OX063-d$_{24}$-MTSL, mOX063-d$_{24}$, and Ox-SLIM. B) Three-dimensional model of E15C/K28C GB1 based on the wild-type GB1 crystal structure (PDB:2QMT). The side chains of the mutated cysteines are represented as lines. Reproduced from Hasanbasri, *JPC* 2021.

Methods

Synthesis of mOX063-d$_{24}$. OX063-d$_{24}$ trisodium salt (112 mg, 0.077 mmol, 1eq.), synthesized using our previously reported protocols[83], was dissolved in anhydrous dimethylformamide (DMF) (100 mL) under argon at room temperature. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (28 mg, 0.054 mmol, 0.7 eq.) in DMF (1 mL) was added; the green solution turned into a red-brown colored solution.Then, N-(2-aminoethyl)maleimide trifluoroacetate salt (23 mg, 0.09 mmol, 1.2 eq.) in DMF (1 mL) and N,N-diisopropylethylamine (DIEA) (26.8 μL, 1.4 mmol, 2eq.) were added. The solution turned back to green. The reaction mixture was diluted 20x with deionized water and acidified to approximately pH~2 with trifluoroacetic acid. The crude product was loaded into a C18 cartridge and purified by reverse-phase chromatography using a C18 column with a gradient of water/acetonitrile (both containing 0.1 % TFA) 95/5 to 85/15. The purified product was freeze-dried, then dissolved in water, titrated to pH=7 with NaOH, and freeze-dried again to provide 48 mg (40%) of mOX063-d$_{24}$ as a disodium salt. The purity assessed by HPLC reached >95% as shown in Figure S1. HRMS characterization is shown in Figure S2.

GB1 labeling protocol.   E15C/K28C GB1 and E15C GB1 expression and purification were performed as previously described[84]. The GB1 mutant was reacted with tris(2-carboxyethyl)phosphine (TCEP) overnight at 4 °C to reduce any disulfide formation. To label the protein, GB1 was run through four 5 mL GE Healthcare Hitrap desalting columns, to remove any TCEP, directly into a solution of mOX063-d$_{24}$. The final solution of 10:1 of mOX063-d$_{24}$:GB1 was allowed to react overnight at 4 °C. The spin-labeled protein was concentrated using Sartorius VivaSpin Turbo 4 centrifugal filter units with a molecular weight cutoff of 5 kDa to remove the unreacted label. The final solution was prepared in PBS pH 7.4. Concentration and labeling efficiencies were calculated from UV-Vis measurement using Nanodrop2000 Spectrophotometer from Thermo Scientific. The extinction coefficient of GB1 was obtained from the ProtParam tool (https://web.expasy.org/protparam/).

Cellular extracts and oocyte microinjection. Oocytes were obtained from Carolina Biological Supplies. The cytosol was extracted following previously published protocol[85]. Cytosol sample was prepared at 50 μL containing

doubly labeled mOX063-d$_{24}$-GB1 (200 μM spin concentration) and drawn into Pyrex capillary tubes (I.D. = 0.8 mm) for room-temperature CW experiments. For *in-cell* pulsed-ESR experiments, 50 nL of doubly labeled mOX063-d$_{24}$-GB1 (2 mM spin concentration) was microinjected into 12 oocytes following previously published protocol[85]. The microinjected oocytes were inserted into a Quartz Q-band sample tube (2 mm I.D. and 3 mm O.D.) and incubated at room temperature for 30 minutes before being flash-frozen in liquified methylacetylene-propadiene propane (MAPP) gas. The Q-band *in-cell* sample was estimated to be 60 μL of ~20 μM bulk spin-concentration.

ESR measurements. Room-temperature continuous-wave (CW)-ESR experiments were performed on a Bruker ElexSys E680 CW/FT X-band spectrometer using a Bruker ER4122 SHQE-W1 high-resolution resonator. CW samples were prepared in Pyrex capillary sample tubes. CW experiments were run at a center field of 3520 G with a sweep width of 20 G, a microwave frequency of ~9.87 GHz, modulation amplitude of 0.07 G or 0.005 G, and modulation frequency of 100 kHz or 300 kHz[83] for a total of 1024 or 2048 data points using a conversion time of 30.01 ms.

