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Oral Presentations
Solutions for High-End Research to Routine Analytical Tasks

Arthur Heiss

EPR Division, Bruker BioSpin Corp., Billerica, MA 01821

Arthur.Heiss@bruker.com

One of the main drivers for new developments in EPR is the demand for higher signal-to-noise. With a number of recent and upcoming product introductions we have made significant progress in this field.

The high power Q-Band pulse-EPR setup allows running DEER experiments with dramatically improved sensitivity, e.g. the measurement time of 22h in X-Band is reduced to 25min in Q-Band, thus increasing sample throughput by more than a factor of 50. To achieve this, a combination of a high power pulse amplifier (150W) and a large volume resonator are used allowing short inversion pulses of 10ns at 150MHZ resonator bandwidth.

Limitations in excitation bandwidth are a severe handicap in pulse-EPR. The availability of high speed arbitrary waveform generators allows new methods in EPR based on pulse shaping for larger bandwidth excitation. With shaped broad band inversion pulses, the DEER modulation depth can be improved by a factor 2 – 3 and HYSCORE spectra can be measured with much higher S/N.

An ongoing project to substantially increase S/N is the development of a rapid scan unit. The direct registration of the EPR spectra via Rapid Scan method allows recording of absorption spectra, exciting complete spectrum in single shot. In addition due to the short time during which the spins are exposed to microwave field, the saturation effect is less pronounced compared to cw-EPR. This allows use of high microwave fields and consequently increases signal amplitude.

Currently Direct Rapid Scan (DRS) EPR Accessory is being designed by Bruker to further extend the functionality and performance of ELEXSYS and EMX plus systems. The preliminary information about accessory specification will be presented and its performance demonstrated with experimental results.

For a routine analytical tasks in a commercial use all technological advances have to be combined with a strong focus on usability. This is exemplified by the EMXnano bench-top spectrometer which features full instrument calibration with respect to field and signal amplitude, dedicated workflows and requires from the user only little technical knowledge.
The evolution of biomedical EPR (ESR)

Lawrence J. Berliner, Department of Chemistry and Biochemistry
2190 E Iliff Ave, University of Denver, Denver, Colorado USA.

Abstract: Although the first EPR/ESR spectrum of a paramagnetic substance was observed over 70 years ago, technical improvements did not evolve until World War II with the advent of radar technology. The approaches to biomedical problems started somewhat later with the real burst with the spin label technique about 50 years ago. The applications to proteins, then membranes and nucleic acids, and later applications to cells and eventually in-vivo on small animals and now humans. This talk gives an overview of EPR/ESR studies of biomedically related systems. It also presents a personal historical perspective and reflects on possible future directions.
Fluorescent Proteins such as GFP Catalytically Generate Superoxide and H$_2$O$_2$

Douglas Ganini, Fabian Leinisch, Ashutosh Kumar, JinJie Jiang, and Ronald P. Mason

Free Radical Biology, Immunity, Inflammation & Disease Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA.

e-mail: mason4@niehs.nih.gov

Fluorescent proteins are an important tool that has become omnipresent in life sciences research. They are frequently used for localization of proteins and monitoring of cells. Green fluorescent protein (GFP) was the first and has been the most used fluorescent protein. Enhanced GFP (eGFP) was optimized from wild-type GFP for increased fluorescence yield and improved expression in mammalian systems. In fact, many GFP-like fluorescent proteins have been discovered, optimized or created, such as the red fluorescent protein, TagRFP. Here we show that immature intermediates of eGFP induce oxidative stress in biological systems by reacting with NADH. eGFP and TagRFP catalytically generate the free radical superoxide anion (O$_2^{•–}$) in an NADH-dependent manner. We detected for the first time the generation of the free radical O$_2^{•–}$ in samples of eGFP and NADH using ESR spin trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The spectrum was a superimposition of the spin adducts DMPO/$\cdot$OOH and DMPO/$\cdot$OH, was present for over 30 min and was totally dependent on eGFP and NADH.

DMPO/$\cdot$OOH is known to decay to DMPO/$\cdot$OH by a number of pathways. The signal was sensitive to superoxide dismutase, but not to catalase, as expected. We demonstrate that the free radical O$_2^{•–}$ and consequent H$_2$O$_2$ generated by eGFP in the presence of NADH affects the gene expression of cells. Many biological pathways are altered, such as a decrease in HIF1α stabilization and activity. The biological pathways altered by eGFP are known to be implicated in the pathophysiology of many diseases associated with oxidative stress. However, since cells inevitably experience oxidative stress when fluorescent proteins are expressed, the use of this tool for cell labelling and in vivo cell tracing also requires validation using alternative methodologies.

Carbonaceous particulate matter (PM), mostly produced by combustion processes, has been extensively investigated because of its dangerous effects on human health. It is known the correlation between the presence of inorganic substances (mostly salts of metals and nanoparticles of silica deposited or grafted on the surface of the carbonaceous particles) and the production of reactive oxygen species (ROS) which play a role of *prima donna* in inducing biological damages, such as inflammation and oxidation of DNA in cells exposed to PM [1, 2, 3]; nevertheless, it is still debated whether ROS production is caused only by these inorganic components, or the carbonaceous core also can give a contribution.

We have then focused our attention on the superoxide production reactions in the presence of a series of materials made by pure carbon: both nanographites produced by ball milling (ordered structure samples) and commercial nanocarbons (disordered-structure sample) were considered as a model for the pure carbonaceous core of PM's. A sample of diesel soot was considered as reference material. Spin trapping and Raman measurements in aqueous suspension show a clear-cut enhancement of the production rate of superoxide ions. The reactivity of the particles is affected by the presence of the so-called *edge-states*. The states are described in terms of limited-dimension orbitals localized at the edges of the graphitic platelets, which are populated by unpaired electrons at the Fermi level of graphite; they have been carefully characterized with EPR methods by some of us [4, 5].

The production of ROS species in the presence of carbon nanoparticles was also tested by spin trapping method and by fluorescence measurements; in this case human bronchial epithelial cells were used.

**Bibliography**

Nitrone spin traps for the EPR detection and characterization of transient free radicals

M. Hardy, G. Casano, F. Poulhès, D. Bardelang, P. Tordo, H. Karoui, O. Ouari

Institut de Chimie Radicalaire (ICR), Aix Marseille University and CNRS, Marseille, France.

olivier.ouari@univ-amu.fr

Our recent results on the design and tailoring of nitrone spin traps for improved detection of superoxide radical by spin trapping technique will be reported. In the past decades, spin trapping has shown to be a powerful and reliable technique to study transient free radicals, but its application to biological systems has been hampered mainly by the low trapping rate of superoxide radical and the fast reduction of the generated spin adducts. A series of spin traps has been synthesized to better understand the parameters driving the spin trapping properties and new spin traps have been prepared with improved superoxide trapping rate and enhanced resistance to reduction by glutathione and enzymes.1-3 Also, the development of dinitroxides as polarizing agents for Dynamic Nuclear Polarization / ssNMR at high field will be presented.4-6

References

Use of Electron Paramagnetic Resonance (EPR) to Elucidate Pathologic Responses

Vincent Castranova

Dept. of Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, WV 26506
vcastran@hsc.wvu.edu

Transient inflammation in response to an acute biological insult, such as a microbial exposure or trauma, plays a vital role in the resolution of infection and in wound healing. However, in response to a severe or chronic biological insult, sustained inflammation is associated with the initiation and progression of chronic diseases, such as fibrosis, diabetes, cardiovascular disease, cancer, emphysema, etc. This pathogenesis involves a positive feedback loop between inflammation and oxidant stress, where inflammation induces the generation of reactive oxygen species (ROS), which in turn causes oxidant damage to DNA and activates the further production of inflammatory, fibrogenic, and proliferative mediators.

This presentation describes three examples in which EPR has been used effectively to elucidate the pathogenic process following particle inhalation. These examples include the use of EPR to:

1) Provide a mechanistic explanation for the enhanced pulmonary response to freshly fractured vs. aged crystalline silica - Results indicate fracturing crystalline silica particles, as would occur in rock drilling, grinding or sandblasting, significantly increases the generation of hydroxyl radicals as measured by EPR. This increase in radical generation results in an increase in oxidant damage and pulmonary inflammation in animal models. These results accurately predict that workers exposed to freshly fractured silica would exhibit lung disease (acute silicosis) more rapidly than workers exposed to aged silica, who develop chronic silicosis over decades of exposure.

2) Rank the biological activity of various metal and metal oxide nanoparticles on the lung - Results indicate that hydroxyl radical production from alveolar macrophages exposed to various metal and metal oxide nanoparticles is a strong predictor of the degree of lung inflammation observed in rats after pulmonary exposure to these nanoparticles.

3) Determine whether carbon nanotubes cause pulmonary fibrosis via mechanisms similar to asbestos – Results indicate that, unlike asbestos, purified carbon nanotubes do not generate hydroxyl radicals in the absence of alveolar macrophages, yet both cause lung fibrosis. These results suggest that carbon nanotube-induced fibrosis does not involve the mechanism of persistent oxidant injury and inflammation as is the case with asbestos.
Biomarkers of oxidative stress: Reinterpreting the best biomarker of oxidative stress in toxicity and disease.

Maria B. Kadiiska, Thomas J. van’t Erve, Ronald P. Mason.
National Institutes of Health/ National Institute of Environmental Health Sciences
111 T.W. Alexander Dr., Research Triangle Park, NC 27709, USA.
Kadiiska@niehs.nih.gov

Investigation of oxidative stress biomarkers is a subject of continuously increasing interest, both in science and in commerce. The field is evolving and there is a great need not only to validate but also to understand biomarkers at a chemical and enzymatic molecular-mechanism level. Antioxidants and oxidized lipids, proteins and DNA levels in plasma and urine of experimental animals exposed to varied kinds of exposures were measured as a method-validation study.

The goal of this international and multi-laboratory study known as the BOSS (Biomarkers of Oxidative Stress Study) is to identify a biomarker for CCl4-, ozone- or LPS -induced oxidative stress and to assess whether inconsistent results often reported in the literature might be due to the limitations of the available methods for measuring the various types of oxidative products.

The BOSS study compared data from measurements with more than 30 different techniques to estimate plasma and urinary oxidative products and antioxidants in all three models of oxidative stress in order to conclude which oxidized molecules or antioxidants fulfilled the oxidative stress biomarker criterion of significant effects measured in biological fluid and seen at two doses at more than one time point. It is determined that measurements of lipid oxidation products 8-iso-PGF2α in plasma and urine is the potential candidate for general biomarkers of oxidative stress. Therefore, currently, the best accepted biomarker of oxidative stress is the lipid oxidation product 8-iso-prostaglandin F2α (8-iso-PGF2α), which has been measured in over a thousand human and animal studies. 8-iso-PGF2α generation has been exclusively attributed to nonenzymatic or chemical lipid peroxidation (CLP). However, 8-iso-PGF2α can also be produced enzymatically by the prostaglandin-endoperoxide synthases (PGHS) in vivo. When failing to account for PGHS-dependent generation, 8-iso-PGF2α cannot be interpreted as a selective biomarker of oxidative stress.

We established that the 8-iso-PGF2α/PGF2α ratio can be used to distinguish chemical from enzymatic lipid peroxidation and therefore the ratio of 8-iso-PGF2α to prostaglandin F2α (PGF2α) is recommended as a quantitative measure to distinguish enzymatic from chemical lipid peroxidation in vitro, in animal models, and in humans. It is concluded that the 8-iso-PGF2α/PGF2α ratio accurately determines the source of 8-iso-PGF2α and provides an unbiased measure of oxidative stress in vivo. This study, for the first time, offers specific approaches to measure, calculate, distinguish, and correctly identify the differences between the effects of oxidative stress and inflammation.

Even though inflammation and oxidative stress are discussed separately in many publications, lectures, and past SOT presentations, the complex cross-talk and importance to distinguish between the two mechanisms is rarely investigated. Although inflammation, free radical damage and oxidative stress are not “diseases”, distinguishing among them in vivo and in human disorders could lead to better interceptive strategies and correct interpretation of the results in former and future studies.
Electron Paramagnetic Resonance in determining metal oxide toxicity.

Stephen S. Leonard

Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV 26505, USA.

Occupational and environmental exposures to metals are associated with many forms of toxicity and the development of various cancers. A wide variety of metals have been reported to act as mutagenic and carcinogenic agents in both human and animal studies. Although toxicity caused by metals has been intensively investigated, the mechanisms of action, especially at the molecular level, are still unclear. Accumulating evidence indicates that reactive oxygen species generated by metals may play an important role in the etiology of disease. Electron paramagnetic resonance (EPR) is a valuable tool in examining metal-induced generation of reactive oxygen species, the mechanisms involved, their effects, possible treatments and can be an important first step in examining metal or metal containing compound possible toxicity. Using EPR as a research tool is a fast and relatively inexpensive method to screen compounds for their possible toxic effect on biological systems. EPR can also determine if compounds have impurities of reactive metals and screen a large number of samples down to a smaller sample size of critical interest. Using chemical, enzymatic and cellular methods, samples can be quickly measured for reactivity and production of hydroxyl and superoxide radicals. Our investigations of occupational exposures have used EPR for reactive oxygen species-related toxicity determination in various metals, metal oxides, nanoparticles, agricultural dust, airborne sand from Iraq, fumes and smoke samples. After serving to help screen samples for the more reactive types, EPR can then be used to define type, and strength of radicals involved as well as their generation pathways. EPR can also be used to investigate possible treatments and ways of reducing free radical generation due to occupational exposures. Due to the extensive information it can provide, its adaptability, speed, and relative low cost EPR is a valuable method in research of toxicity in occupational exposures.

Disclaimer: The findings and conclusions in this report are those of the author and do not necessarily represent the views of the National Institute for Occupational Safety and Health.
Fluorescence Imaging of Subcellular-Targeted Spin Traps

Villamena, Frederick A. and Headley, Colwyn

Department of Biological Chemistry and Pharmacology, College of Medicine, The Ohio State University, 473 W. 12th Ave. Columbus, OH 43205

villamena.1@osu.edu

Spin traps are nitrone-based compounds that have found application in the detection of free radicals in chemical and biological systems. Moreover, spin traps have been employed as therapeutic agent in ameliorating the effects of oxidative stress in diseases. Current limitations for their use in radical detection include short adduct half-life, slow reactivity to superoxide, and poor target specificity, and by improving the last two limitations could also increase spin trap’s pharmacological efficacy. In this presentation, efforts in spin trap development that address all the three limitations will be presented with emphasis on subcellular compartmentalization.
I will describe recent developments at our Cornell-ACERT research center.

Over the years we have developed the theory of slow motional ESR, and we have provided many experimental studies based on it. In recent years we have applied it for a new methodology to study the initial step in viral attack of a cell for many types of viruses. In a current study we have shown that the same transmembrane protein (HAP2) mediates the fusion of sperm and egg cells, and does the same for viral attacks of cells for virus types such as Dengue and Zika.[1] This raises the question: What came first—the virus or the egg?

The method of Pulse Dipolar Spectroscopy (PDS-ESR) that ACERT helped to pioneer has gained widespread use. We have been studying the complex mechanism of bacterial chemotaxis signaling; it involves a sensor that senses food or poison in its path, and its signal is then sent a distance of over 200 Å by receptor proteins to the active protein units which then signal the flagellum motor, telling it whether to move forward or away. Our extensive PDS studies[2] have elucidated key details of all these steps in the signaling chain.

We have pioneered Fourier transform Two-Dimensional Electron-Electron Double Resonance to study room-temperature dynamics in fluids and proteins. In recent work at 95 GHz with 1.2 kW pulses[3], we have studied the exchange of a spin probe between aqueous and lipid membrane phases. We see 2D cross-peaks emerge between the signals from the two phases. The rate of exchange was quantitatively measured in the microsecond range, which is a range not readily available by other techniques. But this is the range of protein conformational changes, and we are adapting the method to this objective.

Finally, I address how to proceed when one’s experiments, after much struggle, do not provide adequate SNR. We have developed a new denoising method, based on wavelet transforms, that enables one to improve the SNR by about two orders of magnitude while preserving the fidelity of the signal. Examples of retrieving signals from noisy ESR spectra will be shown.[4]

References:


Biocatalyst reactant-protein-solvent dynamical coupling revealed by multiple EPR probes and techniques

Meghan Kohne, Benjamen Nforneh, Neslihan Ucuncuoglu, Chen Zhu and Kurt Warncke

Department of Physics, Emory University, Atlanta, GA 30322
kwarncke@physics.emory.edu

The comprehensive microscopic understanding of enzyme-catalyzed reactions requires a description of the choreography of protein configurational fluctuations involved in the chemical step of substrate-to-product conversion, and the role of the solvent environment as a stochastic, bi-directional dynamical modulator. To reveal the effects of native protein configurations and inter-configurational motions on reaction chemistry, the configurational transition rates, displacement amplitudes (extents), or both, must be perturbed, so that the reaction rate is impacted. We have achieved this, and developed the necessary approaches to the resolution and characterization of individual chemical steps in enzyme catalytic sequences,[1] by measuring reaction kinetics in the cryogenic range of 190-250 K, by using time-resolved, full-spectrum electron paramagnetic resonance (EPR) spectroscopy in fluid cryosolvent [2,3] and frozen solution systems.[4, 5] In the B12-dependent ethanolamine ammonia-lyase (EAL) from Salmonella typhimurium the substrate radical rearrangement step is studied over the T range of 190-230 K. The low-T kinetics are marked by two abrupt changes in Arrhenius dependence with descending T, at 220 K (bifurcation) and 217 K (kinks). These changes in the free energy landscape indicate transformation from a dependence of the reaction on native collective fluctuations in the physiological reaction regime (220-295 K), to a dependence on local fluctuations in the regime below the transition (203-214 K). The bifurcation and kink transitions are proposed to represent the effective quenching of two distinct sets of specific native protein collective configurational fluctuations, that (1) reconfigure the substrate radical state and enable it for reaction, and (2) execute the chemical step of rearrangement. To address the role of protein-solvent dynamical coupling in the reaction, the motional properties of the protein-associated and mesodomain [6] solvent components around EAL are resolved and studied over comparable temperature (T) ranges by using the spin probe, TEMPOL. In parallel, protein surface dynamics were addressed by using 4-maleimido-TEMPO (4MT) spin-label attached to a surface cysteine side chain of EAL. The T-dependence of the rotational dynamics of TEMPOL and 4MT components as a function of T are revealed by the EPR line shape. The T-dependences of the TEMPOL and 4MT motional and phase partitioning properties are compared with the T-dependence of the rearrangement reaction kinetics, to identify the roles of solvent-protein motional coupling in the reaction. Supported by NIH R01DK054514.


Oral Presentation 11
Structure determination of oligomeric proteins in lipid bilayers by combining solid state NMR and long-range DEER constraints

S. Milikisyan,1 S. Wang,2 R. A. Munro,3 M. Donohue,1 M. E. Ward,3 D. Bolton,3 L. S. Brown,3 T. I. Smirnova,1 V. Ladizhansky,3 A. I. Smirnov1

1Department of Chemistry, North Carolina State University, 2620 Yarbrough Drive, Raleigh, NC, 27695, USA; 2 Beijing Nuclear Magnetic Resonance Center and College of Chemistry and Molecular Engineering, Peking University, 5 Yiheyuan Road, Haidian; Beijing National Laboratory for Molecular Sciences (BNLMS), Beijing, People's Republic of China; 3Department of Physics and Biophysics Interdepartmental Group, University of Guelph, 50 Stone Rd E., Guelph, Ontario, N1G 2W1, Canada

E-mail: Alex_Smirnov@ncsu.edu

Oligomerization of integral membrane proteins is a common biophysical phenomenon that is often the final step of protein folding into functional assemblies. Stability of protein oligomers varies. While some oligomeric assemblies could persist under a broad range of experimental conditions including solubilization in detergents, it is not uncommon for the oligomeric structures to be affected by the membrane lipid composition and even sample preparation procedures. These considerations warrant further development of experimental methods suitable for quantitation and structure determination of protein oligomers in the native lipid milieu.

Here, we combine spin-labeling Double Electron-Electron Resonance (DEER) at Q-band (34 GHz) and solid-state NMR (ssNMR) spectroscopy to refine the structure of a membrane-embedded trimer (~81 kDa) formed by seven-helical transmembrane photoreceptor Anabaena Sensory Rhodopsin (ASR, ~27 kDa) from Anabaena sp. PCC7120. ASR is a notable example of a membrane protein whose oligomeric state depends on the membrane mimetic environment. While ASR monomers have been shown to form stable trimers in cellular E.coli membranes1 and preserving the trimeric structure upon subsequent solubilization in detergents and upon reconstitution in lipids,2 the crystallization conditions appear to promote formation of dimers observed in the ASR crystals.3 An essential feature of a combined DEER + ssNMR approach employed here is that it provides structural distance restraints spanning a range of ca. 3-60 Å, while using the same sample preparation, such as site-directed mutations, paramagnetic labeling, and reconstitution in lipid bilayers, for both ssNMR and DEER. We also describe direct modelling of the multispin effects on the DEER signal to determine the oligomeric order and obtain long-range DEER distance restraints between the ASR trimer subunits that were used to refine solid-state NMR structure of ASR. The improved structure of the ASR trimer revealed a more compact packing of helices and side chains at the intermonomer interface when compared to the structure determined using solely the ssNMR data. The extent of the refinement is significant when compared with typical helix movements observed for the active states of homologous proteins. We believe that our combined approach of using complementary DEER and NMR measurements for the determination of oligomeric structures would be widely applicable to membrane proteins where paramagnetic tags can be introduced. Such a method could be used to study the effects of the lipid membrane composition on protein oligomerization as well as to observe structural changes in protein oligomers upon drug, substrate, and co-factor binding.

Insights into the mechanism of LPS transport in *E. coli* using site-directed spin labeling EPR spectroscopy

KM Schultz, M Fischer, CS Klug*

*Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI, USA

*candice@mcw.edu

Gram-negative bacteria such as *Escherichia coli* have an inner membrane (IM) and an asymmetric outer membrane (OM), which protect the cytoplasm and act as a highly selective permeability barrier for the cell. Lipopolysaccharide (LPS) is the major component of the outer leaflet of the OM and is essential for the survival of nearly all Gram-negative bacteria. LPS is transported across the periplasm to the outer leaflet of the OM by the Lpt (LPS transport) system, which in *E. coli* is composed of proteins LptA, LptC, LptDE, and LptFGB2. Structures of four of the seven proteins have been solved, and the structure and functional dynamics studies presented here focus on using experimental data using site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy techniques such as continuous wave (CW) EPR, double electron electron resonance (DEER), and high pressure (HP) EPR.

A major strength of the SDSL EPR spectroscopy technique is its ability to detect and follow changes in local protein structure due to inter- and intramolecular conformational changes or dynamic interactions with other proteins or substrates based on spin label mobility changes or distance changes between two spin labels. The R1 group, which is formed by the attachment of a small nitroxide spin label to a cysteine residue, is an excellent reporter of local structure and protein-ligand and protein-protein interactions. The ability to monitor the environment of spin labels at specific positions in a protein enables the detection of changes such as the oligomeric and protomeric structure of LptA, the identification of the location and quantification of LPS binding to LptA and LptC, and the characterization of LptA binding to its critically important binding partners LptC and LptDE.

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Pulsed -ESR techniques that reliably measure interspin separations in the order of 1.5-10 nm - even in non-crystalline samples - to ultimately provide an “amino-acid-level” picture of structure and structural transitions, have impacted biophysical research. The talk will discuss our efforts in developing Cu$^{2+}$-ion based pulsed-ESR distance methods and illustrate how they can potentially be used to understand structure function relationships in proteins. The talk will focus on restriction endonuclease EcoRI, which binds to the specific DNA sequence GAATTC with an affinity that is 50,000-90,000-fold greater than that of a miscognate site that differs by only one base pair. In the presence divalent metal ions, such as magnesium, EcoRI the specific sequence of viral DNA with a high specificity. We will describe the insights gained in regard to the high specificity as well as cleavage chemistry from ESR distance measurements. Finally, the talk will describe recent efforts to bind Cu$^{2+}$-ions site selectively at $\alpha$–helical and $\beta$–strand sites in protein. The spin probe is assembled in situ from natural amino acid residues and a metal salt, and requires no post-expression synthetic modification. Initial results show that the resultant Cu$^{2+}$-probe potentially provides distance distributions that are five times narrower than the common protein spin label – the approach, thus, has the potential to significantly overcome the inherent limitation of the current technology which relies on a spin label with a highly flexible side-chain.
Alamethicin and trichogin GA IV belong to the family of naturally occurring peptides that display antibiotic properties. The interest to this new type of drug is based on its ability to modify the permeability of biological membranes. Above a threshold concentration, these peptides form pores across the membrane, providing a mechanism of its antimicrobial action. However this mechanism requires rather high peptide concentration, sometimes unrealistic, as compared with the known therapeutic doses. Recently we have shown [1] that at a small concentration which is below the threshold value, alamethicin participates in formation of nanoscale lipid-mediated clusters of guest lipid-like molecules in the lipid bilayer. The similar results were then obtained also for the trichogin GA IV embedded in the bilayer. These data were obtained by electron spin echo (ESE) technique – a pulsed version of electron paramagnetic resonance (EPR) – on spin-labeled stearic acid in a model phospholipid bilayer with peptides added at 1/200 peptide-to-lipid ratio or even lower. ESE decay measurements are interpreted assuming that stearic acid molecules in the membrane are assembling around the peptide molecule. The peptide capturing effect on the guest lipid-like molecules may disturb membrane functioning, which presents an alternative mechanism of the peptide antimicrobial action and which could be effective for the peptides antimicrobial activity at the low peptide concentration in the bacterial membranes.

Electrostatics in silica-lipid hybrid structures

Erkang Ou, Maxim A. Voinov, Alex I. Smirnov, Tatyana I. Smirnova

Department of Chemistry, North Carolina State University, 2620 Yarbrough Drive, Raleigh, NC, 27695, USA

E-mail: tismirno@ncsu.edu

Interfacing biological and artificial systems at the nano-scale level is essential for developing novel living-nonliving biotechnology platforms for applications in biology and medicine as well as in design of biosensors. Although impressive progress was achieved in creating new bio-nano hybrid systems, needs remain high to understand the influence of a nanostructured support and confinement on structure and properties of the membrane-protein interface.

In this work we report on spin-labeling EPR studies to 1) evaluate the effect of anionic lipid surface charge density on effective pKa of membrane-burred ionisable sidechains and 2) assess effects of solid inorganic interface, specifically, silica support, on the phospholipid membrane surface potential and on effective pKa of the membrane-burred ionisable sidechains. The change in the protonation state of the pH-sensitive ionisable nitroxide label was directly observed by CW EPR allowing for determination of the effective pKa of the probe. We have shown that the effective pKa of the probe site-specifically positioned at the interface of transmembrane α-helical WALP peptide and phospholipid bilayer increases by more than 2 pH units in depth-dependent manner upon replacing zwitterionic PC with anionic PG lipids. Almost 80% of that pKa shift was observed upon replacing only half of the PC with PG lipids. We have also investigated the effect of placing a phospholipid bilayer with integrated transmembrane α-helical WALP peptide on the surface of silica nanoparticles on the peptide dynamics and the effective pKa of the probe. Novel EPR active pH-sensitive lipids IMTSL-PE and IKMTSL-PE [1-3] were used to assess the phospholipid membrane surface potential. We have shown that placing POPC or POPC/POPG bilayers on silica nanoparticles increases the negative electric potential at the membrane surface with the potential of mixed bilayer being more sensitive to the silica support. Supported by NSF 1508607 to TIS.

Energy Transduction and Alternating Access of the Mammalian ABC Transporter P-Glycoprotein

Reza Dastvan, Brandy Verhalen, Sundarapandian Thangapandian, Yelena Peskova, Hanane A. Koteiche, Robert K. Nakamoto, Emad Tajkhorshid, Hassane S. Mchaourab

1 Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232, USA. 2 Department of Biochemistry, Center for Biophysics and Quantitative Biology, and Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA. 3 Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908, USA. †Present address: Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA.

E-mail: reza.dastvan@vanderbilt.edu

ATP binding cassette (ABC) transporters of the exporter class harness the energy of ATP hydrolysis in the nucleotide binding domains (NBDs) to power the energetically uphill efflux of substrates by a dedicated transmembrane domain (TMD) [1-4]. Although numerous investigations have described the mechanism of ATP hydrolysis and defined the architecture of ABC exporters, a detailed structural dynamic understanding of the transduction of ATP energy to the work of substrate translocation remains elusive. Here we used double electron–electron resonance (DEER; also called PELDOR) [5-8] and molecular dynamics simulations to describe the ATP- and substrate-coupled conformational cycle of the mouse ABC efflux transporter P-glycoprotein [3] (Pgp; also known as ABCB1), which has a central role in the clearance of xenobiotics and in cancer resistance to chemotherapy [9]. Pairs of spin labels were introduced at residues selected to track the putative inward-facing to outward-facing transition. Our findings illuminate how ATP energy is harnessed in the NBDs in a two-stroke cycle and elucidate the consequent conformational motion that reconfigures the TMD, two critical aspects of Pgp transport mechanism. Along with a fully atomistic model of the outward-facing conformation in membranes, the insight into Pgp conformational dynamics harmonizes mechanistic and structural data into a novel perspective on ATP-coupled transport and reveals mechanistic divergence within the efflux class of ABC transporters.

I my talk, I will first guide you through the evolution of continuous wave electron paramagnetic resonance (CW EPR) from the last millennium analog techniques to the 21st century digital methods. Multi-harmonic (MH) [1] and rapid-scan (RS) [2] EPR methods will be described in detail. Limitations and advantages of the methods will be demonstrated. In the second part of my talk, I will touch upon the new step in the EPR development, which is the convergence of two largely independent evolutionary branches: CW RS EPR and pulsed spin echo[3, 4].


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Low levels of molecular oxygen—hypoxia— in mammalian cells, both normal tissues and malignant tumors has been known to cause local resistance to radiation therapy for over a century. This stimulated clinical trials to decrease tumor hypoxia. This assumed similar levels of tumor hypoxia in all human tumors. There were no means to measure such levels. Despite suggestive results, these trials failed to demonstrate conclusive therapeutic improvement. Highly reductive hypoxic tumor tissue selectively retails nitroimidazoles. However, \(^{18}\)F-labelled nitroimidazole positron emission tomography (PET) images are plagued by confounding variability and repeatability.

Spin lattice relaxation (SLR) based pulsed electron paramagnetic resonance (EPR) \(pO_2\) images are nearly absolute images of quantitative voxel measures of molecular oxygen partial pressures in tissues and tumors of mammals. These have resolutions of ~ 1 torr \(pO_2\) at low \(pO_2\), avoiding confounding variation from the presence of unpaired electrons other than those of oxygen. This ensures that the exchange with unpaired electron spins of the trityl probe do not confound the sensitivity of the image to the electrons of molecular oxygen unpaired spins. For the longitudinal relaxation rate exchange of magnetization with another spin probe does not affect the loss of the magnetization since the exchange a probe spins maintains the longitudinal spin probe system energy. \(2/3\) mm linear resolution is also achieved.

\(T2\) weighted MRI are registered with EPR \(pO_2\) images defining the boundaries of a C3H mouse leg Fibrosarcoma in the EPR \(pO_2\) image. We identify all tumor voxels with \(pO_2\) less than a threshold of 10 torr. Using this protocol, tumors were treated to a whole tumor dose of sufficient to cure 15% of tumors (TCD\(_{15}\)), as determined in separate experiments. Digitally reconstructed radiographs of all hypoxic (>10 torr) voxels were generated. A boost of extra dose delivered to 100% of hypoxic voxels was compared to delivery of the same volume delivered to well oxygenated voxels. The hypoxic boost and the boost treatment avoiding the hypoxia used novel conformal 3D printed tungsten blocks. This protocol has produced, for the first time in a century, highly significant data demonstrating the efficacy of treating mammalian tumor hypoxia.
The First-ever Multi-Institutional Clinical Trial for Tumor Oximetry using EPR with OxyChip


Dartmouth-Hitchcock Medical Center, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756

*email: kuppu@dartmouth.edu

The objective of this work was to establish a novel and robust technology, based on EPR oximetry, as a practical clinical tool for measurement of tumor oxygen in the clinical setting. This will enable clinicians to make patient-individually and informed treatment-decisions based on the status of pre-, during, and post-treatment tumor oxygen status. We have developed an implantable oxygen sensor, called OxyChip, consisting of LiNc-BuO microcrystals encapsulated in a biocompatible polymer matrix. Preclinical measurements established that the OxyChip is robust and capable of making direct and repeated measurements of pO₂ for a year or longer without toxicity or change in oxygen sensitivity. We have designed the first-ever clinical studies to establish the safety and efficacy of the EPR technology to obtain tumor pO₂ data in cancer patients undergoing surgery, radiation, or chemo treatments. Following implantation into tumor tissue within 25 mm from skin surface, pO₂ measurements were performed noninvasively using an external RF coil working at 1.2 GHz. Repeated measurements of pO₂ were performed for several weeks after OxyChip implantation. The OxyChips were removed when the tumors were surgically resected, as is standard of care therapy for these patients. Post-operative assessment of explanted OxyChips and pathology evaluation of the implanted site in the tumor tissue were performed to establish the safety of the procedure.

To date, we have performed studies on a total of 10 patients. The tumors types included lipoma, melanoma, squamous cell carcinoma (SCC) of the head and neck, and basal cell carcinoma (BCC) with the duration of OxyChip in the tumor for up to ~5 weeks. Where possible, we carried out measurements on the same patient during multiple visits. The figure shows the pO₂ data from a patient with SCC tumor measured on multiple days.

The resected tissues showed no signs of infection or inflammation beyond the expected trauma along the needle track from the implantation itself. In addition, the patients had no clinical signs of reaction to the OxyChip and the procedures were well tolerated throughout the duration of study enrollment. We did not observe any anticipated or unanticipated adverse effects to report. The explanted OxyChips were morphologically intact and the oxygen-sensing calibration of the explanted chips was unchanged from the pre-implant calibration. In conclusion, the ongoing EPR oximetry trial using OxyChip addresses a clinically relevant and timely need to enable reliable and repeated pO₂ measurements in tumors.

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**Trityl radicals in perfluorocarbon emulsions as stable, sensitive, and biocompatible oximetry probes**

Ilirian Dhimitruka, Yasmin Alsayed Alzarie, Craig Hemann, Alexander Samouilov, and Jay L. Zweier

Department of Internal Medicine, Davis Heart & Lung Research Institute, College of Medicine, The Ohio State University, Columbus Ohio 43210, USA

E-mail: jay.zweier@osumc.edu

EPR oximetry with the use of trityl radicals can enable sensitive O$_2$ measurement in biological cells and tissues.$^{1,2}$ However, in vitro cellular and in vivo biological applications are limited by rapid trityl probe degradation or biological clearance and the need to enhance probe O$_2$ sensitivity. We have synthesized novel perfluorocarbon (PFC) emulsions, ~200 nm droplet size, containing esterified perchlorinated triphenyl methyl (PTM) radicals dispersed in physiological aqueous buffers.$^3$ These formulations exhibit excellent EPR signal stability, over 20-fold greater than free PTM probes, with high oxygen sensitivity ~17 mG/mmHg enabling pO$_2$ measurement in the hypoxic region, in aqueous solutions or cell suspensions with sensitivity > 0.5 mmHg. We used these formulations to follow cellular respiration, and the resulting oxygen depletion. Thus, PFC-PTM probes hold great promise to enable combined O$_2$ delivery and sensing as needed to restore or enhance tissue oxygenation in disease.

References


Three-dimensional oxygen mapping using a pair of isotopic nitroxy radicals and CW-EPR-based single-point imaging

Harue Kubota,1 Denis A. Komarov,1 Shingo Matsumoto,1 Kumiko Yamamoto,2 Hironobu Yasui,3 Osamu Inanami,2 Igor A. Kirilyuk,4 Valery V. Khramtsov5 and Hiroshi Hirata1

1 Division of Bioengineering and Bioinformatics, Graduate School of Information Science and Technology, Hokkaido University, North 14, West 9, Kita-ku, Sapporo, 060-0814, Japan
2 Laboratory of Radiation Biology, Graduate School of Veterinary Medicine, Hokkaido University, North 18, West 9, Kita-ku, Sapporo, 060-0818, Japan
3 Central Institute of Isotope Science, Hokkaido University, North 15, West 7, Kita-ku, Sapporo, 060-0815, Japan
4 Novosibirsk Institute of Organic Chemistry, Novosibirsk, 630090, Russia
5 Department of Biochemistry, West Virginia University, Morgantown, WV 26506, USA

hhirata@ist.hokudai.ac.jp

CW-EPR-based visualization of the partial pressure of oxygen (pO2) and the concentrations of nitroxy radicals is reported. Since the concentration of a spin probe affects its EPR linewidth, measurements of pO2 have traditionally required simultaneous estimation of the probe concentration. Methods for measuring pO2 using monohydrogenated CTPO (mHCTPO) were developed in the 1990s to overcome this dependence on the concentration of the probe [1–3]. We revisited this problem to visualize pO2 and the probe concentrations in a three-dimensional (3D) subject. To simultaneously measure unknown parameters, we used a pair of isotopic nitroxy radicals, such as 14N- and 15N-labeled dicarboxy-PROXYLs (2H-DCP and 2H,15N-DCP) as oxygen-sensitive spin probes [4]. First, we established simultaneous equations to express the effects of the self-broadening of 2H-DCP and 2H,15N-DCP, cross-broadening between 2H-DCP and 2H,15N-DCP, and oxygen-broadening on the linewidths of the probes. To estimate the linewidths of the probes, we used a CW-EPR-based single-point imaging (SPI) modality [5,6]. Linewidth maps could be obtained from T2* maps measured from a mixture of 2H-DCP and 2H,15N-DCP. The concentrations of the probes and pO2 could then be calculated by solving the simultaneous equations. In addition to the method, cytotoxicity and in vivo clearance of DCP probes were clarified [7]. We also show that in vivo 3D T2* and pO2 mapping of a mouse tumor-bearing leg is feasible with CW-EPR-based SPI using DCP probes.

References

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Development of High Resolution OMRI Scanner for Small Animal imaging

Kazuhiro Ichikawa
Faculty of Pharmaceutical Sciences, Nagasaki International University, Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan.
Email: ichikawak@niu.ac.jp

A class of aminoxyl (nitroxy) radicals has been frequently used for detecting reduction-oxidation (redox) status, pH or partial oxygen pressure in vivo, based on its free radical status, absorption lineshape. The spatial resolution of the radical distribution detected by ESR technique is in the range of few to dozen mm and is difficult to directly compared with corresponding organ structure.

Overhauser effect, i.e., dynamic nuclear polarization (DNP) is one of spin polarization techniques based on different spin-spin system. Electron spin has been utilized to hyperpolarize nuclear spin, such as proton spin, to transfer “electron information” to proton, achieving free radical imaging in high spatial resolution in principle. For in vivo observation, static magnetic field of OMRI is frequently in the range of 5 to 10 mTesla, to ensure good microwave penetration into small animal. The S/N ratio of conventional OMRI images was low and the physical resolution was practically limited.

In this study, a 0.04 Tesla OMRI was constructed to improve S/N ratio of OMRI imaging for small animal. Specification of main magnet was as follows; 0.04 Tesla electromagnet; gap size 400 mm; homogenous volume 40 mm. The OMRI resonator was designed and constructed in a configuration of NMR solenoid coil (40mm length) and ESR loop-gap excitation coil. The resonant frequencies and Q values were 1.4 MHz/100, 1 GHz/80 for NMR and ESR, respectively. The Q values were comparable with those of conventional low field OMRI resonators at 15 mTesla. We also incorporated eddy current compensation algorithms to improve overall throughput. As the results, the MRI S/N ratio and overall throughput was improved by factor of 5 and 10, respectively.

The 0.04 Tesla OMRI system was utilized for imaging mouse brain. A solution of methoxycarbonyl PROXYL was intravenously injected an immediately 3D OMRI images were obtained in less than 2 min to be processed for OMRI images; voxel size 0.25x0.25x1.5-3.0 mm, 16 slices. The OMRI image quality was useful for detailed comparison with anatomical images obtained with 1.5 T MRI. In conclusion, the result of preliminary evaluation of 0.04 Tesla OMRI system indicated the system would be useful for free radical imaging in small animal.


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Pulse EPR 25 mT Oxygen Imager For In Vivo Applications

Boris Epel, Subramanian V. Sundramoorthy, and Howard J. Halpern

Center for EPR Imaging In Vivo Physiology; Department of Radiation and Cellular Oncology, University of Chicago, USA

e-mail: bepel@uchicago.edu

Higher magnetic field and operation frequency is one of the methods for increasing of the imager signal to noise ratio (SNR). We expect that a new imager with the magnetic field of 25 mT will demonstrate at least 3-fold improvement of the SNR in comparison to the currently used 9 mT instrument. High SNR will provide us the possibility of improving image resolution, precision and acquisition time.