All pulsed experiments were performed on a Bruker ElexSys E680 CW/FT X-band spectrometer equipped with a Bruker ER5106-QT2 resonator for Q-band and a 300 W amplifier. The temperature was controlled using an Oxford ITC503 temperature controller and an Oxford CF935 dynamic continuous-flow cryostat connected to an Oxford LLT 650 low-loss transfer tube. Echo decay experiments used a two-pulse sequence, $\pi/2$-t-π, where t was increased by a step size of 8 ns for 1024 points. T_m values were obtained by fitting the Echo decay results with a stretched exponential decay. The time point where the signal is $1/e$ of the original intensity is the reported T_m value. Inversion recovery experiments followed a three-pulse sequence, π-t_1-$\pi/2$-t_2-π, where t_2 was 400 ns and t_1 was increased by a step size of 1 μs or 10 μs for 1024 points. Fitting of inversion recovery data is detailed in the Supporting Information. Double Quantum Coherence (DQC)[22,86] was performed at the field with the maximum signal intensity of mOX063-d$_{24}$. The DQC experiments followed a six-pulse sequence, $\pi/2$-t_p-π-t_p-$\pi/2$-t_1-π-t_1-$\pi/2$-t_2-π, where t_p and t_2 were increased and decreased respectively by 10 ns for 136 points. The initial parameters were set as $t_p = 1.3\ \mu s$, $t_1 = 50\ ns$, and $t_2 = 1.5\ \mu s$. To remove unwanted echo signal, the 64-step phase cycle was implemented[22,23]. The DQC time traces were then analyzed using DeerAnalysis[87] by Tikhonov Regularization.

SNR was calculated from the raw DQC time traces using previously published method[88]. In summary, the raw DQC time trace was fitted to a 5$^{\text{th}}$-order polynomial. The fit was subtracted from the time trace to isolate the noise of the time trace. The noise was used by the software SnrCalculator to calculate the final SNR.

Results and Discussion

Scheme 1. Synthesis of mOx063-d₂₄ from Ox063-d₂₄. Reproduced from Hasanbasri, *JPC* 2021.

Our recent report of the synthesis of OX063 triarylmethyl radical and its deuterated analogues OX063-d$_{24}$[83] enables the synthesis of OX063 derivatives such as spin labels. A short maleimide linker was conjugated to OX063-d$_{24}$ using PyBOP peptide coupling reagent as depicted in Scheme 1. The mOX063-d$_{24}$ was isolated in 40% yield after purification on C18 alongside with 10% of the di-maleimide derivative.

We overexpressed and labeled E15C GB1 with mOX063-d24 through a reaction between cysteine residues and the maleimide linker. The labeling reaction occurred by incubating E15C GB1 and mOX063-d24 in PBS pH 7.4 overnight. The solution was filtered through a centrifugal filter with a molecular weight cut-off of 5kDa to remove free mOX063-d24. We first performed ESI-MS to confirm the covalent attachment of mOX063-d24 to E15C GB1. The data is shown in Figure S3. The MS showed peaks corresponding to singly labeled and non-

labeled E15C GB1. This result is expected because of the detachment of the spin label during sample preparation in trifluoroacetic acid for ESI-MS and has been reported before using a maleimide-linked FT[78].