We present a 25 mT pulse electron paramagnetic resonance imager for in vivo applications constructed at the University of Chicago. This imager is used to measure molecular oxygen concentration in tissues of rodents and has an innovative design.

The main features of the imager are:

- permanent 25.6 mT magnet with 1 mT field offset coils
- gradient system with 30 mT/m X-Y-Z gradients
- 19 mm and 25 mm loop-gap and Alderman-Grant resonators, animal support platform
- arbitrary waveform generator in the excitation arm
- 4KW pulse amplifier and transmit-receive switch for high-bandwidth pulses
- digital detection on the carrier frequency (720 MHz)
- optional mixer for the frequency down-conversion
- SpecMan4EPR IM 2.5 imaging software

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Monitoring oxygen partial pressure (pO2) in tissue is of significance for diagnostics and proper treatment of many clinical conditions, such as cardiovascular disease, stroke, wound healing, and cancer. Currently, the capability of clinical tissue pO2 measurements is mostly limited to skin tissue using cumbersome electrochemical methods. Although a number of methods have been proposed for deep tissue measurement of oxygenation, they lack the ability to provide reliable and repeated measurements for frequent monitoring of tissue oxygenation. Some of the technologies (e.g., pulse oximetry, which measures blood oxygen saturation) currently used in the clinic for monitoring oxygen are only useful in areas with sufficient blood flow. Magnetic resonance methods, such as blood-oxygen-level-dependent (BOLD MRI) and tissue oxygen-level dependent contrast imaging, have very desirable features including their unique capability for deep-tissue noninvasive measurements and imaging; however, they are too expensive, complex, and immobile, to be used as a routine procedure. Therefore, there is still an unmet need for methods that can measure pO2 in patients with a reasonable degree of accuracy (especially in the hypoxic range), reliability, and robustness.

Oximetry based on ESR offers certain unique advantages over other methods, including direct detection and high sensitivity and specificity to molecular oxygen. Unfortunately, the adaptation of ESR oximetry for useful clinical measurements is also faced with limitations, most notably due to restrictions that arise from the existing hardware. The conventional ESR systems are large, bulky units with restrictive spacing between the magnet poles (for patient placement) and require the patient to be transported to the ESR facility for routine measurements.

In recent years, we have introduced and validated several design concepts, with the aim of miniaturizing the entire ESR setup for addressing specific clinical needs. Our ultimate goal is to have a complete compact “pocket-sized” ESR spectrometer, coupled with application-specific compact ESR probehead that includes the magnet and the resonator, to address timely clinical applications such as tissue pO2 monitoring.

In the talk, we will present two new application-specific probeheads, one aimed at skin pO2 measurements and the other on deep tissue pO2 monitoring, each with its own unique mechanical and electromagnetic characteristics. These probeheads operate in pulsed mode in conjunction with oxygen-sensitive paramagnetic particulates that have linear response between 1/T2 and pO2. The probeheads are driven by advanced pulsed ESR spectrometer, with arbitrary-shaped pulses, enabling short dead times required for such pO2 measurements at a wide range of O2 concentrations.
Spin probes and traps - molecular spies for EPR spectroscopy and imaging

Valery V. Khramtsov

1In Vivo Multifunctional Magnetic Resonance center, Robert C. Byrd Health Sciences Center, West Virginia University, and Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, USA; valery.khramtsov@hsc.wvu.edu.

With a few rare exceptions concentrations of endogenous free radicals in biological systems is below detection limit. Therefore a majority of biological EPR applications are based on the use of exogenous spin traps, probes and labels. EPR spin trapping is often considered to be a gold standard for detection and identification of biologically-relevant reactive radical species (RRS). The most frequently used nitrone and nitroso spin traps are diamagnetic compounds that react with the short-lived radicals to form and accumulate more stable paramagnetic spin adducts for further analysis with EPR spectroscopy. EPR spectral pattern of a particular spin adduct depends on the specific spin trap used and free radical trapped, and serve as specific marker of a particular free radical species. Alternatively, a number of cyclic hydroxylamines have found applications as RRS-sensitive probes: oxidation of hydroxylamines in the reaction with reactive radicals results in the formation EPR-detectable nitroxide product. In particular, hydroxylamines have advantage in comparatively high rate constant of the reaction with the superoxide, \( \text{O}_2^- \) \((10^3-10^4 \text{ M}^{-1}\text{s}^{-1})\), compared to nitrone spin traps. Stable organic radicals, nitroxides and trityl radicals, have been also used to detect free radical reactions that result in EPR signal decay due to formation of diamagnetic products. An exception present nitronyl nitroxides used for detection of important free radical, nitric oxide (NO\(^\cdot\)): they react with NO\(^\cdot\) with formation of another EPR-detectable radical, iminonitroxide. Arguably, the most popular EPR probes for NO\(^\cdot\) detection are iron-dithiocarbamate probes.

Nitroxide and trityl probes are two major classes of soluble organic radicals used for functional EPR applications. Spectral sensitivity of these radicals to local physical properties of the medium such as viscosity, polarity and temperature has been used in numerous biophysical and biomedical applications including studies of biological macromolecules and biomembranes, in particular via site-directed spin labeling technique. Nitroxides have advantages in well-developed chemistry resulted in variability of structure, solubility, functionality and ability to be targeted. On the other hand, trityl radicals have advantages over NRs in extreme stability toward tissue redox processes, longer relaxation time and narrower line width making them particularly attractive for imaging applications. Table 1 represents particulate oxygen-sensitive probe and soluble paramagnetic probes for assessment of chemical tissue microenvironment, namely trityl probe for concurrent in vivo monitoring of tissue oxygenation (pO\(_2\)), extracellular pH (pH\(_e\)) and interstitial inorganic phosphate, Pi, and nitroxide probes for pH\(_e\), GSH and reducing capacity measurements. Advantages of particulate probes for EPR oximetry are high functional sensitivity, stability in living tissue, and minimal toxicity enabling repeated measurements of tissue pO\(_2\) for up to several weeks after implantation. On the other hand, soluble probes possess multiple functionality and provide an opportunity for spatial-resolved measurements using EPR-based imaging techniques.

Table 1. Functional paramagnetic probes for assessment of chemical tissue microenvironment.

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EPR/MRI Imaging Biomarkers to Guide Treatment in Tumor Bearing Mice

Murali Krishna Cherukuri
Radiation Biology Branch, NCI, NIH, Bethesda, MD, USA
murali@helix.nih.gov

The tumor microenvironment in solid tumors is characterized by regions of poor perfusion, hypoxia and low pH. Biochemically, tumor cells, both in vitro and in vivo display the aerobic glycolysis phenotype. Imaging techniques which provide biomarkers reporting on these features will be useful in: a) providing diagnostic/prognostic information; and b) developing appropriate treatments based on a priori information of the tumor microenvironment.

In this presentation, Electron Paramagnetic Resonance Imaging (EPRI) which provides non-invasively quantitative pO$_2$ maps and metabolic MRI using hyperpolarized $^{13}$C labeled pyruvate which provides biochemical profiles of tumors will be used to probe the microenvironments of three tumor xenografts in mice to characterize their metabolic and physiologic status. These tumor bearing mice will be treated with radiation therapy or with hypoxia activated pro-drugs to evaluate the potential of the imaging biomarkers to predict treatment response.

Data will be presented to support the capability of EPRI as well as the lactate/pyruvate from $^{13}$C MRI can predict difference in the benefit from oxygen-dependent anti-tumor treatments in individual pancreatic tumor cell lines that may help properly choose the best treatment in patients with pancreatic cancer.
Redox Active Anticancer Pro-drugs: Synthesis, Properties, Mechanisms of Action, Cells Delivery and Cells Toxicity in the Dark and Under Light

1J.Furso, 1M.Olchawa, 1T.Sarna, 1M.Elas, 2P.Richard, 2I.Craciun, 2C.Palivan, 3L.Fedenok, 3N.Polyakov, 3T.Leshina, 3I.Slepneva, 4I.Kirilyuk, 5L.Weiner

1Department of Biophysics, Jagiellonian University, Krakow, Poland, 2Department of Chemistry, University of Basel, Switzerland, 3Institute of Chemical Kinetics and Combustion, Novosibirsk, 4Novosibirsk Institute of Organic Chemistry, Russia, 5Weizmann Institute of Science, Rehovot, 76 100 Israel,

email: lev.weiner@weizmann.ac.il

Redox active compounds, namely quinones capable of chelating metal ions, were synthesized and used for generation of reactive oxygen species (ROS) in cancer cells. Stability constants of the quinones were determined for a number of metals. Linking with metals significantly lowers the redox potentials of our quinones (electrochemical measurements). Thus, quinones-chelators can be reduced to semi-quinones by biological reductant molecules: glutathione, NAD(P)H and ascorbic acid. These quinones are easily reduced during interaction with electron transfer chains in cells. EPR and the spin trapping method were used to study generation of ROS by quinones in solutions and in cancer cells. For several lines of cancer cells the values of IC50 were estimated. Toxicity of our quinones-chelators is significantly higher than that of the commonly used redox active anti-cancer drug- doxorubicin (adriamycin). As found, the compounds can also generate ROS under irradiation by visible light too. Their toxicity effect on cancer cells under light was estimated. Delivery of these compounds into cancer cells by means of original nano particles and polymers was carried out and experimentally verified.

The derivatives of these compounds containing functional groups were obtained and their conjugates with peptide hormone GnRH were produced. The conjugates were shown to have high affinity for receptors on cancer cell surfaces.

We believe that the proposed compounds, i.e. conjugates of our quinones-chelators with peptide hormones and/or monoclonal antibodies, in combination with specific methods of delivery, will allow avoiding numerous toxic side effects and will work as a new type of addressed antitumor drugs.
Design and Synthesis of Nitroxide Radicals for Biophysical and Biomedical Applications
Andrzej Rajca, Joseph T. Paletta, Zhimin Yang, Hui Zhang, Shengdian Huang, Ping Du and Suchada Rajca
Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304
Email: arajca1@unl.edu

This presentation will focus on the design and synthesis of new nitroxide radicals with tailored properties for biophysical and biomedical applications. The distinct structural feature of these nitroxides is that they do not contain gem-dimethyl groups. For biophysical applications, these new nitroxide structures circumvent the dynamic averaging effects associated with the methyl group rotation that limited the application of the common nitroxide spin labels. Our goals are to design and synthesize spin labels with the following characteristic properties: (1) hydrophilic radicals with small molecular size that permit EPR distance measurements (DEER and DCQ) at temperatures up to ambient and (2) biostable radicals (and unnatural amino acids) that allow extended in vivo studies of cells and mitochondria. Towards that end, we have recently prepared gem-dicarboxylate, spirocyclic and diazaadamantane frameworks. Several laboratories, Drs. Sandra and Gareth Eaton (University of Denver), Dr. Jack Freed (ACERT & Cornell University), Dr. Hassane Mchaourab (Vanderbilt University), and Dr. Enrica Bordignon (Ruhr University) have investigated biophysical properties of these nitroxides and are in the process of developing protocols for applying them as useful probes for investigation of proteins. For biomedical applications, we have designed and synthesized polynitroxides and demonstrated their potential as an organic radical contrast agent (ORCA) for MRI. Our goal is a practical metal-free MRI contrast agent that provides high quality in vivo MR images for clinical use. The requisite radicals within polynitroxide frameworks should possess slow bioreduction rates and strongly enhanced 1H water relaxivity. In collaboration with Dr. Murali Cherukuri (NIH), we are working on improving the properties of ORCAs and with Dr. Jeremiah Johnson (MIT), we take advantage of our nitroxides in the development of a new class of polymer nanoparticles dual-modality organic radical contrast agents–ORCAFluors–for combined magnetic resonance and near-infrared fluorescence imaging in vivo.

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Electron Spin Relaxation of Nitroxides Designed for DEER Experiments
Sandra S. Eaton and Gareth R. Eaton
Department of Chemistry and Biochemistry, University of Denver, Denver, Colorado 80210
Email: Sandra.Eaton@du.edu

DEER (double electron electron resonance) is a powerful EPR method for measurement of interspin distances in biomolecules. Experiments typically are carried out at temperatures of about 50 – 70 K using expensive liquid helium, or with lower sensitivity at 80 K using liquid nitrogen. The temperature constraints on DEER experiments are due to rotation of the gem-dimethyl groups in commonly-used nitroxides at rates that are comparable to the inequivalent proton couplings to the unpaired electron. This process enhances the rate of spin echo dephasing, 1/T_m, between about 80 and 300 K. Faster 1/T_m relaxation decreases the intensity of the spin echo that is used in the DEER measurements, which degrades signal-to noise (S/N) and decreases the upper limit for distances that can be measured.[1] For commonly-used nitroxides DEER measurements are not possible at temperature above about 100 K because 1/T_m is so fast. The spin-lattice relaxation rate, 1/T_1, is also important for the DEER experiments because it determines how quickly the signal averaging can be repeated. Faster 1/T_1 improves S/N for DEER. The S/N in the DEER experiment is much more strongly dependent on T_m than on T_1.[2]

Several types of nitroxides that do not contain gem-dimethyl groups have been synthesized in the laboratory of Dr. Andrzej Rajca, University of Nebraska. These include nitroxides with cyclohexyl groups, carboxylate groups, and an adamantyl framework.[2, 3] The goal is radicals that permit DEER measurements at temperatures up to ambient. Hydrophilicity of the radicals also is important for studies of biopolymers. The temperature dependence of 1/T_m and 1/T_1 for these nitroxides was studied in water:glycerol mixtures and in sugar glasses with softening temperatures well above ambient. For nitroxides that do not contain gem-dimethyl groups, values of 1/T_m in rigid lattices are sufficiently long at ambient temperature to permit DEER measurements in strongly immobilizing glasses and up to about 160 K in water:glycerol. The effect of nitroxide structure and of solvent on the relaxation rates will be discussed.

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Designing Spin Probes with Reduced Membrane Permeability

Nathaniel D. A. Dirda, Eric A. Legenzov, Joseph P. Y. Kao

Center for Biomedical Engineering & Technology, and Department of Physiology, University of Maryland School of Medicine, 111 S. Penn St., Baltimore, MD 21201

jkao@umaryland.edu

We have developed immunoliposomes to deliver molecular probes to tumor cells with high selectivity to enable imaging.\textsuperscript{1,2} For this purpose, molecular probes are encapsulated in the liposomes at as high a concentration as possible, subject to the constraint of physiological osmolarity ($\sim$300 mM). In order to be stably encapsulated in liposomes, the imaging probes must have low membrane permeancy. Moreover, low membrane permeance also deters leakage of the probe molecules once they have been delivered into tumor cells. We have shown that incorporating ionic functional groups in the probe molecules very effectively decreases membrane permeance and improves intracellular retention.\textsuperscript{3} However, the requirement for electroneutrality means that counter-ions are needed for charge balance and thus limits the maximum concentration of encapsulated probe. For example, for a probe bearing 5 carboxylates (with suitable counter-ions such as Na\textsuperscript{+}), the physiological osmolarity constraint limits the maximum encapsulation to 30 mM, which may be unacceptably low for some applications. Therefore, as an alternative strategy to achieve membrane impermeance, we have conjugated probe molecules to highly water-soluble moieties that are known or expected to be membrane-impermeant. In this study, we examine the preparation and properties of the new probe conjugates.

Nitroxides are widely used in biology as spin labels, functional spin probes for pH, oxygen and thiol levels, and tissue redox status imaging. In addition, potential therapeutic applications of nitroxides attract much attention due to their unique antioxidant activity. The main challenges in biological applications of nitroxides are related to their fast bioreduction to EPR-silent hydroxylamines and rapid clearance. Site-specific targeting may strongly increase biological activity of nitroxides. Recently we have described antihypertensive activity of mitochondria-targeted piperidine and pyrrolidine nitroxides with triphenylphosphonium cationic groups.1 In this work we have studied cellular accumulation of nitroxides of pyrrolidine and piperidine series, including those with dialkylamino and acetoxymethoxycarbonyl groups. The later are known to undergo hydrolysis with cellular esterases to hydrophilic carboxylate derivatives.2

It was found that nitroxides containing either dialkylamino or acetoxymethoxy carbonyl groups were rapidly absorbed by cells from the media, 4-(dimethylamino)-TEMPO (CAT2), 3,4-bis-(acetoxymethoxycarbonyl)-PROXYL (DCP-AM)2 and 3-(2-(bis(2-(acetoxymethoxy)-2-oxoethyl)amino)acetamido)-PROXYL (DCAP-AM)2 showing the strongest EPR signal of cellular fraction. Remarkably, the EPR parameters of 3,4-dicarboxy-PROXYL (DCP) and its mono- and di-acetoxymethyl esters are different, and consequent intracellular hydrolysis of acetoxymethoxycarbonyl groups in DCAP-AM can be followed by EPR. To elucidate intracellular location of the resultant DCP, the mitochondrial fraction has been isolated. EPR measurements showed that mitochondria were the main place where DCP was finally accumulated. TEMPO derivatives showed expectedly much faster decay of EPR signal in cellular fraction, compared to pyrrolidine nitroxides. It was found that supplementation of endothelial cells with 50 nM of DCP-AM completely normalized the superoxide level measured by mitochondria-specific probe MitoSOX and HPLC. Moreover, administration of DCP-AM to mice (1.4 mg/kg/day) resulted in substantial nitroxide accumulation in the tissues and significantly attenuated hypertension. We tested if hydroxylamine derivatives of dicarboxyproxyl nitroxides such as DCP-AM-H can be used for the detection of cellular superoxide in vitro and in vivo using xanthine oxidase system, cultured endothelial cells and angiotensin II model of hypertension. In contrast to DCP-AM, reduced form DCP-AM-H has rapidly accumulated in cells and reaction with cellular superoxide lead to accumulation of persistent dicarboxy-PROXYL nitroxide signal. Our data demonstrate that acetoxymethoxy-carbonyl containing nitroxides accumulate in mitochondria and demonstrate site-specific antioxidant activity.

References

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Preparation of Robust Metal-Free Magnetic Nanoemulsions Encapsulating Low-Molecular-Weight Nitroxide Radicals and Hydrophobic Drugs Directed toward MRI-Visible Targeted Delivery System

Rui Tamura,1* Kota Nagura,1 Yusa Takemoto,1 Satori Moronaga,1 Satoshi Shimono,1 Yoshiaki Uchida,2 Akihiko Shiino,3 Tsukuru Amano,3 Fumi Yoshino,3 Kenji Tanigaki,4 Yohei Noda,5 Satoshi Koizumi,5 Naoki Komatsu,1 Tatsuhisa Kato,1 Jun Yamauchi,1
1Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606-8501, Japan; 2Osaka University, Japan; 3Shiga University of Medical Science, Japan; 4Shiga Medical Center, Research Institute, Japan; 5Institute of Quantum Beam Science, Ibaraki University, Japan.

E-mail: tamura.rui.8c@kyoto-u.ac.jp

We reported that chiral all-organic rod-like liquid crystalline (LC) compounds with a five-membered cyclic nitroxide unit in the central core position exhibited a sort of spin glass-like inhomogeneous ferromagnetic interactions in the LC phases by application of low magnetic fields at high temperatures.1 With a view to extending such a unique magnetic phenomenon, referred to as ‘positive magneto-LC effects’, to other organic radical soft materials, we have prepared robust metal-free magnetic nanoemulsions (mean particle sizes of 17 nm) composed of the biocompatible non-ionic surfactant 1 and the hydrophobic nitroxide radical (±)-2 in (-)-PBS.2 The structure of the nanoemulsions has been characterized by EPR spectroscopy, and DLS and SANS measurements. The nanoemulsions showed high colloidal stability, low cytotoxicity, enough reduction resistance to excess ascorbic acid, and sufficient contrast enhancement in the proton longitudinal relaxation time (T1)-weighted MR images in (-)-PBS in vitro and in vivo. Furthermore, additional hydrophobic anticancer drugs such as paclitaxel could simultaneously be encapsulated inside the nanoparticles, and the resulting drug-loaded nanoemulsions were efficiently incorporated into HeLa cells to suppress the cell growth. We expect that such drug-loaded nanoemulsions can be used as a theranostic nanomedicine for MRI-visible targeted drug delivery system.

In vivo EPR, preclinical and clinical: challenges and opportunities

Harold M. Swartz

Geisel School of Medicine at Dartmouth, Radiology Dept., Lebanon, New Hampshire, USA; harold.swartz@dartmouth.edu

The goal is to provide an overview of the state-of-the-art of in vivo EPR in several very active and somewhat related fields that has been pursued for 50+ years by the speaker and his incredible colleagues. The focus is on our group’s accomplishment, but the strength of the work derives also from the important developments by many other groups, several of which are represented at this meeting.

EPR can be an incredibly effective and versatile tool, ranging from dating fossils millions of years old and identifying sources of fossil fuels such as petroleum to applications in living systems, including clinical measurements. The key to productivity is to identify those areas where EPR can provide insights and measurements better than and/or complementary to alternative approaches. With the development of capabilities of making measurements in human subjects it became feasible to leverage this capability with the substantially demonstrated capability of EPR techniques to measure oxygen in living biological systems. These capabilities extend from measurements of gradients in oxygen within cells in culture to direct repeated measurements of tissue pO2. This capability has advanced to the point where EPR oximetry for use in clinical measurements has been awarded a program project from the NCI to determine its clinical potential. Another major application of in vivo EPR is for dosimetry. Tooth dosimetry has advanced to where it has the capability to make measurements needed for urgent medical triage and at the site where a large scale radiation event has recently occurred. Additional technical developments underway have the potential to further improve the effectiveness of tooth dosimetry and enable it to also become a potential gold standard for measurements required for guidance of medical care. In vivo nail dosimetry has the potential, in conjunction with other dosimetry, to address more complicated dosimetry needs such as whether exposure was homogeneous. All of the EPR dosimetry techniques have an important advantage of being unaffected by trauma and burns. These and other capabilities for dosimetry can be particularly important for the deployed military, which can have very different needs than those for exposures of a large civilian population.

Conclusion: Biological Applications of EPR is a growing and increasingly important field. The future, especially for direct applications in human subjects to advance clinical effectiveness in several fields of application, looks even brighter.

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Conflict of Interest: The author is part owner of a company, Clin-EPR, LLC, which markets clinical EPR systems.
Extracellular acidification is necessary and sufficient for metastasis
Gillies R.J.

Department of Cancer Imaging and Metabolism, H.Lee Moffitt Cancer Center and Research Institute, Tampa, FL, 33612, USA.

It is axiomatic that, in order for cancers to evolve into clinically relevant disease, they have to solve a number of challenges, such as local invasion and immune evasion. We present evidence here that extracellular acidification is necessary and sufficient to drive local invasion and metastasis and, at the same time, helps cancers evade immune surveillance. Aerobic glycolysis (the Warburg Effect) is a hallmark of cancer that, in combination with poor perfusion, results in acidification of the microenvironment of solid tumors. We have shown in vitro and in vivo that acidosis is associated with local invasion and metastasis in some human cancers. Extracellular acidification is mediated by upregulated H+ transporting systems, for example carbonic anhydrase IX (CA-IX). In a recent study (Lloyd et al, Cancer Res 2016) we showed, using IHC of locally invasive ductal carcinoma, there were significant phenotypic differences between the invasive edge and the core of the tumor. In particular, we observed significantly higher staining for CA-IX at the edge compared to the core, suggesting protons produced by CA-IX result in a more proliferative, invasive, metastatic phenotype as well as inhibition of CD8+ T-cell function. In mouse models, we also recently showed (Estrella et al, Cancer Res 2013) that acidosis was spatially associated with local invasion. Importantly, in this system, we also showed that invasion could be inhibited with Buffer therapy (200 mM NaHCO3) that directly and specifically increases the extracellular pH. In a number of other studies, we have shown that buffer therapy was effective in inhibiting spontaneous and experimental metastasis in vivo.

To test whether acidosis is sufficient to induce metastasis, we have transfected lowly glycolytic and non-metastatic MCF-7 breast adenocarcinoma cells with two different proton transporting systems. In the first model, we used the yeast plasma membrane proton ATPase 1 (PMA1); a Type 1 P-type ATPase, which pumps protons into the extracellular milieu. PMA1 was stably over-expressed in MCF7 clones and expression of PMA1 transformed the cells into a more aggressive phenotype with increased glucose consumption, lactate production, and proton production. Phenotypically, the transfected clones acquired a mesenchymal phenotype, and had elevated rates of migration and invasion in vitro, as well as and increased rates of spontaneous and experimental metastasis in vivo. In the second model we used CA-IX; an exofacial carbonic anhydrase that is highly expressed in invasive cancers and known to maintain enzymatic function at acidic pH. CA-IX is upregulated in multiple cancer types with minimal expression in normal tissue, and is therefore an attractive and biologically relevant therapeutic target. To test if extracellular protons produced by CA-IX could induce metastasis, we stably overexpressed CA-IX in MCF7 cells and again this resulted in a stable, glycolytic phenotype with increased glucose consumption, lactate production, and proton production. Furthermore, when injected into SCID mice, these clones formed gross metastases, while mock transfected and parental MCF-7 cells did not.

Hence the triad of evidence (association, inhibition, induction) has been satisfied to support the theorem that acidosis is necessary and sufficient for local invasion and metastasis. It is worthwhile noting that this is not universal across all cancers, as some are resistant to inhibition (Bailey et al, Neoplasia 2014).
Investigating the role of macrophages in TME regulation using an EPR approach

Steinberger K, Bobko AA, Gross AC, Evans R, Marsh CB, Khramtsov VV, Eubank TD

IMMR center, Robert C. Byrd Health Sciences Center, West Virginia University, 1 Medical Center Drive, Morgantown, WV 26506.

eMail: tdeubank@hsc.wvu.edu

Oxygen, pH, intracellular glutathione (GSH) and redox potential are volatile parameters unique to a chemical tumor microenvironment (cTME) relative to normal tissues. These parameters are significant factors underlying the regulation of metabolism and pathophysiology in solid tumors, regardless of their cell type or origin. For example, oxygen tension can play a dual role in tumor progression. While oxygen is required for tumor cells to proliferate, reduced oxygen activates hypoxia-induced pathways via hypoxia-inducible transcription factors (HIFs) that regulate drug resistance, cell metabolism, tumor progression, and metastasis. Low extracellular pH, the reverse of normal cells, has been shown to enhance tumor invasion. Increased levels of tumor intracellular GSH is protective against chemotherapy and increases the antioxidant capacity and resistance to oxidative stress observed in many tumor cell types.

The chemical tumor microenvironment is not the only non-tumor cell regulator of progression. Infiltrating macrophages or tumor-associate macrophages (TAMs) are immune cells which have been shown to portend a poor prognosis. In fact, the higher number of TAMs in the tumors of breast cancer patients the worse the outcome. Interestingly, we have previously shown that specific stabilization of HIF-1α or HIF-2α disparately regulates macrophage function towards tumor angiogenesis and tumor growth. In our current study, we used transgenic LysMcre (wild type), LysMcre/HIF-1αfloxed(fl)/fl (HIF-1α-deficient macrophages) and LysMcre/HIF-2αfl/fl (HIF-2α-deficient macrophages) mice with orthotopically implanted late-stage polyoma middle-T antigen (PyMT)-overexpressing murine breast tumors and investigated macrophage function on the regulation of tumor pO₂, pH, and GSH. We used LiNcBuO microcrystal uptake into PyMT tumor cells before implantation or intratumoral administration of soluble nitrooxide radicals for longitudinal and real-time EPR analysis. Further, we investigated the effects of macrophage HIF-1α and HIF-2α deficiency on docetaxel effectiveness of inhibiting tumor growth. Ongoing experiments include spontaneous forming MMTV-PyMT tumor-bearing mice crossed with mice having wild type, HIF-1α or -2α-deficient macrophages in combination with the soluble EPR probes to understand the role of macrophage HIFs in regulating these parameters during tumor initiation, staging, the malignant switch, and tumor metastasis.
Modulation of tumor microenvironment and targeting lung metastasis by antagonizing A2B adenosine receptor

Jason Evans1, Andrey Bobko2, Stephanie Lewis1, Charles Martin1, Mohammad Rahman1, Sara Cole1, Elena E. Tchekneva1, Anwari Akhter1, Anneliese Antonucci1, Valery V. Khramtsov2, Mikhail M. Dikov1
1Ohio State University, 460 W. 12th Ave., BRT 484, Columbus, OH 43210
2West Virginia University, 1 Medical Center Dr., IMMR Center, Morgantown, WV 26506
Mikhail.Dikov@OSUMC.edu

Our work addresses two poorly understood areas of tumor metastases; the first is how tumor-conditioned immune cells initiate and drive premetastatic niche evolution and secondary tumor establishment and secondly, how the tumor microenvironment (TME) conditions shape the tumor immune response and function.

We have developed a model that is fully capable of addressing these biological questions through in vivo EPR monitoring of the primary TME allows simultaneous measurements of tumor pO2, pH, and inorganic phosphate (Pi) levels, which are parameters implicated in tumor metastasis, and demonstrates how the TME contributes to metastasis. In combination, we employ an in vivo immune/tumor cell imaging platform in which mice are fitted with cutaneous window chambers containing syngeneic lung tissue transplant to create a metastatic site in which differentially-labeled tumor and immune subsets will be imaged via multiphoton microscopy.

We show that our EPR methodology accurately monitors TME changes that occur with tumor growth as well as their modulation due to pharmacological inhibition of the A2B adenosine receptor giving reason to the use of specific A2B receptor inhibitors as anti-tumor and anti-metastatic therapeutics. A2B inhibition prevented the accumulation of Pi in the tumor interstitial space for every tumor model tested, which includes lung adenocarcinoma, breast adenocarcinoma, colon carcinoma, and melanoma. The exact role this plays in tumor initiation and progression is not completely elucidated but correlates with the reduction of tumor lung metastases and, in the case of PBF-1129, tumor growth. Secondly, our window chamber model enables spatiotemporal analysis of premetastatic niche enrichment, individual tumor cell recruitment, and subsequent secondary tumor growth with specific focus on metastatic lung disease. To our knowledge, no model exists capable of unifying these aspects of tumor biology and immunity.

The project will lead to understanding a key process of metastasis and thus allow targeted immunotherapies to block metastasis and thus eliminate, or greatly reduce, the lethal aspect of cancer. Future work will also examine the potential anti-tumor therapeutic strategy of using specific A2B adenosine receptor antagonists PSB-603 and PBF-1129. Of which, PBF-1129 is undergoing pre-clinical and IND-enabling and demonstrates high efficacy, suggesting the possibility for clinical trials with A2B antagonists for cancer therapy in the nearest future. Lastly, our methodology is targeting a glaring hole in the understanding of tumor metastasis, meaning the forthcoming information from our work holds great promise to identify novel therapeutic strategies aimed at greatly diminishing the chief cause of cancer morbidity.
EPR imaging a synergetic adventure in mathematics, spectroscopy and chemistry.

Yves-Michel Frapart

LCBPT, UMR 8601 CNRS – Paris Descartes University – Sorbonne Paris City University, 45, rue des Saints Pères, 75270 Paris cedex 06, France

yves.frapart@parisdescartes.fr

EPR imaging and localized spectroscopy open a new era in science especially in biomedicine. Even if this field is the source of many outstanding results, many difficulties must be overcome.

1) EPR is a molecular imaging modality, one need an anatomical or structural co-modality to localized the obtained signal.
2) Routine utilization lack the existence of available and reproducible stable “contrast agents” or molecular probes regarding the acquisition time.
3) Those molecular probes as to be controlled using comparative modality.
4) Algorithm proposed to determine characteristic parameters leading to biomarkers and algorithm used to transform spectrum in image may be improved.
5) Standardization and multi-sites studies have to be performed.

In this presentation we will present our most recent results to optimize EPR and EPR imaging using various approach:

- Multimodality co imaging (Bezières et al, Mol.Img. 2012).
- Synthesis of new chemical molecular probes and co characterization of the chosen biomarker (Boutier-Pischon et al, FRR, 2015)
- Formulation of triaryl methyl molecular probes and its application in vivo (Abbas-Bennai, JMR, 2016)
- Utilization of Total Variation algorithm for image reconstruction (Kerebel et al, Inverse Problem, submitted), and source separation of two different radical in a sample (Kerebel et al, FRR Submitted).
- Application of Maximum Likelyhood Estimates theory for EPR parameters g, signal amplitude and linewidth (Tran-Duc et al, IEE submitted)

All proposed published algorithm are available on an EPR virtual machine platform under request.
Continuous flow chemistry with in-line EPR monitoring for the synthesis of multifunctional biocompatible triarylmethyl spin probes

Benoit Driesschaert¹, Martin Poncelet¹, Valery Khramtsov¹.

¹In Vivo Multifunctional Magnetic Resonance center, Robert C. Byrd Health Sciences Center, West Virginia University, and Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, USA; Benoit.driesschaert@hsc.wvu.edu.

Water soluble triarylmethyl (TAM) spin probes represent a unique family of stable radicals which have found numerous in vivo biomedical magnetic resonance applications. Their use as hyperpolarizing agents of $^{13}$C labeled metabolites (such as $[^{13}C]$-pyruvate) allows to monitor in real time the biochemistry of living organisms, including humans, by MRI. The long relaxation times (narrow linewidth) of TAMs provide them with an unprecedented (multi)functional sensitivity to important physiological parameters such as oxygen, pH and inorganic phosphate (Pi) for in vivo EPR applications. In this talk, we will describe the recent TAM developments carried out at the In Vivo Multifunctional Magnetic Resonance center of the West Virginia University. First, a click chemistry platform allows for a rapid and convenient PEGylation of the TAM core improving biocompatibility and tuning the pharmacokinetics of the probe. Second, we will present a flow chemistry system with an in-line EPR monitoring allowing for a rapid optimization of the synthesis and a fully automatic continuous production of our lead multifunctional $pO_2$, pH and Pi trityl pTAM probe.

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Boronic acid–based trityl probes for diol recognition.

Andrey A. Bobko\textsuperscript{a,b}, Benoit Driesschaert\textsuperscript{a,b}, Martin Poncelet\textsuperscript{a,b}, Marissa Fletcher\textsuperscript{c} and Valery V. Khramtsov\textsuperscript{a,b}

\textsuperscript{a} In Vivo Multifunctional Magnetic Resonance center, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506, United States
\textsuperscript{b} Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, United States
\textsuperscript{c} West Virginia University, Morgantown, WV 26506, United States

andrey.bobko@hsc.wvu.edu

Glucose plays an important role in cellular metabolism in both normal and pathophysiological conditions. Fluorescent and absorbance spectroscopies of boronic acid-based probes are very popular for diol recognition (especially, glucose) in many biological applications. We have synthesized a set of boronic acid trityl probes for diol concentration assessment using electron paramagnetic resonance. Here we report and discuss the EPR spectra of mono-, bi- and three-boronic acid substituted trityl radicals, the pKa value of boronic acid residue and stability constants of ethylene glycol/boronic acid complex formation. We have shown that stability of ethylene glycol/boronic acid complex strongly depends on its hydrolysis. For the first, this allows us to determine all equilibrium constants for boronic acid–diol interaction. In summary: boronic acid-based trityl radicals represent a new class of multifunctional paramagnetic probes (for glucose, pH and oxygen) that might find applications in \textit{in vivo} studies of physiological and pathophysiological processes in live objects.

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Detection of Hyperoxia-induced Oxidants in Murine-transformed Clara Cells

Qian Li, Trent E. Tipple

Neonatal Redox Biology Laboratory, Division of Neonatology, Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama 35233

qianli@uab.edu

Introduction: Oxygen toxicity contributes to lung injury in newborn and adult humans. We have shown that stimulation of endogenous antioxidant responses attenuates lung injury in newborn and adult models\(^1,2\). Using lung epithelial murine-transformed Clara cells (mtCCs), our previous study indicated the importance of excessive oxidation in the pathology of hyperoxic lung injury\(^3\); however, the specific identities of reactive oxygen intermediates including hydrogen peroxide, superoxide (O\(_2^-\)) and hydroxyl radical have not been defined. Compared to other methods, electron paramagnetic resonance (EPR) combining spin trap/probe provides specific and sensitive detection for these oxidants. Spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (CM-H) detects both cytosol and mitochondrial oxidants (not specific for O\(_2^-\)) and has been used in both in vitro and in vivo studies.

Hypothesis: Hyperoxia stimulates the generation of CM-H-detectable oxidants in mtCCs.

Methods: 5 × 10\(^6\) mtCCs were cultured on 10 cm plates for 48 hrs. After changing to fresh medium, cells were exposed to either hyperoxia (HO; 85% O\(_2\)) or room air (RA; 21% O\(_2\)) with 5% CO\(_2\) at 37ºC for 3, 20 and 24 h. Cells were washed with DPBS and 2 ml of Krebs-Hepes buffer (pH 7.4) containing 100 µM diethylenetriamine pentaacetic acid (DTPA), 2.5 µM diethyldithiocarbamate (DETC), and 1 mM CM-H were added. Following 10 min incubation in RA and 5% CO\(_2\) at 37ºC, cells were collected and the cell suspension was injected into Aqua-X sample cell for EPR measurement using a Bruker Elexsys E500 system. Due to the autooxidation of CM-H, EPR spectra were recorded exactly at 15 min following CM-H preparation for all samples. EPR settings were: power 20 mW, modulation amplitude 2G, time constant 40 ms, conversion time 80 ms, and 1 scan.

Results: In RA, mtCCs produced CM-H-detectable oxidants at all time points. This is consistent with oxidant generation under RA conditions and also indicates the sensitivity of CM-H as a detection probe. HO stimulated oxidants generation. Brief quantitation by averaging the peak to trough values of the three peaks of the oxidized CM-H (CM\(^{˙-}\)) shows that CM-H-detectable oxidants generation increased 3.6 fold in HO-exposed cells at 3h, 4.1 fold at 20h, and 3.2 fold at 24h when compared to their respective RA-exposed controls.

Conclusion and Future Direction: Our data revealed that HO increases CM-H-detectable oxidant generation by 3-4 fold in mtCCs and as early as 3h. Further quantitation will be performed by comparing to known concentrations of CM\(^{˙-}\). We will perform experiments using SOD knockdown and overexpression in mtCCs to test whether the detectable oxidant is O\(_2^-\). To verify mitochondrial specificity of these oxidants, we will utilize a mitochondrial specific spin probe 1-hydroxy-4-[2-(triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine (mitoTEMPO-H). Future applications using these probes in our murine models for ex vivo EPR detection will provide both mechanistic and therapeutic insights into hyperoxia-induced lung injury.