The final product was characterized using UV-Vis to assess spin-labeling efficiency. This data is shown in Figure 2. The spectrum features two distinctive peaks at 280 nm and 469. Only mOX063-d$_{24}$ contributes toward the 469-nm peak[89], while both mOX063-d$_{24}$ and GB1 contribute toward the 280-nm peak. The UV-Vis spectrum was analyzed using the deconvolution method, as depicted in Figure 2[78], which fits the mOX063-d$_{24}$-GB1 spectrum using GB1's UV-Vis spectrum (dash-dotted line) and mOX063-d$_{24}$'s UV-Vis spectrum (dotted line). The deconvolution allowed us to obtain the absorbance of GB1 at 280 nm (ε_{280}= 9970 M^{-1} cm^{-1}) and mOX063-d$_{24}$ at 469 nm (ε_{469}= 16000 M^{-1} cm^{-1})[89], which were used to calculate their concentrations. The final concentrations of GB1 and mOX063-d$_{24}$ in the sample are 165.0 µM and 161.9 µM, respectively. Therefore, the ratio of GB1: mOX063-d$_{24}$ purified is about 1:0.98. Overall, our UV-Vis results indicate efficient mOX063-d$_{24}$ labeling of cysteines on GB1.
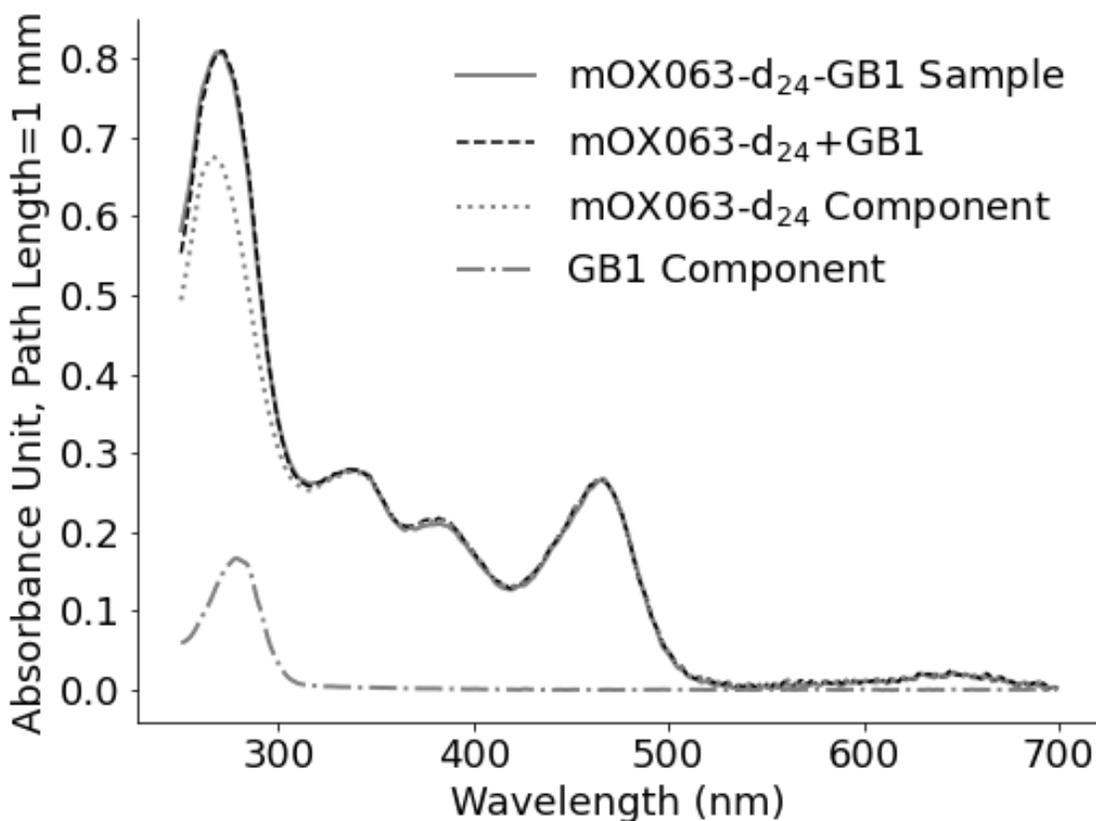
Figure 2. UV-Vis spectrum of mOX063-d$_{24}$-GB1 sample (gray line). The mOX063-d$_{24}$-GB1 spectrum was deconvoluted into its GB1 (dash-dotted line) and mOX063-d$_{24}$ (dotted line) components. The sum of the two components (dashed line) fits well with the mOX063-d$_{24}$-GB1 spectrum. Reproduced from Hasanbasri, *JPC* 2021.