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Measurement of cerebral oxygen in neurological disorders

Ke Jian Liu
Department of Pharmaceutical Sciences, Health Sciences Center, University of New Mexico, Albuquerque, NM 87131, USA
(kliu@salud.unm.edu)

Oxygen is required to maintain neuronal metabolism and function in human brain. Cerebral ischemia causes heterogeneous changes in tissue oxygenation and cellular metabolism, with a region of decreased blood flow, the penumbra, surrounding a severely damaged ischemic core. Because oxygenation is central in ischemic neuronal death and vascular damage, it is critical to understand exactly what actual changes occur in interstitial oxygen tension (pO2) in ischemic regions during stroke. Cerebral ischemia induces a complex series of molecular pathways involving signaling mechanisms, gene transcription, and protein formation. Free radicals and oxidative stress have been suggested to be involved in each of the steps in the injury cascade. We have developed techniques to measure tissue oxygenation (pO2) in vivo, as a function of time in specific focal regions of the brain, using Electron Paramagnetic Resonance (EPR) techniques. Both absolute values and temporal changes of localized interstitial pO2 have been measured in rats following ischemia and reperfusion. Our results showed that penumbral pO2 level could be modulated by changing the percentage of oxygen content in the breathing gas, and that 95% O2 given to rats was able to raise penumbral interstitial pO2 close to the physiological (pre-ischemic) value during ischemia. Most importantly, normobaric hyperoxia treatment immediately after an ischemia not only increased tissue pO2, but also decreased free radical generation, contrary to common expectation. Furthermore, hyperoxia treatment during ischemia reduces infarction volume, alleviate blood brain barrier damage, and improves the neurological function of the animal. We have carried out extensive studies to investigate the underlining molecular and cellular mechanisms associated with the neuroprotection afforded by hyperoxia treatment. These results support the notion that tissue oxygenation, and the resulting oxidative stress, play important role in ischemic brain injury, and that oxygen-based treatment could be a viable strategy to minimize the detrimental effects caused by ischemic stroke.
In vivo MRI-detectable free radical trapping detection assessment in neurological diseases

Rheal A. Towner

Advanced Magnetic Resonance Center, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104 USA

In vivo free radical imaging in disease pre-clinical models over the past decade has become a reality. For over half a century, free radicals were characterized by electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy coupled with spin trapping. Nitrone spin traps, such as PBN, DMPO or 4-POBN, are most commonly used for biological systems, and have been administered in vivo in various pre-clinical disease models for several decades. The disadvantage of the ESR/EPR approach is that the spin adducts (spin trapping agent – free radical adducts) are short-lived due to reductive and/or oxidative processes in biological systems. Immune-spin trapping (IST) involves the use of an antibody that recognizes macromolecular DMPO spin adducts, regardless of the oxidative/reductive state of the trapped radical adducts. We extended the in vitro/ex vivo IST approach to an in vivo approach that involves the use of IST in conjunction with molecular magnetic resonance imaging (mMRI). This involves the use of a spin-trapping agent, DMPO, which is used to trap free radicals in a disease model, and administration of a mMRI probe, called an anti-DMPO probe, that combines an antibody against DMPO-radical adducts and a MRI contrast agent, resulting in targeted free radical adduct mMRI. The contrast agent used in our approach, includes an albumin-Gd-DTPA-biotin construct, where the anti-DMPO antibody is covalently linked to the cysteine residues of albumin, forming an anti-DMPO-adduct antibody-albumin-Gd-DTPA-biotin entity. We have been able to use the combined IST-mMRI approach in several disease models, including multi-tissue assessment in diabetic mice with further assessment of cardiomyopathy, amyotrophic lateral sclerosis (ALS)-like mice, glioma-bearing mice, and mice with septic encephalopathy (cecal ligation and puncture and LPS-induced models). In all disease cases, trapped free radical levels were significantly higher when compared to appropriate controls (disease controls (e.g. wildtypes or shams), non-DMPO controls (i.e. administered saline instead of DMPO), and/or mMRI probe controls (i.e. a non-specific IgG was covalently bound to the albumin of the MRI contrast agent construct instead of the anti-DMPO antibody). The advantage of this approach is that heterogeneous levels of trapped free radicals can be detected directly in vivo, and be used to pinpoint where high levels of free radicals are formed in different tissues. The approach can also be used to assess possible therapeutic agents that are either free radical scavengers or generate free radicals. For example, we have used this approach to assess the free radical scavenging ability of an anti-cancer agent, OKN-007, in a rat glioma model. Some of the disadvantages with the methodology include limited access to pre-clinical MRI systems, availability of the anti-DMPO antibody, and the radical source that is being trapped. The focus of the talk will be on the use of the in vivo free radical imaging approach in various neurological disease models – past and current studies.
Oxidative Stress or Redox Signaling: ESR Spectroscopy in the Duty of Human Health.

Bruno Fink¹, Christoph Centner², Denise Zdzieblik², John M. Hunter³, Boris V. Nemzer³,⁴, Daniel Koenig²

¹ Noxygen Science Transfer & Diagnostics GmbH, Lindenmatte 42, 79215 Elzach, Germany; ² University of Freiburg, Department of Sport Science, Schwarzwald Str. 175, 79175 Freiburg, Germany; ³ VDF FutureCeuticals, Inc., 2692 N State Rt. 1-17, Momence, IL 60954 USA; ⁴ University of Illinois at Urbana-Champaign, 1201 W. Gregory Dr, Urbana, IL 61801 USA

Corresponding author: bruno.fink@noxygen.de

BACKGROUND: Generation of reactive oxygen species (ROS) is a continues life process and is often misinterpreted as oxidative stress. Excessive generation of ROS causes damage to biomolecules, maintenance of a physiological level of oxidative challenge, termed oxidative eustress, is essential for regulation of life processes through redox signaling. Recent interest has been focused on the maintenance of healthy levels of redox signaling and the related oxidants, which are crucial for providing us with concrete nutritional targets in order to better understand and maintain “optimal health”.

OBJECTIVES: Following this hypothesis we performed a pilot crossover, placebo controlled, single dose study on the dose dependent effects of SPECTRA7™ (25 and 50 mg), a dietary supplement, affecting the cellular metabolic index (CMI), the extent of cellular generation of reactive oxygen species and oxygen consumption in healthy human participants (n = 8).

DESIGN: The measurement of the CMI (ex-vivo intra- and extracellular formation of reactive oxygen species (ROS, O₂•⁻, H₂O₂, OH⁻) in whole blood, respiratory activity of blood cells, as well as mitochondrial dependent ROS formation and respiratory activity), was performed using the benchtop EPR spectrometer NOXYSCAN, spin probe CMH and oxygen label NOX-15.1, respectively. Furthermore, we investigated the ability of single dose of SPECTRA7™ to modulate ex-vivo cellular inflammatory resistance induced by stimulation with exogenous TNF-alpha, effects on blood glucose level and followed changes in circulating NO concentrations as parameter of endothelial function.

RESULTS: In this pilot study, we demonstrated that the administration of SPECTRA7™ resulted in statistically significant, long-term, dose dependent inhibition of mitochondrial and cellular ROS generation by as much as 9.2 or 17.7 % as well as 12.0 or 14.8% inhibition in extracellular NADPH system-dependent generation of O₂•⁻, and 9.5 or 44.5% inhibition of extracellular H₂O₂ formation. This was reflected with dose dependent 13.4 or 17.6% inhibition of TNF-alpha induced cellular inflammatory resistance and also 1.7 or 2.3-times increases of bioavailable NO concentration. Additionally, we observed significant up to 25 mg/ml decrease in physiological hyperglycemia.

CONCLUSIONS: For the first time, we demonstrated the ability of multifunctional natural supplement to effect cellular redox signaling and cellular metabolic activity. The unique design and activity of the plant-based natural supplement, in combination with the newly developed “Cellular Metabolic Index” test, demonstrates the potential of using dietary supplements to modulate redox signaling, which is considered by many researches as oxidative stress. This also opens the door to future research into the use of natural supplements for supporting the prevention of metabolic dysbalance in more than 60% of the population in industrial nations with two or more symptoms of “Metabolic Syndrome”. 

Oral Presentation 44
Swapping one free radical for another to address metabolic/cardiovascular dysfunction allied to obesity

Nadiezhda Cantu-Medellin¹,², Hunter C. Champion¹,², Jeff Baust¹,², Claudette St. Croix¹,³ and Eric E. Kelley⁴,⁵

¹University of Pittsburgh ²Vascular Medicine Institute and ³Center for Biological Imaging, ⁴University of West Virginia, Department of Physiology and Pharmacology and ⁵In Vivo Multifunctional Magnetic Resonance Center

Recent reports have identified a substantial source of elevated reactive species and uric acid (UA) in human and rodent obesity/diabetes to be the enhanced expression and activity of xanthine oxidoreductase (XOR). XOR catalyzes the terminal two steps in purine catabolism: oxidation of hypoxanthine to xanthine and xanthine to UA while concomitantly reducing O₂ to H₂O₂ and O₂⁻. While ROS and UA have been independently implicated in the pathogenesis of obesity, insulin resistance, dyslipidemia/steatosis, systemic inflammation and cardiovascular disease; the extent to which either ROS or UA formation in obesity is causative of these conditions remains ill-defined. This may be, in part, due to the linkage between the enzymatic production/catabolism of UA and reactive species generation as well as the potential for alternative substrates for XOR that result in overall diminution of both UA and ROS production. For example, under hypoxic/inflammatory conditions, similar to those encountered in obese/diabetic tissues, XOR can catalyze the reduction of nitrate (NO₃⁻) to nitrite (NO₂⁻) and NO₂⁻ to nitric oxide (NO); a total, two-electron transfer process that, by default, significantly diminishes capacity to generate both reactive species and UA while elevating levels of salutary NO. To garner a more clear understanding of interplay between these processes, we examined the effects of manipulating XOR product identity on metabolic and cardiopulmonary dysfunction allied to diet-induced murine obesity (60% HFD for 20 weeks). Treatment of obese mice (C57Blk/6J) with the XOR-specific inhibitor febuxostat (Uloric®) for the last 13 weeks of the 20 week diet decreased XOR activity, abolished obesity-mediated elevation in circulating UA levels, diminished systemic oxidative stress (45%), reduced fasting blood glucose, improved impaired glucose tolerance and improved cardiopulmonary hemodynamics (RVESP, PAP, PVR and Tau). Treatment with NaNO₂ produced similar yet, more pronounced beneficial effects than febuxostat whereas treatment with both NaNO₂ and febuxostat abolished protective effects observed with NaNO₂ alone, suggesting XOR dependence. Ex Vivo experiments confirmed XOR dependence of NO₂⁻-mediated effects by demonstrating diminution (65-75%) of NO generation upon specific inhibition of XOR or genetic ablation of XOR (xdh⁻⁻). When combined these data suggest that diminution of both ROS and UA as well as the production of NO leads to beneficial outcomes. As such, these results coalesce to identify a novel salutary role XOR that can be leveraged for clinical benefit.

Oral Presentation 45
Free radical lipid oxidation – out of hand or on a tight leash?
Valerian E Kagan
University of Pittsburgh, Pittsburgh, PA, USA
Kagan@pitt.edu

Amphiphilic polyunsaturated lipids are essential for life as structural building blocks of biological membranes and as signaling molecules. Coordination of numerous metabolic reactions and pathways requires high diversification of lipids which is achieved, to a large extent, via oxygenation of polyunsaturated lipids. This fundamental role of oxygenated polyunsaturated lipids in regulation is associated with a risk of their injurious effects via aberrant reactions of hydrophobic electrophilic carbonyl compounds – aldehydes, ketones, epoxides – with essential nucleophilic sites in proteins. These secondary reactive lipid electrophiles are generated from the common hydroperoxy-precursors, the primary molecular products of lipid peroxidation reactions. Therefore, control of the hydroperoxy-lipids is operated by the key intracellular redox regulatory system – thiols and their discoordination leads to ferroptosis, a non-apoptotic, iron dependent form of regulated cell death. We will present new data identifying hydroperoxy-phosphatidylethanolamines generated by 15-lipoxygenases as the proximate ferroptotic death signals in cells controlled by tocopherls and tocotrienols.
Overhauser MRI of free radicals
David J. Lurie
School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, AB25 2ZD, Scotland, UK
d.lurie@abdn.ac.uk

It is almost 30 years since the Overhauser effect was first employed as a method of imaging the spatial distribution of free radicals [1]. The basic method is known as Proton-Electron Double-Resonance Imaging (PEDRI) or, equivalently, as Overhauser MRI (OMRI). The sample’s EPR is irradiated during the acquisition of a proton NMR image; parts of the sample containing unpaired electrons exhibit altered image intensity due to the Overhauser transfer of polarisation from electron to proton spins, revealing the location of the free radical. Low magnetic fields are usually employed in order to achieve adequate penetration of the EPR irradiation and to avoid overheating the sample through non-resonant absorption; for example, in vivo PEDRI experiments have been performed on rats at 10 mT (237 MHz) [2] and on mice at 20 mT (564 MHz) [3]. Nevertheless, Massot et al. have demonstrated in vivo experiments at significantly higher fields and frequencies (194 mT, 5.43 GHz), apparently without adverse effects [4].

The disadvantage of using ultra-low magnetic fields is the inherently low signal-to-noise ratio (SNR) of the NMR experiment. Fortunately, magnetic field-cycling can be employed to improve SNR. In Field-Cycled PEDRI (FC-PEDRI) the magnetic field is switched between a low value (the evolution field, \( B_0^E \)) and a high value (the detection field, \( B_0^D \)) during the pulse sequence [5]. EPR irradiation takes place at \( B_0^E \) (~4 mT) at low frequency (~100 MHz) and lasts for \( 3 \times T_1 \approx 500 \) ms. The field is then switched to \( B_0^D \) and the NMR detection pulse(s) and magnetic field gradients for imaging are applied.

In our laboratory we constructed two FC-PEDRI systems, both of which employed dual, coaxial magnets. In the first system a large (60 cm bore) permanent magnet provided a vertically-oriented detection field of 59 mT [6]. An internal, resistive, field-offset coil generated an opposing field, so that the value of \( B_0^E \) could be selected. The second system used a 450 mT superconducting primary magnet, with a coaxial resistive, actively-shielded field-offset coil (12 cm bore) [7]. An interesting, alternative approach to field-cycled PEDRI has been demonstrated by Utsumi and colleagues, which involves rotating the sample through low-field (20 mT) and high-field (1.5 T) regions for EPR irradiation and signal detection, respectively [8].

Applications of Overhauser techniques to date have included the study of exogenous free radicals as contrast agents [2,3], the use of probes of pH [9,10] and for monitoring redox status [11] or tissue oxygen concentration [12]. The main advantage of Overhauser methods over “direct” EPR imaging is that the spatial resolution is independent of the linewidth of the free radical. Furthermore, a spatially-registered proton MR image comes “for free” with OMRI/PEDRI and can be used to display anatomy.

Development of New Field-cycling DNP-MRI for Free Radical Imaging

Hideo UTSUMI$^{1,2}$, Toshiki MASUMIZU$^{1,2}$, Ryoma KOBAYASI$^{2,3}$, Hidenori KAJIWARA$^4$, Atsushi IIKURA$^4$, Fuminori HYODO$^{2,5}$, Tomoko TAHIRA$^{2,6}$

$^1$School of Pharmaceutical Sciences, The University of Shizuoka, Yada, Suruga-ku, Shizuoka 422-8526 Japan, $^2$Kyushu University, $^3$Osaka University, $^4$FUJI ELECTRIC CO., LTD., $^5$Gifu University, $^6$Kinjo Gakuin University

E-mail: utsumih@u-shizuoka-ken.ac.jp, utsumi@remi.jp

DNP-MRI (dynamic nuclear polarization magnetic resonance imaging), a new imaging method for free radical species in vivo, was first reported by Lurie, et al. (1987). The major advantage of DNP-MRI is that the spatial resolution of free radical imaging is similar to that in MRI. We succeeded in simultaneous dual images of nitroxyl radicals labeled with $^{14}$N and $^{15}$N nuclei by changing the EPR irradiation in DNP-MRI.$^1$

We then tried to demonstrate the imaging of redox status$^2$, whole body-pharmacokinetics$^3$ and free radical intermediates produced from endogenous molecules, such as CoQ10 and FAD$^4$.

The special resolution of free radical imaging in these work is very poor due to the low magnetic field of MRI (0.01-0.02T) and we have developed a high sensitive DNP-MRI scanner by transporting the sample between EPR (20 mT) and MR magnets at 1.5 T, the spatial resolution of which was less than 0.2 mm.

Here, we developed DNP-MR system (magnet rotation type) applicable to human hand. To secure the stability and safety of the system, the magnets (5mT) for ESR and (0.3T) for MRI are stored in the jacket. MR Imaging were carried out with the gradient echo sequence, 2-6sec/cycle of magnets rotation, with/without 1.3-2sec of ESR excitation. Using this system, we demonstrated the clear images of the phantoms containing the redox intermediate radical of FAD and melanin with less than 0.5 mm of the spatial resolution. The newly developed DNP-MRI could become a promising technique to add metabolic/biochemical dimensions to anatomic images, if endogenous free radical were imaged,


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Continuous-flow DNP polarizer for *in-vivo* MRI applications at 1.5 T


1 Institute of Physical and Theoretical Chemistry and Center for Biomolecular Magnetic Resonance, Goethe University, Frankfurt am Main, Germany
2 Comprehensive Heart Failure Center, University Hospital Würzburg, Würzburg, Germany
3 Institute of Diagnostic and Interventional Radiology, University Hospital Frankfurt, Frankfurt am Main, Germany

* E-mail: vasyl@prisner.de

Proton signal enhancements for more than 20-fold were achieved with a newly designed DNP polarizer exploiting a multimode microwave resonator placed inside the bore of a 1.5 Tesla clinical scanner (Siemens Aera) and used for Overhauser dynamic nuclear polarization of protons in water. Different from other approaches in our setup the hyperpolarization is achieved continuously [1] by water flowing through the resonator under continuous microwave excitation with flow rate up to 1.8 ml/min, which should be high enough for DNP MR angiography applications in small animals like mice. The hyperpolarized substrate cooled to physiological temperature can be routed with help of a mechanical switch to a quartz capillary for injection to the target object [2]. This approach allows hyperpolarization of protons without the need of an additional magnet and avoids the losses arising from the transfer of the hyperpolarized solution between magnets. Performance of the polarizer will be demonstrated with various phantoms of blood vessels, and on ex-vivo models.


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Developments of Multi-Extreme THz ESR: Towards the Biological Application

Hitoshi Ohta\textsuperscript{1,2}, Tsubasa Okamoto\textsuperscript{2}, Hideyuki Takahashi\textsuperscript{3}, Eiji Ohmichi\textsuperscript{2}, Susumu Okubo\textsuperscript{1}, Takahiro Sakurai\textsuperscript{4}, Shigeo Hara\textsuperscript{4}

\textsuperscript{1} Molecular Photoscience Research Center, Kobe University, Kobe, 657-8501 Japan.
\textsuperscript{2} Graduate School of Science, Kobe University, Kobe 657-8501, Japan.
\textsuperscript{3} Organization of Advanced Science and Technology, Kobe University, Kobe, 657-8501, Japan.
\textsuperscript{4} Research Facility Center for Science and Technology, Kobe University, Kobe 657-8501, Japan.

hohta@kobe-u.ac.jp

First our developments on multi-extreme THz ESR will be overviewed. The specifications of our multi-extreme THz ESR are as follows; 1) frequency region between 0.03 and 7 THz covered by Gunn oscillators, multipliers, backward wave oscillators (BWO) and FIR laser [1], 2) temperature region between 1.8 and 300 K [1], 3) magnetic field region up to 55 T using the pulsed magnetic field [1], 4) pressure region up to 1.5 GPa [2]. As these multi-extreme conditions can be achieved simultaneously, we call it as the multi-extreme THz ESR. Recently we have extended the pressure region up to 2.7 GPa by introducing the hybrid-type pressure cell, while the field region was limited to 10 T [3]. Some applications of high pressure THz ESR will be shown

Secondly developments of our micro-cantilever ESR, which enables measurements of micro-meter size single crystal [4], will be reported. Recently we have achieved the micro-cantilever ESR measurement up to 1.1 THz [5], which is the world record for such mechanical ESR detection. As the micro-cantilever ESR has a very high sensitivity, it will enable us to detect ESR of the metal protein with the reasonable amount of sample from the point of view of biology. As an example, its application to the ESR measurement of hemin [6], which is the model substance of Myoglobin, will be presented.

In our future work we are aiming to observe ESR between the ground state and the first excited state of the metal protein, such as the Myoglobin, by our micro-cantilever ESR system. This direct measurement of the zero-field splitting will enable us to understand the electronic state of the metal protein in more details

Multi-Frequency EPR of Bio-hybrid Systems for Hydrogen Production


Chemical Sciences and Engineering Division, Argonne National Laboratory, Argonne, IL 60439, USA
§ Department of Chemistry and Physics, Chicago State University, Chicago, IL 60619, USA

Email: jniklas@anl.gov

To generate molecular hydrogen as solar fuel, two electrons are needed to reduce protons to one hydrogen molecule. These electrons can be provided by natural and artificial photosensitizers. Using natural Photosystems allows taking advantage of nature’s optimized light-harvesting and electron-transfer capabilities. In addition, the incorporation of the hydrogen catalysts in protein surrounding has the potential to protect and stabilize them. To achieve sustainable hydrogen generation, the catalysts should not be rare and expensive, but use earth abundant elements like first row transition metals. The catalytic properties of these systems depend not only on the chemical structure of the complexes but also on the local surrounding and in particular on the direct ligands to the metal ion(s) as provided by the associated protein. Knowledge of the electronic properties is important for an in-depth understanding of the catalytic properties of the complexes. Multi-frequency Electron Paramagnetic Resonance (EPR) spectroscopy at X-band (9 GHz), Q-band (34 GHz), and D-band (130 GHz) has been used to determine for transition metal catalysts like cobaloxime in different surroundings the electronic g-tensors and hyperfine interaction with various magnetic nuclei like $^{59}\text{Co}$, $^{14}\text{N}$, $^1\text{H}$. The experimental results are supplemented with DFT calculations. The knowledge gained by these model studies is used to characterize the binding of the hydrogen catalyst to proteins. The bio-hybrid complexes are capable of light-induced molecular hydrogen generation with high yield.

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Multi-Frequency Resonator Development

Jason W. Sidabras, Edward J. Reijerse, Wolfgang Lubitz
Biophysical Chemistry, Max Planck for Chemical Energy Conversion, Striftstr. 34-36, Mülheim an der Ruhr, Germany
Email: jason.sidabras@cec.mpg.de

Over the last decade the use of finite-element modeling software to calculate Maxwell’s Equations have become a popular way to design and implement microwave resonators for Electron Paramagnetic Resonance (EPR). We present the development of a series of new resonators and describe the methods needed to compare resonators across a variety of different geometries and frequencies. With the use of computer optimization guided by analytical analysis and human intuition, the next generation of EPR probes can be realized. Resonator geometries between X-band and 244 GHz will be discussed.

For instance, a novel Q-band Uniform Field resonator has been designed to provide a strictly uniform microwave field over a 10 mm region of interest and increase the efficiency over the current TE011 geometry. Methods to produce a uniform rf field in cavity resonators were introduced by Mett et al. [1–3] for cavity and re-entrant geometries and later for loop-gap resonators [4]. Uniform field cavities are defined as resonant structures at cut-off with a strictly uniform field over a region-of-interest transverse to the propagation vector of the waveguide. The re-entrant TE01U magnetic field was calculated to be 89% uniform, compared to 50% in a cylindrical TE011. The re-entrant TE01U resonator is designed for pulse experiments that need coherent pulses (such as ESEEM and HYSCORE). Uniform field resonators provide the same magnetic field excitation along the entire sample volume improving the modulation depth and resonator efficiency.

References
A prototype combined PET-EPRI scanner: initial testing

Alexander V. Stolin1, Mark Tseytlin2, Andrey Bobko2, Oxana Tseytlin2 and Raymond R. Raylman1

1Department of Radiology, Box 9236, One Medical Center Dr., Morgantown, WV, USA
2Department of Biochemistry, Box 9214, One Medical Center Dr. Morgantown, WV, USA

Presenting Author email: astolin@hsc.wvu.edu

Objectives: Electron paramagnetic resonance (EPR) enables interrogation of electron spins of free radicals to detect relatively stable compounds. EPR-based techniques in combination with paramagnetic contrast agents are accurate methods for measuring characteristics of the extracellular physiologic environment (oxygen saturation and pH, for example). Whereas, positron emission tomography (PET) imaging uses radiolabeled pharmaceuticals systemically administered in pM concentrations to quantify mainly intracellular processes (glucose metabolism and amino acid incorporation, for example). The combination of these two methods has the potential to enable unique investigations studying the dynamics between cellular physiology and tissue microenvironment. Our objective in this investigation is to explore the interactions between PET and EPR systems when they are combined in to a single scanner, and demonstrate the potential utility of a PET-EPRI system.

Methods: The PET scanner used in this study is a portable ring of twelve detector modules, each consisting of an array of LYSO detector elements (1.5mmx1.5mmx10mm) coupled to an array of silicon photomultipliers. EPR component was comprised of a permanent magnet equipped with DC coils to generate magnetic field 270G, the RF bridge/resonator and the RS-coil (operating at up to 100kHz and peak-to-peak field modulation up to 40G). Three gradient coils (x, y and z) were used to produce 3D projects used to reconstruct images with a filtered backprojection algorithm. The RS coil and resonator were mounted inside the bore of the PET scanner. The combined system was then inserted into the magnet. Solution containing mixtures of EPR contrast agent (3mM and 4.5mM water solution of stable trityl radical) and FDG (0.531μCi/μl and 0.266μCi/μl) was placed in four 2.5mm-diameter cylinders in an acrylic block, and positioned at the center of the PET-EPR scanner. Simultaneous PET image data and EPR spectra were then acquired 10min.

Results: PET and EPRI images of all cylinders were successfully obtained. The relative EPR and PET concentrations were related to intensities in the images. No artifacts were observed in either image set.

Conclusion: A combined PET-EPR system has the promise to enable unique and potentially important studies exploring the relationship between intracellular function and the extracellular microenvironment in cancer and cardiac tissues, potentially leading to new insights into the physiologic dynamics of these cells. This initial investigation demonstrated that there are no significant impediments to the melding of these two techniques. The next step is to construct a higher resolution, high detection sensitivity PET scanner optimized for use with EPRI.
EPR of Ionizable Nitroxides at Cryogenic Temperatures: Pros and Cons

Maxim A. Voinov,1 Christina T. Scheid,1 Olga N. Bulgakova,2 Igor A. Kirilyuk,3 Alex I. Smirnov1

1Department of Chemistry, North Carolina State University, 2620 Yarbrough Drive, Raleigh, North Carolina, 27695-8204, USA; 2Kemerovo State University, Krasnaya Str. 6, Kemerovo 650043, Russia; 3N. N. Vorozhtsov Novosibirsk Institute of Organic Chemistry SB RAS, Lavrentiev Ave. 9, Novosibirsk 630090, Russia.

E-mail: Maxim_Voynov@ncsu.edu

Many biophysical studies benefit from stabilizing biological samples at cryogenic temperatures in glassy matrixes. Examples include cryogenic DNP (dynamic nuclear polarization; DNP is typically combined with magic angle spinning solid-state NMR), cryo electron microscopy, EPR and DEER (double electron-electron resonance). The cryogenic temperatures in EPR experiments are typically employed to (i) increase the electronic relaxation times, (ii) minimize effects of molecular motion, (iii) slow down the kinetics of electron transfer in redox chains, and (iv) stabilize short-lived radicals trapped in inert matrices. However, detailed examination of optical absorption spectra of polarity indicator dyes revealed that the polarity of frozen organic solvent glasses is substantially larger than that of liquid solvents at room temperature.1 In structural biology, a concern has been raised regarding the crystallographic data obtained in a vitrified bulk solvent being not representative of biological molecules at thermodynamic equilibrium.2,3 Currently, only scarce data are available on how properties of solute molecules are affected by structural rearrangement of the proximal solvent molecules upon vitrification. Spin-probe EPR has proved to be a sensitive technique for studying various aspects of local solvent structure such as local polarity and hydrogen bonding. We propose that a unique structural insight on vitrified solutions could be gained from EPR of nitroxides with pH-dependent EPR spectra. The ionization constants (pK_a) of such nitroxides have been shown to be very sensitive to the parameters of local environment such as electrostatic potential and effective dielectric constant.4,5 Here we present proof-of-concept results on a comparative EPR titration of small-molecule pH-sensitive probe IKMTSL-ME and spin-labeled phospholipid IKMTSL-PTE at room temperature and 77 K.

![Diagram](image)

Right: Fraction, f, of the nonprotonated form of IKMTSL-ME in buffer solutions containing 50% v/v of i-PrOH, calculated from the fast-motion X-band A_iso titration data (17 °C, ●) and from the rigid-limit X-band EPR titration data (77 K, ○) vs. pH measured at 17 °C; Left: Chemical structures of IKMTSL-ME and IKMTSL-PTE.

Metabolic imaging of energy metabolism in traumatic brain injury using hyperpolarized [1-13C]pyruvate

Stephen J. DeVience1, Xin Lu1, Julie Proctor2, Parisa Rangghran2, Elias R. Melhem1, Rao Gullapalli1, Gary M. Fiskum2, and Dirk Mayer1

1. Department of Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, 22 S. Greene St., Baltimore, MD 21201
2. Department of Anesthesiology and the Center for Shock, Trauma, and Anesthesiology Research (S.T.A.R.), University of Maryland School of Medicine, 22 S. Greene St., Baltimore, MD 21201

Email sdevience@som.umaryland.edu

Traumatic brain injury (TBI) is a leading cause of death and disability in people under age 45 and can lead to cognitive impairments, mood disorders, and neurodegenerative diseases. TBI is known to cause perturbations in the energy metabolism of the brain, including a disruption of oxidative phosphorylation manifested as decreased activity of pyruvate dehydrogenase complex (PDH), the enzyme complex that links glycolytic with oxidative metabolism by converting pyruvate to carbon dioxide and acetyl-CoA. In this work, we use magnetic resonance spectroscopy of hyperpolarized 13C-pyruvate as a direct, non-invasive method for detecting these changes following traumatic brain injury.

TBI was induced in the left parietal lobe of healthy adult male rats using a controlled cortical impact (CCI) device. At 3.5-4 hours post injury, hyperpolarized 13C-pyruvate imaging was performed using a clinical GE 750w 3T MRI scanner and a doubly tuned (1H/13C) quadrature small animal coil. Spectrally-resolved imaging of the brain was initiated 30 s after injection of hyperpolarized [1-13C]-pyruvate. Pyruvate, lactate, alanine, and bicarbonate were quantified from their spectral peaks in each voxel.

We found both the bicarbonate signal and the ratio of bicarbonate to lactate signal to be sensitive to traumatic brain injury. At the site of the injury, there is a noticeable locus of relatively high lactate signal and relatively low bicarbonate signal (Fig. 1). The resulting 13C-bicarbonate signal was found to be 24 +/-6% lower in the injured hemisphere compared with the non-injured hemisphere (p=0.02), while the hyperpolarized bicarbonate-to-lactate ratio was 33 +/-8% lower in the injured hemisphere (p<0.01, Fig. 2). In control and sham surgery groups, there was no significant difference between sides of the brain.

For the first time, hyperpolarized metabolic imaging with 13C-pyruvate was applied to TBI. The presented data demonstrate a significant change in brain energy metabolism following CCI injury in rats.
Oxygen release by ultrasound sensitive O$_2$ microbubbles in solution and in vivo: temporal study

Agnieszka Drzal$^1$, Anthony Delalande$^2$, Chantal Pichon$^2$, Martyna Elas$^1$

$^1$Jagiellonian University, Department of Biophysics - Kraków, Poland, agnieszka.drzal@doctoral.uj.edu.pl, martyna.elas@uj.edu.pl
$^2$Centre de Biophysique Moléculaire, CNRS UPR4301, University of Orléans - Orléans, France, anthony.delalande@cnrs.fr, chantal.pichon@cnrs.fr

Email: agnieszka.drzal@doctoral.uj.edu.pl

Introduction: Hypoxia is a major determinant of human tumor sensitivity to radiation therapy [1]. Ultrasound sensitive microbubbles filled with oxygen may act as a short-term tissue radiosensitizer. Oxygen from microbubbles can be released locally by an ultrasound impulse, leading to a controlled, local increase in pO$_2$ [2]. The aim of this study was to investigate oxygen release from O$_2$ microbubbles both in solution, as well as in tumor tissue in vivo after intravenous and intratumoral administration.

Methods: Anionic pegylated microbubbles composed of DSPC and DSPE-PEG2000 were used. For oxygen release study solutions of three oxygen sensitive EPR spin probes were applied: CP (3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, 0.1 mM), CTPO (3-Carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy, 0.2 mM) and (15)N-mHCTPO (4-hydro-3-carbamoyl-2,2,5,5-tetra-perdeuteromethyl-pyrroline-1-(15)N-oxyl-d(12), 0.1 mM). CTPO, due to the lack of sensitivity at high oxygen concentrations, was only used for the experimental setup. EPR measurements were performed independently using X-band (Bruker BioSpin EMX, Germany) and L-band (Bruker Elexsys-II E540, Germany) CW EPR spectrometers. Either line width of EPR spectra or superhyperfine structure was measured in solutions leveled with air, deoxygenated with argon, after oxygen microbubbles addition for 10 minutes and after 2W/cm$^2$ ultrasound impulse application (Vevo SoniGene, VisualSonics, Canada). Microbubbles filled with nitrogen were used as a control. In vivo EPR oximetry was conducted with L-band CW spectrometer (Bruker Elexysy-II E540, Germany) in a 4T1 tumors growing in a mammary fat pad of Balb/c mice. For oxygen mapping right after microbubbles injection CP was used as a spin probe. Temporal changes in tumor oxygenation was measured 3, 6, 24 and 48 hours after microbubbles injection with OxyChip as an oxygen sensor. Independently, hemoglobin oxygen saturation was imaged with photoacoustic (Vevo LAZR, VisualSonics, Canada) in the same tumor model. The effectiveness of intravenous and intratumoral administration of O$_2$ bubbles was compared.

Results: EPR oximetry shows that oxygen microbubbles release some of O$_2$ without ultrasound impulse. However, after the impulse application oxygen content increase in solution to a level close to air. Such changes are not seen in nitrogen filled microbubbles.. In vivo, microbubbles injection (both i.v. and i.t.) and ultrasound impulse cause an increase of areas with higher pO$_2$ within imaged tissues. Photoacoustic imaging confirmed release of O$_2$ in the tumor. Enhancement in tumor pO$_2$ decreased gradually during 24 hours after microbubbles injection.

Conclusions: Ultrasound sensitive O$_2$ microbubbles have a potential to effectively increase the oxygenation of tissues.

Optimization of particulate sensors for EPR oximetry

Maximilian v. Groening1, Juliane Frank1, Daniel Gündel2, Simon Drescher1, Oliver Thews3, Karsten Mäder1

1Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, D-06120 Halle (Saale), Germany; 2Department of Nuclear Medicine, Ernst-Grube-Str. 40, D-06120 Halle (Saale), Germany; 3 Julius-Bernstein-Institute of Physiology, Martin Luther University Halle-Wittenberg, Magdeburger Str. 6, D-06112 Halle (Saale), Germany.
E-Mail: maximilian.von-groening@pharmazie.uni-halle.de

Lithium octa-n-butoxynaphthalocyanine (LiNc-BuO) is a valuable compound for EPR oximetry, which was developed by Kuppusamy’s group [1]. The compound shows a high oxygen sensitivity, however, drawbacks include the existence of different crystal polymorphs [2] and the tendency to form aggregates. Furthermore, own observations show that LiNc-BuO partially interacts with biological surroundings. Therefore, we aimed to improve the performance of LiNc-BuO by coating with bioinert oxygen permeable polymers. We selected polyvinylacetate (PVAc) as a suitable polymer. In vitro investigations did show that the coating has a negligible impact on the oxygen sensitivity (Fig. 1).

PVAc coated LiNc-BuO was injected with tumor cells into rats. The impact of the breathing gas on the tumor oxygen content (normoxia, hyperoxia and hypoxia) was studied in vivo and compared to other oxygen measurements [3]. The results indicate a reliable and reproducible performance of the coated, non-agglomerated particles. Current studies focus on the development of nanosized coated particles for improved biodistribution, which will enable novel applications for both in vitro and in vivo experiments.

Technical Improvements for Practical In Vivo Oximetry Measurements

Wilson Schreiber, Victoria A. Wood, Maciej M. Kmiec, Sergey V. Petryakov, Benjamin B. Williams, Ann Barry Flood, Harold M. Swartz

Geisel School of Medicine at Dartmouth
1Medical Center Drive – Williamson Building (Level 7) – Lebanon, NH 03756

wilson.schreiber@dartmouth.edu

There is a well-recognized need to be able to make reliable and repeatable oxygen measurements in tissues in the context of routine care in clinical settings. Given the profound effect that oxygen tension has on the relative radiosensitivity of hypoxic tumors [1], the efficacy of radiation therapy could be greatly enhanced with the advent of a technology in which oxygen measurements can be readily and non-invasively made. It is known that EPR (electron paramagnetic resonance) oximetry uniquely has the potential to provide direct, reliable, and accurate measurements on a temporal basis (repeated measurements) over long periods of time [2].

The overall concept and design of the EPR oximetry system that we have developed builds on EPR spectrometer magnets, bridges and resonators that have previously been developed for in vivo use [3] [4]. While these previous systems had functioned adequately for research purposes, it is our intent is to make the EPR oximetry measurement process fast, accurate, and more suitable for clinical acceptance through advances in hardware, software, and overall operator and measurement subject experiences.

We have developed a series of flexible resonator detectors which can be used for a variety of oxygen measurements in vivo, including surface measurements, measurements within the mouth, and various intracavity measurements. The design of this resonator facilitates a wide coupling range that accommodates critical coupling for surface measurements and closed-cavity measurements. This flexible resonator ameliorates the risk of pressure restricting blood flow to tissues, which can result in more accurate oxygen estimations. The flexibility of this resonator also allows for more versatile placement on a variety of measurement locations on the human anatomy.

The EPR data acquisition software has also been refined through the introduction of several routines and algorithms to report and plot oxygen levels while the tissues are being measured. These routines also include functions that dynamically optimize EPR spectrometer acquisition parameters for oximetric data acquisition, exclude poor-quality data using pre-defined spectral criteria, and include automatic controls and monitoring of patient and instrumental parameters.

Clinical EPR oximetry systems are currently available at three medical centers (Dartmouth-Hitchcock Medical Center in Hanover, New Hampshire; Emory University Medical Center in Atlanta, Georgia; and the medical center at Université Catholique de Louvain in Brussels, Belgium), and we are planning to effectuate several other clinical systems to medical centers in the near future.

Through these and other advancements we have made, we believe that we are closer than ever to bringing practical oximetry measurements into the clinic in order that the medical field takes fuller advantage of the utility of oxygen in tissues by providing an easy and straightforward means to measure it.

References


Acknowledgements

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Poster Presentations
EPR detection of hydroxyl radicals and cytotoxicity of zinc nanoparticles in RAW 264.7 cells

AM Morris¹, AB Stefaniak², KM Dunnick¹, MA Badding¹, NS Olgun¹and SS Leonard¹

1.) Health Effects Laboratory Division, NIOSH, 1095 Willowdale road, Morgantown, WV 26505
2.) Respiratory Health Division, NIOSH, 1095 Willowdale road, Morgantown, WV 26505
YRK9@cdc.gov

Handling nanoparticles presents novel hazards to human health, especially when used commercially before possible toxic effects may be evaluated. Zinc nanoparticle use is expanding and exposures are possible during the manufacture of concrete, rubber, food products, sunscreen, and paint. The toxicity of zinc nanoparticles has been investigated but little is known regarding zinc nanowires, a material with properties that make it ideal for solar cells and electronics. In this study, zinc metal nanoparticles (MNP), zinc oxide nanoparticles (NP), zinc oxide micron particles (MP), and zinc oxide nanowires (NW) were comparatively investigated. Potential toxic effects were studied using RAW 264.7 mouse macrophage cells. The particles were characterized by shape, diameter, and percentage of zinc in the sample. A CellTiter-fluor assay was used to determine cell viability and a CytoTox assay was used to determine lactate dehydrogenase (LDH) release following 4 h and 24 h exposures for three different particle doses (10, 25, and 50 µg/ml). Electron Paramagnetic Resonance (EPR) was used to determine hydroxyl radical (OH) production in both acellular and cellular experiments. There was a trend towards decreased viability at 4 h with 50 µg/ml and at 24 h with 25 µg/ml. At 24 h, a significant decrease in viability was observed at 50 µg/ml for all particle types. LDH levels in cell culture media were significantly increased with MNP treatment at 50 µg/ml for 4 h. After 24 h, all particles at 25 and 50 µg/ml caused significant LDH release. EPR results indicated that MNP stimulated significantly greater ·OH production than NP, MP, and NW upon reaction with hydrogen peroxide and in the presence of RAW 264.7 cells. Our results demonstrate that while MNP stimulated the most ·OH production, all of the zinc particles decreased cell viability, suggesting multiple mechanisms for zinc nanoparticle cytotoxicity.
Cytotoxicity and Pro-Inflammatory Mediated Responses by Hydraulic Fracking Sand Dust in Murine Macrophage Cells

Nicole S. Olgun, Anna M. Morris, Kristen A. Russ, Jeffrey S. Fedan, Stephen S. Leonard

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV

Hydraulic fracturing is used in the majority of natural gas wells across the United States. Water, sand, and chemicals are delivered at high pressure to drilled wells to cause fractures in the shale formations, allowing for the release of natural gas. Fracking sand, comprised mainly of silica dioxide (SiO₂), along with water and chemicals, is used to keep these fissures open. Silicosis is a pulmonary disease that affects workers exposed to inhaled silica and is characterized by inflammation and fibrosis, causing a decrease in lung capacity. Fracking sand dust (FSD) is generated during preparation of fracturing fluid for injection. In this study, murine macrophage cells (RAW 264.7) were used to better understand the mechanisms of toxicity associated with inhaled FSD (< 10 µm). We hypothesized that the soluble and insoluble components present in the FSD would each play a unique role in observed pro-inflammatory responses and cytotoxicity. FSD was washed in PBS two separate times, 5 days each time, allowing for any soluble material to be released (5 d and 10 d, respectively). On the 10th day, sand that was twice washed was re-suspended in PBS (10mg/ml) so that comparisons could be made to a freshly prepared, unwashed mixture. Measured with electron paramagnetic resonance (EPR), production of the hydroxyl radical (•OH) was the highest in unwashed sand, followed by PBS from the 5 d and 10 d washes. Unwashed FSD sand also generated the most intracellular ROS and was significantly higher than sand re-suspended after two consecutive washes. Compared to PBS controls, the viability of RAW 264.7 cells decreased by 40% in sand that was washed and re-suspended after 10 days, whereas unwashed sand decreased viability by 30% over a 24 h period. Finally, production of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 were measured using ELISA. While IL-1β and IL-6 production decreased with washing, TNFα production remained elevated. Our results indicate that FSD is cytotoxic to RAW 264.7 cells, as evidenced by decreases in viability, and stimulates intracellular ROS and •OH production. The stark differences in the production between cytokines stimulated by the dust warrants future studies into the pro-inflammatory effects of its soluble and insoluble components.
EPR Real Time quantification of ROS produced by TFD promoted by Up-conversion process.