To further validate the labeling efficiency, the mOX063-d$_{24}$-GB1 sample was characterized using CW-ESR at room temperature.Figure 3A and 3B show the CW-ESR spectrum of mOX063-d$_{24}$ bound to GB1 and free mOX063-d$_{24}$. The free mOX063-d$_{24}$ contained a superhyperfine interaction with the amide nitrogen ($a^N \sim 220\ mG$) on the linker, depicted as a partially resolved triplet splitting of the ESR lineshape. This nitrogen hyperfine is consistent with previously published trityls with ^{14}N-containing linkers[79,90,91]. After mOX063-d$_{24}$ reacted with GB1, the superhyperfine nitrogen were broadened and unresolved due to the slower tumbling rate upon protein binding, seen in Figure 3B[79]. However, the tumbling rate after protein binding is still rapid enough to resolve the satellite ^{13}C peaks in the CW of GB1-bound mOX063-d$_{24}$, seen in Figure 3A. This behavior has been described in a previous report of OX063-d$_{24}$ spin-label[79]. Spin-counting of the mOX063-d$_{24}$-GB1 CW spectrum yields a spin concentration of 190 μM. Given the protein concentration of 194 μM, these results indicate a labeling efficiency of 97%, which agrees with the UV-Vis data.
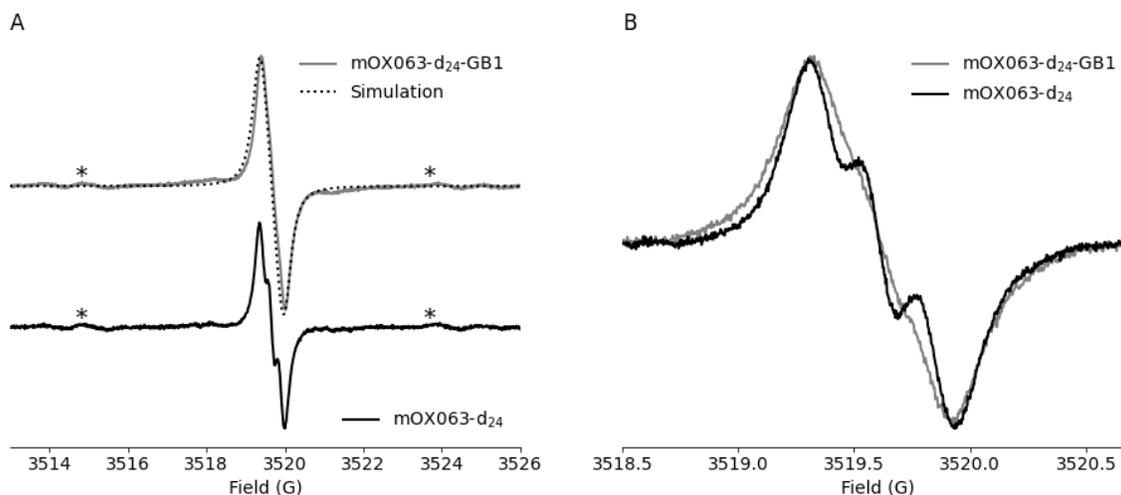
Figure 3. A) CW-ESR spectra of mOX063-d$_{24}$-GB1 (top) and mOX063-d$_{24}$ (bottom). The spectrum of mOX063-d$_{24}$-GB1 can be fitted with a narrow single-component simulation. The ^{13}C satellite peaks are marked with *. B) The CW-ESR spectra of mOX063-d$_{24}$-GB1 and mOX063-d$_{24}$ with the observation window ~2 G at the central lineshape. The nitrogen superhyperfine is partially resolved in the mOX063-d$_{24}$ spectrum but not in the mOX063-d$_{24}$-GB1 spectrum. Reproduced from Hasanbasri, *JPC* 2021.

More importantly, the mOX063-d$_{24}$-GB1 CW spectrum can be fitted with a narrow single-component simulation without a broad component, commonly seen when using FT[74,75,78,92]. FT's broad component has been attributed to aggregated species of FT[76,77] and non-specific binding in proteins[75,76,79,93] and membranes[74]. As a result, when using the simple spin-labeling workflow, FT had labeling efficiencies of 24% to 80% depending on the linker and protein[67,78,79,92]. On the other hand, mOX063-d$_{24}$ is highly soluble and does not bind non-specifically[79]. Therefore, the hydrophilicity of mOX063-d$_{24}$ allows ~100% labeling efficiency using simple protein labeling protocols. Additionally, the labeling efficiency of mOX063-d$_{24}$ is slightly improved from the previously developed hydrophilic trityl spin-label, Ox-SLIM, which reported to have 85% labeling efficiency[80]. Such differences in labeling efficiency could be due to the difference in maleimide linker length between mOX063-d$_{24}$ and Ox-SLIM, and potentially to the differences in solvent accessibilities between the two sites in the two proteins.

Next, pulsed-ESR was used to measure the relaxation times of GB1-bound mOX063-d$_{24}$ since these are critical parameters that dictate the efficacy of the label in pulsed dipolar spectroscopy. These data were acquired at a spin concentration of 5 μM and the sample was prepared in 20 mM PBS buffer at pH 7.4, and contained 20% glycerol. The phase-memory relaxation time (T_m) was measured by echo decay experiments.The measured values of T_m are listed in Table 1 and the data is shown in Figure S4A. The T_m of GB1-bound mOX063-d$_{24}$ is 5.1 μs, 4.3 μs, or 3.6 μs at 80 K, 150 K, or 180 K, respectively. These relaxation measurements provided additional data points to the existing measurements from previous studies of OX063-based spin-labels (cf. Table S1). For comparison, the T_m value of 5.1 μs for GB1-bound mOX063-d$_{24}$ at 80 K is longer then the T_m value of 1.6 μs of protein-bound FT at 80 K[78]. The longest reported T_m of protein-bound FT is 2.9 μs at 50K[79]. Increasing the T_m can both increase the echo intensity for distance measurements and increase the range of feasible temperature of the experiment.