1Nichollas Serafim Camargo; 2Bozena Sikora; 3Przemyslaw Kowalik; 2Anna Borodziuk; 2Ewa Mosiniewicz-Szablewska; 4Paulo César de Morais, 1Zulmira Guerrero Marques Lacava, 1,2Luis Alexandre Muehlmann; 3Fabiane Hiratsuaka Veiga de Souza; 4Paulo Eduardo Narcizo de Souza.

1Laboratory of Nanobiotechnology, University of Brasilia, Brasilia/DF, Brazil, 2Institute of Physics, Polish Academy of Sciences, Al. Lotników 32/46, PL-02-668 Warsaw, Poland, 3Faculty of Ceilândia, University of Brasilia, Brasilia/DF, Brazil, 4EPR Laboratory, Institute of Physics, University of Brasilia, Brasilia/DF, Brazil.

psouza1974@gmail.com

Photodynamic therapy (PDT) is a form of treatment in which monochromatic light is used to activate a photosensitizer applied to a tumor tissue or pathogen. The photosensitizing agent penetrates the cells, upon receiving the radiation of specific wavelength undergoes a chemical transition and becomes toxic promoting the death of the tumor cells. Unlike conventional chemotherapy, PDT has a localized effect in regions that are illuminated and that accumulate the photosensitizers. Most of the photosensitizers in current use are activated by photons of 630-660 nm, but their limited depth of penetration in this wavelength range restricts PDT to skin. One solution for accessing more internal tissues is to use longer wavelengths > 800-1000 nm. However, photons with these wavelengths do not have enough energy to activate the photosensitizers. This difficulty can be overcome by using molecules that promote the up-conversion process (1).

The aim of this project is to produce nanoparticles for drug delivery with their degradation sensitive to reactive oxygen species (ROS). As surface-cross linking agent 2-aminophenyl disulfide (2ASS) was used, aluminum phthalocyanide (AlPHCN) was adsorbed onto a surface of the particle to work as photosensitizer, this system was loaded with up-conversion nanoparticles NaYF4: 2% Er, 20% Yb/ oleic acid and doxorubicin (UpLSN), it was projected to be responsive around 980nm by up-conversion energy transfer from NIR to visible around 650nm. In biological systems, the release profile may change for nonspecific reactions that can lead to different action of ROS. This species may crack a polymeric matrix by oxidation and reduction of the disulfides, allowing the water to access the anhydride rings present at the polymeric matrix core, increasing the drug release, or may have therapeutic effect by local oxidative stress. A solution with 200μM of CMH was prepared in Krebs – Hepes buffer with UpLSN nanoparticles. After 980nm of irradiation, the concentration of ROS was determinate by EPR analysis at real time.

The production mechanisms of excited oxygen species are well known. After photosensitizer excitation by direct or up-conversion energy transfer, triplet exited states from photosensitizer donates energy to triplet molecular oxygen, generating singlet oxygen species, singlet oxygen can decay in different radical species, as superoxide (O$_2^-$), peroxide (H$_2$O$_2$) and peroxynitrite (ONOO-). The experiment was followed by 4 hours [Figure 4 (b)]. At first hour, a basal production was observed, caused by white light exposure (67.0 nM of ROS) (laboratory background), although after 980nm of irradiation, there were a constant and effective production of ROS (183nM/min), proving that the system is governed by radical species of oxygen.


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Increase of reactive oxygen species in different tissues during LPS-induced fever.

Bruna Rafaela Bezerra Gomes; Marina Firmino Lima de Oliveira; Jardeson Saraiva Jorge; Maria Luiza de Oliveira Ferreira; Thays Macêdo Rodovalho; Marcelo Valle de Sousa, Paulo Eduardo Narcizo de Souza; Fabiane Hiratsuka Veiga de Souza.

Laboratory of Protein Chemistry and Biochemistry, University of Brasilia, Brasilia/DF, Brazil, Faculty of Ceilândia, University of Brasilia, Brasilia/DF, Brazil, EPR Laboratory, Institute of Physics, University of Brasilia, Brasilia/DF, Brazil.

fhveigas@gmail.com

Fever is the increase in body temperature that occurs primarily in response to invasion of the body by pathogens, and plays an important role in the acute phase of immune response and in defense against pathogens. However, fever also causes harmful effects as a result of increased metabolic rate and oxygen consumption (1). Thus, the balance between damages and benefits should be considered when deciding whether it is necessary to treat a patient with antipyretics (2). The reactive species of oxygen are generated during physiological and pathological processes, and can act as both signaling molecules and as promoters of oxidative stress (3).

In this study, male Wistar rats received oral pre-treatment with dipyrone, ibuprofen, celecoxib or n-acetylcysteine 30 min prior to intravenous injection of LPS or vehicle, which led to a reduction in febrile response in all treated animals. The concentration of ROS was determined by electron paramagnetic resonance associated with the use of spin probe CMH in the blood, liver, brown adipose tissue (BAT) and hypothalamus of febrile animals treated with antipyretics. Fresh samples were incubated in KHB containing 200 µM CMH for 1 hour. Then, the samples were frozen in liquid nitrogen and kept frozen until the measurements in EPR. The measurements were performed at temperature of 77 K, and the quantification was performed from a calibration curve with known concentration of CP.

Our results demonstrated an increase in the concentration of ROS 5 h after induction of fever in the liver (in µM.g⁻¹, Vehicle: 215.7 ± 21.5; LPS: 322 ± 18.7), BAT (in µM.g⁻¹, Vehicle: 343.7 ± 32.8; LPS: 500.8 ± 62.7) and hypothalamus (in µM.g⁻¹, Vehicle: 212.5 ± 24.7; LPS: 300.2 ± 5.2), and none of the antipyretics used altered the profile of this production. Data from this study suggest that during fever there is a higher concentration of reactive oxygen species in different tissues, which may cause oxidative stress, and antipyretics do not interfere with this production.


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THE USE OF SPIN TRAP IN THE STAGES OF EXPERIMENTAL SEPTIC SHOCK DECREASES THE SURVIVAL IN THE RATS

Sindy J Olvera Vazquez¹, Stephany N Arellano Ahumada¹, Daniel Ramirez Rosales¹, Robert D Kross², Cleva Villanueva López³


²Krosslink Laboratories, Bellmore, USA


sindyrela_10@hotmail.com

It appears that an early increase in NO in the first stage of septic shock compensates for systemic vasoconstriction while a massive production in the late stage is involved in vasoplegia. The half-life of NO is extremely short; To study electron paramagnetic resonance (EPR) is a useful tool to explore the endogenous and exogenous that form the adducts with NO. The objective of this study was to estimate the early and late administration of carbon dioxide (DETC, an exogenous NO spin spatula) on survival and NO production in septic. Septic shock was induced in male Wistar rats by administration of lipopolysaccharide (LPS, 5 mg / kg). 1) isotonic saline solution (ISS, 600 μl ip and 600 μl sc) or 2) DETC (hereinafter 500 mg / kg, diluted in 600 μl ISS, ip) and iron citrate complex Sodium citrate diluted in 600 μl of ISS, sc), the latter administered immediately after the LPS in one group while in the other group was given 3 hours after LPS. Survival times were recorded and differences were assessed using the Mantel Cox test. Two more groups were used for nitrites (NO₃⁻) and nitrates (NO₂⁻). Survival was shorter (p = 0.01) and NO₂⁻ / NO₃⁻ concentration was lower (p <0.001, time 6h) in the DETC group compared to the LPS group. EPR spectra show that NO is trapped by both DETC and hemoglobin. DETC has its greatest effect from 0-3 hrs; But HbNO is always present in the length of the crash. The effect of the control administered at 3 hrs has little effect and at a time that is dominated by the presence of HbNO. We conclude that the administration of the DETC decreases the survival independently of the time that is administered (0 Hrs or 3 Hrs) since the early overproduction of NO is necessary to prolong the survival; While in the late atapa this production is exaggerated so the DETC no longer has effects.
Investigating the mechanism of Samarium reduction via spin-trapping with DMPO and PBN.

Joseph McPeak, Christopher Aretz; Bryan Cowen; Sandra Eaton; Gareth Eaton.

University of Denver, Department of Chemistry and Biochemistry, 2101 E. Wesley Ave. Denver, CO 80210

Joseph McPeak, Joseph.McPeak@du.edu

Samarium(II) iodide is a strong reducing agent that is being used to functionalize carbonyl groups. The single-electron reduction reaction between SmI$_2$ and 5-bromo-6-oxo-6-phenylhexyl-methanesulfonate was studied by spin trapping, to elucidate the mechanism. The spin traps n-tert-butyl-α-phenylnitrone (PBN), 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), and 5,5-dimethyl[2,3,3-2H$_3$]-1-pyrroline 1-oxide ($^2$H-DMPO)$^1$ were used for the identification of reaction intermediates. The possibility of side reactions involving a bromine leaving group were explored with control experiments. Continuous wave and rapid scan EPR were employed to define the time dependence of spin trapped species. Spectra were assigned by literature analogies. The spin-trapped intermediates are different from what was observed previously for reaction of SmI$_2$ with a different starting material.$^2$

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Profiling local water concentration in membranes and proteins down to 2 Å spatial resolution by HYSCORE spectroscopy of nitroxide spin labels

Melanie Chestnut, Sergey Milikisiyants, Amir Koolivand, Maxim A. Voynov, Tatyana I. Smirnova, Alex I. Smirnov

Department of Chemistry, College of Sciences, 2620 Yarbrough Drive, North Carolina State University, Raleigh, North Carolina, 27695-8204, USA
mmchestn@ncsu.edu

Local interactions of water molecules with protein side chains and lipid membranes are the driving forces of self-assembly for these systems and the determinants of their biological function. Currently, the arsenal of biophysical methods to measure local water concentration at specific depth within lipid bilayers or water accessibility of protein residues is severely limited. Accurate determination of water concentration profiles in nano-confined biological systems is even a more difficult task.

Starting with the pioneering work of Griffith et al. to obtain an evidence for water penetration in biological membranes, nitroxide spin labels and EPR have been employed extensively to study local water concentration and effective polarity. These studies expanded significantly with the advent of site-directed spin labelling (SDSL). Many of SDSL EPR methods rely on measurements of nitroxide relaxation enhancement by hydrophilic paramagnetic complexes or evaluation of solvent-dependent nitroxide magnetic parameters. However, both methods provide the data on local water concentration only indirectly. A more direct way of detecting water molecules in the proximity of a nitroxide is electron spin echo modulation (ESEEM) spectroscopy but it has a limited spatial resolution (~ 10 Å). Overhauser DNP combined with SDSL is a very powerful method to study local water dynamics in biological systems but it relies on bulk water polarization and, thus, has also its limitation in spatial resolution and data interpretation. Here, we demonstrate the use of hyperfine sublevel correlation spectroscopy (HYSCORE) to directly measure water molecules interacting with the nitroxide oxygen atom via a hydrogen (H-) bond. HYSCORE is a very sensitive technique for detecting H-bonds formed with a paramagnetic center. In order to convert HYSCORE data into local water concentration we employed a normalization factor for the H-bonded deuteron signal that was taken as intensity of the ESEEM signal measured under identical experimental conditions and spectrometer tuning parameters, which also account for the physical properties of the local media.

We demonstrate that water molecules H-bonded to doxyl-stearic acid (DSA) in lipid bilayers can be accurately measured even in hydrophobic regions of the membrane. A correlation between the observed H-bonded signals and local water concentration has been established using model systems containing mixtures of protonated diglyme and CH3OD, as well as diglyme and deuterated water, with Tempol as the spin probe.

Figure 1. (A) HYSCORE spectrum of 1 mM Tempol in water/diglyme mixture. (B) A zoomed-in area around deuteron Zeeman frequency. Signals from H-bonded deuterons are well resolved and spectrally separated from other signals detected by HYSCORE.

Teasing out metal redox states in coordination complexes, proteins, and cells.

Heather R. Lucas, Ph.D.

Department of Chemistry, Virginia Commonwealth University, 1001 W. Main St., Richmond, VA 23284
hrlucas@vcu.edu

Beyond the metal oxidation state and/or spin state, the coordination environment surrounding a metallocenter has a drastic effect on the EPR signal whether comprised within a ligand scaffold, protein matrix, or cellular medium. The coordination sphere also has a direct effect on the reactivity preferences and/or behavior of the metallocenter. For example, the stability and reactivity patterns of metal-dioxygen species can be steered by the donating ability and structural restrictions imposed by their ligand framework. Namely, the electronic properties of metal-dioxygen binding modes found within coordination species can be tuned to facilitate difficult organic transformation reactions, yet the identity of the reactive species can only be defined by a collection of advanced spectroscopic techniques - including EPR - and synthetic tricks that provide insight into the mechanistic pathway. In many cases, different metals can be housed within identical coordination spheres, altering the O₂ binding preferences and the subsequent reactivity. Such metal switching is not only controlled by synthetic preference and/or the scope of the researcher, but also by environmental factors within a natural system, which can affect protein folding and/or protein translocation. In this work, the catalytic potential of novel metallocomplexes comprised of earth abundant metals such as nickel, cobalt, and copper, will be discussed. Additionally, the effect of biometals such as iron, copper, and manganese on protein folding, aggregation, and protein-membrane interactions will be described, as well as the effect of metals on bacterial virulence. Overall, the scope of this research will highlight the reach of EPR from bioinspired catalysis to metal-dependent biological pathways.

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Spin Probe and Spin Label EPR Spectroscopic Characterization of Solvent Structure and Dynamics Around B_{12}-dependent Ethanolamine Ammonia-lyase

Benjamen Nforneh and Kurt Warncke

Department of Physics, Emory University, Atlanta, GA 30322
e-mail: bnforne@emory.edu

The cryo-temperature dependence of the first-order kinetics of the substrate radical rearrangement step in B_{12}-dependent ethanolamine ammonia-lyase (EAL) from *Salmonella typhimurium*, measured by using time-resolved, full-spectrum EPR spectroscopy, reveals two dynamical regimes and contributions of specific collective protein configurational fluctuations to the reaction. To provide a foundation for addressing the further role of protein-solvent dynamical coupling in the reaction, the properties of the fluid solvent domain (mesodomain) around EAL were studied over the temperature (T) range of 190-265 K in frozen aqueous solutions by using the spin probe, TEMPOL, ±cosolvent dimethylsulfoxide (DMSO). In parallel, protein surface dynamics were addressed by using 4-maleimido-TEMPO (4MT) spin-label at Cys37 of the EutC subunit of EAL. The rotational dynamics of TEMPOL as a function of T were revealed by the EPR line shape and quantified by the rotational correlation time (τ_c) obtained from EPR simulations. For added 1% v/v DMSO, single, rigid component spectra are obtained at T<200 K. Over 210<T≤245 K, two mobile spin populations, with τ_{c,f}, τ_{c,s} ≤10^{-7.0} s, are obtained, with normalized weights, W_f=60, W_s=40 ±1%. At T>245 K, W_f increases relative to W_s to a value of 69% at 265 K. In 1% v/v DMSO-water solution with no EAL, EPR spectra over the same T range were simulated by using a single component, and the T-dependence of the τ_c values was the same as for the τ_{c,f} values. The protein concentration dependence (12−120 μM EAL) of the components showed that W_s shrinks to 8% at 12 μM EAL. The TEMPOL results indicate that the W_c and W_f components correspond to protein hydration layer and the “bulk” solvent mesodomain, respectively. 4MT mobility in the EutC Cys37 surface region of EAL follows trends in DMSO- and T-dependence similar to TEMPOL. Overall, spin probe and label EPR spectroscopy, in conjunction with DMSO addition and T variation, provides an incisive approach to identifying and characterizing solvent-protein-reaction dynamical coupling in EAL. Supported by NIH R01DK054514.

References
The effect of antidepressant drugs on bicellar membranes: an EPR spin labeling study

Dilek Yonar¹ and M. Maral Sünnetçioğlu²

¹Middle East Technical University, Department of Biological Sciences, 06800, Ankara, Turkey
²Hacettepe University, Department of Physics Engineering, 06800, Beytepe, Ankara, Turkey

e-mail: dilekyonar81@gmail.com

Abstract

Bicelles are discoidal phospholipid nanostructures formed by a bilayer of phospholipid with a long hydrocarbon chain closed in the edges by a short-chained phospholipid. Bicelles have appeared as promising membrane models, because of their attractive combination of lipid composition and physicochemical characteristics.¹,² Their lipid composition, small size and remarkable versatility allow them to have the ability of penetrating through the narrow intercellular spaces of the stratum corneum. Thus, bicelles can change the barrier function of skin as enhancer for drug penetration.³

In the current study, it is aimed to observe the bicelle interactions with different kind of antidepressant drugs (clomipramine⁴ and flupentixol⁵) in order to demonstrate its effectiveness for the incorporation of different molecules and their use in the transdermal delivery of the drugs. Bicelle studies were performed with dimyristoyl phosphatidylcholine/diheptanoyl phosphatidylcholine (DMPC/DHPC) bicelles at two different ratios of lipids (2:1 and 4:1) and also in the presence of cholesterol. EPR spectroscopy was employed to study the effect of these drugs on bicellar system properties utilizing 5-doxyl stearic acid (5-DS) spin label as a function of temperature. 2A_max values obtained by direct evaluation of EPR spectra and the order parameter, S, calculated by computer simulation of EPR spectra demonstrated 4:1 DMPC/DHPC bicelle as more ordered system. Moreover, the order parameter, S, of the DMPC/DHPC bicelles increased upon addition of cholesterol. The addition of drug also caused the changes in the order and dynamic of the bicelle.

Our results demonstrated that drug has severe effects on overall bilayer properties. Considering the results, bicelles could be useful lipid systems for future pharmaceutical applications.

References

Use of the Vanadyl Ion as a Structural and Spectroscopic Probe Mimicking the Ferryl Intermediate in Iron-Dependent Enzymes

Alexey Silakov, Ryan J. Martinie, Christopher J. Pollock, J. Martin Bollinger Jr., and Carsten Krebs

Department of Chemistry and Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

e-mail: aus40@psu.edu

The iron(II)- and 2-oxo-glutarate (Fe/2OG) oxygenases catalyze an array of challenging chemical transformations via a common Fe(IV)-oxo (ferryl) intermediate. Although structural information has proven essential in understanding ferryl reactivity, there remains a paucity of structural information concerning the ferryl state of the enzyme. In this work, we demonstrate that the stable vanadyl ion is an excellent mimic of the ferryl intermediate.

We show that vanadyl binds to the model Fe/2OG enzyme TauD, forming a distorted octahedral complex with the protein ligands and succinate reminiscent of the ferryl intermediate. We further demonstrate the efficacy of vanadyl as a structural probe by defining the position of the substrate taurine relative to vanadyl in TauD using advanced pulse electron paramagnetic resonance methods. Structural information, obtained by this and other methods such as x-ray crystallography furnish key insights concerning ferryl reactivity in this important class of enzymes.