At higher temperatures, mOX063-d$_{24}$ also benefit from the shortening of T_1. GB1-bound mOX063-d$_{24}$ has T_1 values of 1.98 ms, 0.175 ms, and 0.112 ms at 80 K, 150 K, and 180 K respectively (Table 1, Figure S4B and S4C). The mechanism for T_1 relaxation of trityl radicals as a function of temperature has been previously studied[94]. As T_1 gets shorter with increasing temperature, the amount of time required for GB1-bound mOX063-d$_{24}$ to completely relax becomes shorter leading to a faster rate of repeating the measurement. For comparison, the T_1 value of 1.98 ms for GB1-bound mOX063-d$_{24}$ at 80 K listed in Table 1 is slightly longer than the T_1 value of 1.7 ms of protein-bound FT at 80 K[78]. However, distance measurements using FT are typically done at 50 K or lower which has T_1 values of 6.3 ms or longer[78]. Therefore, distance measurement using mOX063-d$_{24}$ at higher temperature leads to more scans per unit of time than the distance measurement using FT at the typical temperature of 50 K. Consequently, we expect that distance measurements using mOX063-d$_{24}$ at higher temperatures benefit from a shorter T_1.

Table 1. T_m and T_1 measurements of mOX063-d$_{24}$-GB1 at 80 K, 150 K, and 180 K.

Temperature	T_m	T_1
80 K	5.1 μs	1.98 ms
150 K	4.3 μs	0.175 ms
180 K	3.6 μs	0.112 ms

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To showcase the sensitivity of mOX063-d$_{24}$, DQC experiments at 80 K or 150 K were performed on E15C/K28C GB1 doubly-labeled by mOX063-d$_{24}$ (dmOX063-d$_{24}$-GB1), as shown in Figure 4A. The method to measure SNR is described in the methods section. The DQC time trace achieved
sufficiently high SNR at 150 K within approximately 1.5 hours of runtime ($SNR = 20\ min^{-1/2}$). On the other hand, at 80 K, even after 2 hours of runtime, the DQC time trace ($SNR = 7\ min^{-1/2}$) is noisier than the 150 K DQC time trace. The higher SNR of 150 K DQC than the SNR of 80 K DQC can be rationalized by the following analysis of SNR for pulsed-ESR experiments[95]:

$$SNR(T) \propto \frac{1}{T}\exp\left[-t_{tot}/T_m(T)\right]\sqrt{\frac{1}{T_1(T)}} \text{(Eq.1)}$$

where T is the temperature and t_{tot} is the amount of time the electron coherence evolves until the detection of the echo signal. The $1/T$ term in Eq.1 is due to the Boltzmann factor[96]. Based on Eq.1, the shorter $T_1 = 0.175\ ms$ at 150 K than the $T_1 = 1.98\ ms$ at 80 K (Table 1, Figure S4B and S5C) contributes to 3.36 times improvement in SNR. On the other hand, the increase in temperature from 80 K to 150 K only led to a slight reduction of T_m from 5.1 μs to 4.3 μs. Furthermore, the increase in temperature causes a loss in echo intensity due to the reduction in spin-polarization. Based on Eq.1, the decrease in T_m and spin-polarization reduces the SNR by 0.43 times. As a result, the final SNR at 150 K is $3.36 \times 0.43 = 1.44$ times higher than the SNR at 80 K. We can see the SNR improvement from the DQC echo comparison between 80 K and 150 K shown in Figure S5. Overall, the gain in sensitivity due to T_1 was able to over-compensate the loss of echo intensity from the shortening of T_m and the reduction of spin-polarization. However, increasing the temperature further to 180 K causes the reduction in sensitivity due to reduced spin-polarization and T_m. For example, the sensitivity at 180 K is ~79% of the sensitivity at 150 K based on Eq.1. These comparisons signify the importance of experimentally evaluating the relaxation times at various temperatures, since the values can be different for different systems. While 80 K is not the most optimal temperature for mOX063-d$_{24}$ DQC, its $SNR = 7\ min^{-1/2}$ is comparable to the reported FT's SNR[78] ranging from $7\ min^{-1/2}$ to $8.9\ min^{-1/2}$ at 50 K. These comparison of SNR exemplifies the sensitivity gained from performing mOX063-d$_{24}$ DQC experiments at the optimal temperature.

We analyzed the time traces using the DeerAnalysis2018[87] package and the Tikhonov Regularization method to extract the distance distributions shown in Figure 4B. Expectedly, at both temperatures, the distance distributions were close to identical, with the most probable distance of 3.6 nm. In order to predict the distance distribution, we built an *in silico* model using MTSSLWizard[97]. Since the mOX063-d$_{24}$ spin-label does not exist in the MTSSLWizard package, we first implemented the mOX063-d$_{24}$ model into the MTSSLWizard software. Details are provided in Figure S6. The model predicted that the most probable distance is 3.8 nm, as shown in Figure 4B, which is in reasonable agreement with the DQC results. Furthermore, the experimental results have

a standard deviation of ~0.6 nm, which is on par with the standard deviation of ~0.8 nm obtained using nitroxide on the same GB1 mutant[85].
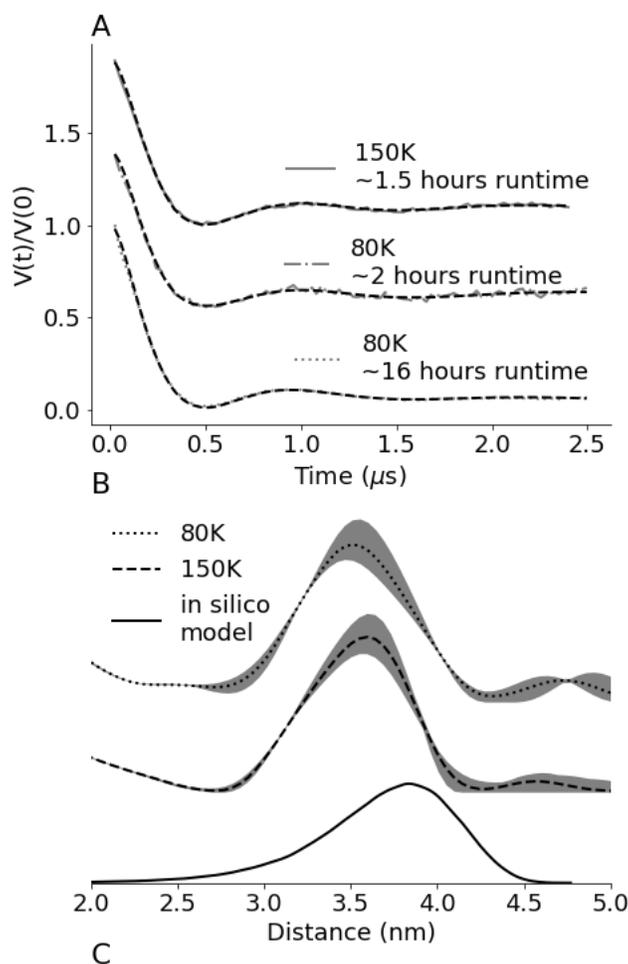
Figure 4. A) DQC time traces of doubly-labeled dmOX063-d_{24}-GB1 at 150 K after 1.5 hours of runtime and at 80 K after 2 hours and 16 hours of runtime. B) Distance distributions obtained from the 150 K and 80 K DQC time traces using DeerAnalysis. The gray regions represent the error obtained from the validation function in DeerAnalysis. Additionally, a distance distribution was also obtained from *in silico* modeling using MTSSLWizard. C) *In silico* model from MTSSLWizard using GB1 (PDB:2QMT) and mOX063-d_{24}. The two clusters represent the space occupied by the radical carbon. Reproduced from Hasanbasri, *JPC* 2021.

After the in vitro experiments, the viability of mOX063-d_{24} for *in-cell* experiments was explored. Specifically, the dmOX063-d_{24}-GB1 (200 μM of spins) was subjected to either ten times excess of ascorbic acid or the cytosol extract of *Xenopus laevis* (African Bullfrog) oocytes. Cytosol was extracted from oocytes using previously published protocol[85]. The signal intensity of mOX063-d_{24} was monitored over time using CW ESR, and the maximum intensity of each CW was plotted against time in Figure 5A. The signal intensity decays to about 97 and 95% of its original intensity in ascorbate and cytosol after 5 hours, respectively. The stability of mOX063-d_{24}

is on par with the stability of other trityls[73,80]. The signal persistence of mOX063-d_{24} showcases the reduction-resistance of mOX063-d_{24} against the cytosolic antioxidants that play a role in reducing radicals *in-cell*[98].

After measuring mOX063-d_{24} stability, dmOX063-d_{24}-GB1 was injected into oocytes and incubated for 30 minutes after injection before flash-freezing the sample. The 30 minutes incubation allows for dmOX063-d_{24}-GB1 to completely diffuse in oocytes[48]. Echo decay experiment measured the T_m of mOX063-d_{24} at 80 K in oocytes to be 4.3 μs as shown in Figure S7, which is shorter than the T_m of mOX063-d_{24} in vitro shown in Table 1 and Figure S4A. The lower GB1-bound mOX063-d_{24} T_m *in-cell* compared to *in vitro* was expected because of the crowded environment *in-cell*. The crowded environment can lead to an increase in the local concentration of protons near the radical which enhances the contribution of electron-nuclei interactions to relaxation. In addition, the presence of paramagnetic metal ions, primarily Mn(II)[99], in the cell can enhance relaxation. However, the T_m of mOX063-d_{24} in oocytes is surprising since previous reports of other organic spin-labels used *in-cell* (nitroxides[50,57,100] and FT[67,72,73]) have T_m values in the range of 0.6-2 μs. Therefore, mOX063-d_{24} also improves the sensitivity of distance measurements *in-cell* due to the T_m that is at least 2 times longer than previously published T_m of nitroxide or FT *in-cell*.

Distance measurements of dmOX063-d_{24}-GB1 in oocytes were done using DQC at 80 K shown in Figure 5B. We observed an artifact that overlaps the desired DQC signal at zero time. Such an artifact has been seen previously and attributed to trityl dimers and to partial labeling of non-cysteines residues such as lysine[101]. We repeated the labeling procedure on WT GB1 that has no cysteine residues. After concentrating the sample, UV-Vis indicates no presence of mOX063-d_{24} as shown in Figure S8. We expected this result since our previous work using GB1 and maleimide-linked nitroxide (5-MSL) did not show over-labeling of the protein[85]. In addition, we did not see an ESR signal from the WT GB1 sample which also excludes the presence of dimers.

We attribute this artifact as due the formation of a small echo generated by the first and the fourth pulses in the DQC 6-pulse sequence.This interference can be readily seen in the 2D contour plot of the DQC signal shown in Figure S9A. As a result, the DQC time trace contained an artifact shown as a sharp feature at the $t = 0$ shown in Figure S9B, which led to improper fitting of the time trace. Additionally, the artifact contributed to a short distance around 2 nm shown in Figure S10A. The artifact seemed to be a result of inefficient phase-cycling in our DQC experiment and is evident in the in-cell data due to the lower SNR.

To support this hypothesis, we performed the DQC experiment using the same parameters on $300 \mu M$ TEMPOL as shown in Figure S11. The same artifact was seen crossing the desired DQC echo at a slanted angle shown in the 2D contour plot in figure S11A. As a result, a sharp feature at t=0 manifested as shown in Figure S11B. The artifact was more prominent in the *in-cell* experiment than in the *in vitro* experiment for two reasons. First, the measured echo in the *in-cell* DQC was half as intense as the measured echo in the *in vitro* DQC. The lower *in-cell* echo intensity is due to the shorter T_m *in-cell* than the T_m *in vitro*. Additionally, reduction of mOX063-d_{24} can still occur due to the contribution of oocytes' membrane-associated factors[85] such as thioredoxin[102] and glutathione reductase[103], which are not accounted for in our cytosol stability measurement. These two contributions led to a less intense measured echo causing the artifact to be prominent in the *in-cell* DQC.

To remove the artifact in the DQC time trace in oocytes, DQC was performed on a sample of free mOX063-d_{24}, which contained only the artifact shown in Figure S9B. The free mOX063-d_{24} DQC time trace was used to subtract the artifact from the time trace of dmOX063-d_{24}-GB1 in oocytes shown in Figure 5B. The artifact-subtracted time trace was used to extract the distance distribution shown Figure 5C, which agrees quite well with the *in vitro* distance measurements in Figure 4B. Furthermore, we were able to repeat the *in-cell* DQC experiment at 150 K, shown in Figure S12, and obtain a similar distribution as the 80 K *in-cell* distribution. Additionally, we repeated our *in-cell* experiments at 80 K using a different batch of oocytes and newly overexpressed and labeled-GB1 to ensure that the *in-cell* results are reproducible. This data is shown in Figure S13. Overall, we obtained a highly sensitive distance measurement in oocytes using mOX063-d_{24}.
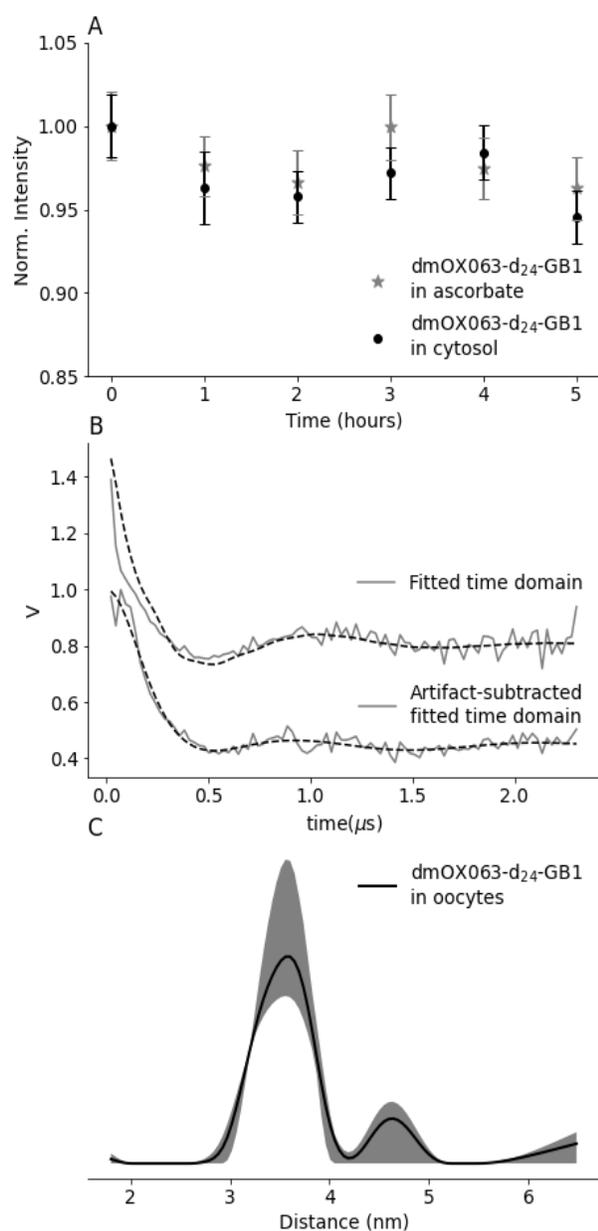
Figure 5. A) A plot of the maximum intensity of dmOX063-d$_{24}$-GB1 vs. time in 10 times excess of ascorbate or cytosol extracted from *Xenopus laevis* oocytes. The height of the vertical bars represents the RMSD in the CW spectrum. B) DQC time trace of dmOX063-d$_{24}$-GB1 at 80 K Q-band before and after artifact subtraction. C) Distance distribution (most probable distance of 3.6 nm) extracted from the artifact-subtracted time-domain signal using DeerAnalysis. The gray region represents the error obtained from the validation function. Reproduced from Hasanbasri, *JPC* 2021.

Conclusions

In conclusion, this work showed that mOX063-d$_{24}$ has a high protein-labeling efficiency of ~97%. Furthermore, we showed that *in vitro* distance measurements of mOX063-d$_{24}$ is more sensitive at higher temperatures. Finally, we obtained distance measurements using mOX063-d$_{24}$ *in-cell* which agree with *in silico* modeling. This work adds to the library of spin labels that can be used for *in-cell* work.In particular, mOX063-d$_{24}$ is similar to Ox-SLIM[80] since both are hydrophilic spin-labels with a maleimide linker, as shown in Figure 1A. However, these two spin-labels differ in their trityl cores and linker lengths. These differences provide variation in the labeling efficiency, T_m, and breadth of distance distribution. In one case, Ox-SLIM's short linker length can provide narrow distance distributions that can readily resolve different protein conformations[73,80]. On the other hand, mOX063-d$_{24}$ provides longer T_m and higher labeling efficiency leading to the sensitivity improvement in the distance measurements. Overall, Ox-SLIM and mOX063-d$_{24}$ are complementary to each other due to their differences.

Supporting Information

HRMS-ESI data of mOX063-d$_{24}$ and mOX063-d$_{24}$-GB1, UV-Vis of mOX063-d$_{24}$ + WT GB1, pulsed-ESR relaxation measurements, details of MtsslWizard modeling, comparison of *in vitro* DQC echo of dmOX063-d$_{24}$-GB1 at 80 K and 150 K, raw 1D and 2D DQC time domains, *in-cell* DQC data at 150 K and 80 K (second trial).

Acknowledgments

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