References:


Investigating the Supramolecular Arrangement of Metal-Binding PNA Duplexes with Double Electron Electron Resonance

Austin Jarvi1; Artur Sargun2; Catalina Achim2; Sunil Saxena1

1Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA

2Department of Chemistry, Carnegie Mellon University, 4400 5th Avenue, Pittsburgh, PA 1521, USA

arj51@pitt.edu

DNA and RNA have long been used to synthesize 3D structures on the nanoscale. Peptide Nucleic Acids (PNAs) are structurally analogous to DNA/RNA, but offer improved stability and specificity. Modification of PNA oligomers with metal binding ligands further enhances the capacity of PNA for arrangement into self-assembling nanostructures. These characteristics make metal-binding PNA oligomers an exciting candidate for biomimetic materials. By selecting paramagnetic metal ions like Cu2+ for use in metal-binding PNAs, these systems become uniquely qualified for study by Electron Paramagnetic Resonance (EPR). Herein we report the use of Continuous Wave (CW) and Double Electron Electron Resonance (DEER) to measure the structure, flexibility and dimerization of a PNA-PNA duplex modified with the metal binding ligands 8-hydroxyquinoline and 5-methylbipyridine. Analysis by DEER produced a bimodal distance distribution, indicating that the PNA-PNA duplexes are bridged by a Cu2+ ion coordinated to the 5-methylbipyridine ligand of each duplex. The most probable distances, 2.3 and 3.7 nm, correspond to an average angle of orientation of 107°. Additionally, the modulation depth parameter contains valuable information regarding the extent of dimerization. We have developed methodology to extract this information from accepted theory, which indicates that approximately 56% of PNA duplexes are dimerized.
In-cell distance measurements using pulsed EPR spectroscopy

Matthew J. Lawless, Sunil Saxena

Department of Chemistry, University of Pittsburgh, 219 Parkman Avenue, Pittsburgh, PA 15260

Email: mjl114@pitt.edu

In-cell distance measurements by pulsed EPR provide unique opportunities to study proteins in a more native environment that is irreproducible in vitro. The in-cell environment is harsh towards the typical nitroxide used in pulsed EPR DEER spectroscopy, however. To better understand the limitations of nitroxide based distance measurements in-cell, we perform an examination of the loss of DEER signal from two contributions: nitroxide radical decay and nitroxide sidechain cleavage[1]. Furthermore, we devise a strategy to extend the lifetime of the nitroxide radical within the cellular environment by use of an oxidizing agent. With the oxidizing agent, we perform DEER distance measurements on double nitroxide labeled GB1, the immunoglobulin binding domain of protein G, at varying incubation times in the cellular environment. By comparing the loss of DEER signal to the loss of signal in CW spectroscopy, we find that cleavage of the nitroxide sidechain, consisting of a disulfide bond, contributes to the loss of DEER signal. This loss is significantly greater in-cell compared to in-cell-extract. Furthermore, local spin concentrations are monitored at these varying incubation times to determine the time required for molecular diffusion of a small globular protein within the cellular milieu. Taken together, these data provide essential knowledge for both designing new in-cell nitroxide-based spin labels and for improving ESR methods in-cell.

Dextran-grafted triarylmethyl radicals

Martin Poncelet\textsuperscript{a}, Benoit Driesschaert\textsuperscript{a}, Valery V. Khramtsov\textsuperscript{a}

\textsuperscript{a} In Vivo Multifunctional Magnetic Resonance center, Robert C. Byrd Health Sciences Center and Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, USA

martin.poncelet@hsc.wvu.edu

Stable tetrathiatriarylmethyl (TAM) radicals are favorite spin probes used in biomedical EPR for the measurement of important physiological parameters, such as oxygen, pH and inorganic phosphate (Pi), \textit{in vivo}. This particular family of water soluble trityl radicals exhibits an unprecedented stability in biological media in combination with long relaxation times, leading to extremely sharp EPR lines. The most representative members of this family are the oxygen probes cTAM, Ox063 and the multifunctional (\(pO_2\), pH, Pi) pTAM probe (Figure 1).

However, the binding of cTAM and pTAM to biological proteins of the plasma (such as albumin) through hydrophobic interactions limits their mode of administration to intra-tissue only. On the contrary, the more hydrophilic structure of OX063 prevents these interactions and therefore allows for its systemic delivery. However, the use of OX063 in EPR imaging has been proven to be challenging due to its rapid clearance. Hereby, we report new dextran-PEG biopolymers of different sizes allowing to tune the probe pharmacokinetics. These macromolecular spin probes were synthesized by grafting TAM radicals on an azide-modified dextran biopolymer using a click chemistry approach (Figure 2). A set of different dextran sizes with different spin probe loadings has been synthesized and the preliminary results of their EPR properties and biocompatibility are reported in this poster.

Figure 1

Figure 2

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Solvent dependence of magnetic parameters of protonatable nitroxides

Maxim A. Voinov, Alex I. Smirnov

Department of Chemistry, North Carolina State University, 2620 Yarbrough Drive, Raleigh, North Carolina, 27695-8204, USA.

E-mail: Maxim_Voynov@ncsu.edu

Magnetic parameters of nitroxide free radicals (i.e., nitrogen hyperfine A tensor and electronic g-factor matrix) have been long recognized to be sensitive to the polarity of the environment. However, the relationship between A and g and solvent polarity is not entirely straightforward. Over the years, several models have been proposed to relate the isotropic nitrogen hyperfine coupling constant $A_{iso}$ to the dielectric permittivity ($\varepsilon$) of the media as a measure of a solvent polarity. The other, less frequently used solvent polarity parameters such as Weinstein-Grunwald Y value, Kosower Z value, and Dimroth-Reichardt $E_T$ (30) value, have also been employed. Although some of the models work satisfactory well for aprotic solvents, for protic media, the $A_{iso}$ contributions arising from hydrogen bonding between the oxygen of the nitroxide (N–O*) group and the solvent have to be taken into account. The problem becomes even more complicated when a nitroxide possesses an additional chemical functionality, such as one capable for reversible protonation, and, therefore, capable for an additional hydrogen bond formation. Here we present the results of our comparative EPR study of the effect of solvents on the magnetic parameters of both protonatable and non-protonatable nitroxides.

The Synthesis and Study of gem-Dicarboxylate Nitroxides

Shengdian Huang, Joseph T. Paletta, Suchada Rajca, and Andrzej Rajca

Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

shengdian@huskers.unl.edu

In combination with site-directed spin labeling (SDSL) technique and double electron electron resonance (DEER) technique, nitroxides can be used as spin labels for probing the structures and dynamics of macromolecules such as proteins, DNAs, and RNAs. But the applications of SDSL technique and DEER technique are related to the structures of nitroxides very tightly. For example, if the nitroxides are hydrophobic, e.g. Spiro-TOAC, the yield of spin labelled peptide is very low.[1] In addition, if the nitroxides, e.g. MTSL, have four methyl groups close to the N-O spin site, the temperature of making DEER measurement is about 50 - 70 K, requiring use of liquid helium, or with lower sensitivity at 80 K with liquid nitrogen.[2] So the aim of this research is to search for hydrophilic new nitroxide spin labels with adequately long T_m for DEER distance measurement at room temperature. Two hydrophilic tetracarboxylate ester pyrroline nitroxides 1 and 2 have already been designed and synthesized.[3] Studies of electron spin relaxation rates in rigid trehalose /sucrose matrices reveal approximately temperature independent values of 1/T_m for 1 and 2 up to about 160 K and modest temperature dependence up to 295 K. Encouraged by the good results, one of the current goals is the synthesis of nitroxide 3, with a methylthiosulfonate (MTS) functional group for selective labelling of cysteins. Another project in progress is the synthesis of gem-dicarboxy nitroxide 4, which is proposed as a promising target with long T_m for DEER measurements in vivo because of large sizes and negative charges of the carboxylate groups.

We thank the National Science Foundation and National Institutes of Health for support of this work through Grants CHE-1362454, NIBIB R01 EB019950-02, and NIGMS U54GM087519-06. We thank Dr. Xinning Liu for his help with the synthesis and Dr. Nolan Gallagher for his help with the Easyspin spectral simulations.

Synthesis and Study of Iodoacetamide Diazaadamantane Nitroxide

Zhimin Yang\textsuperscript{a}, Maren Pink\textsuperscript{b}, Suchada Rajca\textsuperscript{a}, and Andrzej Rajca\textsuperscript{a}

\textsuperscript{a}Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0304, United States
\textsuperscript{b}IUMSC, Department of Chemistry, Indiana University, Bloomington, Indiana 47405-7102, United States

Email: zhimin.yang@huskers.unl.edu

Abstract

Double electron electron resonance (DEER) and double quantum coherence (DQC) are powerful methods for accurate distance measurements owning to minimum perturbation from relatively small size of nitroxides.\textsuperscript{1} These well-established techniques with great advantages in high sensitivity and a wide range of distance, however, are commonly done at temperatures between 50-70 K, due to the dynamic averaging effects associated with \textit{gem}-dimethyl groups rotation in the nitroxides, e.g., in 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-(methyl) methanethiosulfonate label (MTSL).\textsuperscript{2} Low rotation barrier makes the rotation of methyl groups at rates comparable to the anisotropy in the electron-proton hyperfine coupling. This process shortens the spin echo dephasing time $T_m$, making the measurement demanding and expensive. Therefore, the development of new spin labels devoid of methyl groups with sufficiently long $T_m$ for study distances near ambient temperature is an enticing and challenging goal.

In the design of new hydrophilic nitroxides with smaller molecular size and greatest degree of rigidity, the derivatives of 2,6-diazaadamantane are interesting alternatives to the archetypical nitroxides, e.g., diazaadamantane nitroxide diradical (Rassat’s Nitroxide), reported by Rassat and Dupeyere.\textsuperscript{3} Herein, we describe design and synthesis of iodoacetamide diazaadamantane nitroxide (IA-DZD). This new nitroxide is promising as spin label for distance measurement at physiological temperature. Its structure and that of its precursor, chloroacetamide 2,6-diazaadamantane nitroxide, are confirmed by X-ray crystallography.

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**HIF-1α Regulates the Tie2 Receptor on Tie2-Expressing Monocytes in PyMT Breast Tumors and Augments Angiogenic Function and Metastatic Potential**

Kayla Steinberger¹, Mary Forget⁵, Xiaokui Mo⁵, Randall Evans⁵, Amy Gross⁵, Leni Moldovan⁵, Clay B. Marsh⁴, Tim D. Eubank¹²,³,⁴

1 Department of Microbiology, Immunology, and Cell Biology, the Robert C Byrd University Health Sciences Center, West Virginia University, Morgantown, WV 26506
2 West Virginia Clinical and Translational Science Institute, West Virginia University, Morgantown, WV 26506
3 WVU Cancer Institute, West Virginia University, Morgantown, WV 26506
4 School of Medicine, Robert C Byrd University Health Sciences Center, West Virginia University, Morgantown, WV 26506
5 Pulmonary, Allergy & Critical Care Medicine, Davis Heart & Lung Research Institute, The Ohio State University, Columbus, OH 43210

kjp0007@mix.wvu.edu

Tissue oxygenation in the tumor microenvironment serves as a significant parameter in tumor pathophysiology. In fact, normalization of tumor oxygen is associated with inhibition of tumor growth and metastatic capacity. Irregular tumor vessels produced during tumor angiogenesis have low blood perfusion, thus oxygen transport is decreased in tumors. Tie2-expressing monocytes (TEMs) are a distinct subset of pro-angiogenic monocytes selectively recruited to tumors in breast cancer patients. Due to the hypoxic nature of the tumor microenvironment, we investigated if oxygen regulates the trafficking of these cells into tumors or if oxygen regulates monocyte differentiation into TEMs once inside the tumor proper. To understand the differentiation of F4/80+/Tie2- cells to Tie2-positivity, we orthotopically implanted PyMT breast tumor cells containing particulate lithium octa-n-butoxy-naphthalocyanine (LiNcBuO) into the mammary fat pads of LysM-Cre control and HIF-1α[fl/fl]/LysM-Cre mice and evaluated tissue oxygenation by Electron Paramagnetic Resonance (EPR), TEM infiltration, tumor angiogenesis, and metastatic potential. Longitudinal monitoring of physiologic oxygen in tumors using EPR demonstrates the significant role of oxygen-driven TEM regulation in tumor angiogenesis and progression.

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Extracellular Phosphate as a Marker for Tumor Growth

Andrey A. Bobko¹, Timothy D. Eubank¹, Mikhail A. Gavrilin², Yakov Y. Woldman³ and Valery V. Khramtsov¹

¹ Department of Biochemistry, School of Medicine, West Virginia University, Morgantown, WV, 26506
² Ohio State University Wexner Medical Center, Columbus, OH, 43210
³ Valdosta State University, Valdosta, GA 31698

Electron paramagnetic resonance (EPR) was used for in vivo assessment of concentration of interstitial phosphate (P\textsubscript{i}) using hydrophilic trimethylaryl radical [1]. P\textsubscript{i} measurements were performed in a mouse model of breast cancer as tumors progressed to malignancy and during application of metabolically-active drugs. We observed early increase in the P\textsubscript{i} content in tumors compared with the level in normal mammary tissue during malignant progression, therefore identifying P\textsubscript{i} as a potential prognostic factor in tumorigenesis. Strong negative correlation in normal mammary glands vs. no correlation in breast tumors was found between interstitial pO\textsubscript{2} and P\textsubscript{i} measured in PyMT mice in vivo, therefore supporting tumor reliance on glycolysis independent of oxygen availability. The opposite effects of glucose oxidase/glucose (pO\textsubscript{2} decrease and [P\textsubscript{i}] increase) and 2,4-dinitrophenol (pO\textsubscript{2} increase and P\textsubscript{i} decrease) measured in vivo indicate a relationship between these parameters and metabolic alterations. Experiments on cell culture show effusion of phosphate from the cells into extracellular medium being significantly higher for normal epithelial cells (MCF-10A) than for breast adenocarcinoma cells (MDA-MB-231). In both cases effusion increases in the presence of respiratory inhibitors (KCN and 2,4-dinitrophenol). In summary, in vivo interstitial P\textsubscript{i} measurements may provide a tool for assessment of cancer progression. Supported by NIH grants EB014542, CA194013.

Microenvironment Imaging of Cancer Cells as a Phenotypic Biomarker

*Mehdi Damaghi*¹ and *Robert J. Gillies*¹.

**Authors’ Affiliations:** ¹Department of Cancer Imaging and Metabolism, Moffitt Cancer Center and Research Institute, Tampa, Florida, USA.

Early carcinogenesis is an avascular and hypoxic disease that makes tumor cells export metabolically derived acid through glycolysis. Even in presence of oxygen cancer cells keep the glycolytic phenotype (Warburg effect) that acidifies the environment more and constantly. Hence the physical microenvironment of solid tumors is profoundly acidic. Pre-malignant cells within this niche must adapt to acidosis in order to survive this hostile microenvironment. We think pre-malignant cells adapt to acidosis to not only survive in this hostile microenvironment but also to acquire an advantage that helps them to outcompete the others and eventually help them to invade. Here we also show how these cells use matrix remodeling as a strategy of acid adaptation to construct and engineer their niche in their own favor for growth and proliferation. We found collagen as a major protein of extra cellular matrix (ECM) being used by cancer cells in that manner. The unique mechanical properties of fibrillar collagen are mainly controlled by its structure and any change in it can lead to ECM induced tumor growth. ECM unique compositions and topographies are generated through a dynamic biochemical and biophysical interplay between the various cells and the evolving microenvironment. In this study we developed the window chamber and intra vital microscopy to study the collagen remodeling lively. Later we used 3D cell culture and co-culture to validate our findings. Finally we used proteomics to understand molecular mechanism behind the changes in acid adapted versus non-adapted cancer cells.

In conclusion, there are evolutionary advantages behind acid production and adapting to it for cancer cells. We discuss how acquiring these advantages can helps us to design new therapeutics against these unique phenotypes.
Dynamic Nuclear Polarization Properties of phosphonated trityl radical: imaging of pH, oxygen and inorganic phosphate

Artem Gorodetskii1,2,3, Andrey Bobko1,2, Benoit Driesschaert1,2, Martin Poncelet1,2, Valery Khramtsov1,2

1In Vivo Multifunctional Magnetic Resonance center, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506, USA
2Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, USA
3Novosibirsk State University, Novosibirsk, Russia

artem.gorodetskii@hsc.wvu.edu

Electron Paramagnetic Resonance (EPR) spectrum of phosphonated trityl radical is sensitive to oxygen, pH and concentration of inorganic phosphate1. In this research, we used this radical as contrast agent for Overhauser-Enhanced Magnetic Resonance Imaging (OMRI). Using several OMRI images obtained with different pre-selected frequencies and powers of EPR irradiation is possible to map oxygen, pH and concentration of phosphate simultaneously.

Phosphonated trityl radical in water solutions has protonated and deprotonated forms which have EPR spectra with different hyperfine splitting constants. The ratio of enhancements of signal measured at two different EPR frequencies corresponding to resonances of protonated and deprotonated forms allows to calculate pH values. Proton exchange of radical with phosphate results in coalescence of two resonance lines at high phosphate concentrations. It has been shown that values of enhancement in the middle point strongly depends on phosphate concentration. Developed approach allows to map pH and phosphate using three OMRI images obtained upon EPR irradiation at three different EPR frequencies. Oxygen increases relaxation rates of radical resulting in decrease of enhancement of signal. Applying theory of polarization transfer including Heisenberg spin exchange we have found that it is possible to calculate oxygen concentration using two OMRI images obtained at low and high powers of EPR irradiation at frequencies of protonated or deprotonated forms2.

In summary, OMRI with the use of phosphonated trityl radical allows to map oxygen, pH and concentration of inorganic phosphate using five OMRI images obtained with short acquisition times.

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Construction of 0.15 Tesla DNP-MRI

Y. Tokunaga\textsuperscript{a}, M. Nakao\textsuperscript{b}, T. Naganuma\textsuperscript{b} and K. Ichikawa\textsuperscript{c}

\textsuperscript{a} Innovation Center for Medical Redox Navigation, Kyushu University, Japan
\textsuperscript{b} Japan Redox Ltd. Japan
\textsuperscript{c} Nagasaki International University

\texttt{tnaganuma@jrx.co.jp}

Abstract: Dynamic Nuclear Polarization MRI (DNP-MRI) is one of free radical imaging technologies and has been used in biomedical researches such as partial oxygen measurements in tumor, redox status in acute oxidative diseases. External magnetic field of DNP-MRI is frequently in the range of 5 to 10 mTesla, to ensure microwave penetration into small animal and the S/N ratio is limited. In this study, a 0.15 Tesla DNP-MRI was constructed and tested to improve S/N for small sample or skin measurement. Specification of main magnet was as follows; 0.15 Tesla permanent magnet; gap size 160 mm; homogenous volume 80 mm. The DNP-MRI resonator was designed based on TE101 cavity mode and machined from phosphorus deoxidized copper block for ESR excitation and solenoid transmission/receive resonator for NMR detection. The resonant frequencies and Q values were 6.38MHz/150, 4.31-4.41GHz/120 for NMR and ESR, respectively. The Q values were comparable with those of conventional low field DNP-MRI resonators at 15 mTesla. The MRI S/N ratio was improved by factor of 30, as expected. Triplet dynamic nuclear polarization spectra were observed for \textsuperscript{14}N carboxy-PROXYL, along excitation microwave sweep. The DNP spectra intensity depended on electron B1 and excitation time and enhancement factor (E) was ca. 0.5 in current setup. In conclusion, the result of preliminary evaluation indicated that the 0.15 Tesla DNP-MRI would be useful for free radical measurement for small samples.

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Noninvasive EPR Oximetry in Human Subjects using SPOT Chip


Department of Radiology & Medicine, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756; #Schulich Faculty of Chemistry Technion - Israel Institute of Technology, Haifa, Israel

*email: kuppu@dartmouth.edu

The overall objective of this study was to establish EPR oximetry as a noninvasive, reliable, and safe method for measuring transcutaneous oxygen tension (tc-pO₂) in humans. Tc-pO₂ provides information about blood perfusion in the tissue immediately below the skin. The data will be valuable in the diagnosis of wound healing and peripheral vascular/arterial diseases, where regional or local blood-flow may be compromised. It can also be used as a prognostic marker of disease progression and response to therapy, identify responders and non-responders. Toward this goal, we have developed a method using EPR oximetry with an oxygen-sensing paramagnetic chip, termed as SPOT (Superficial Perfusion Oxygen Tension) chip. The SPOT chip has been designed as a thin (~120 µm) circular film with a diameter of 3 or 6 mm. The chip is composed of 20% (w/w) of microcrystals of lithium octa-n-butoxy naphtalocyanine (LiNc-BuO) encapsulated in polydimethylsiloxane (PDMS), a biocompatible polymer approved by FDA for use in human. One side of the chip is made impermeable to molecular oxygen, while the other side is permeable to oxygen. The sensor (chip) is covered with an oxygen barrier material and secured to the skin by a medical transfer adhesive tape. This covering is necessary to ensure that only oxygen that diffuses through the skin surface is measured, and not the oxygen from the ambient environment. The chip is placed on the skin with the oxygen-permeable side in contact with the skin. This allows transcutaneous oxygen to diffuse into the chip. The SPOT chip is placed on the skin at the location of interest prior to measurement and removed and disposed after the measurements are complete. No surgical procedure is required for placement of the SPOT chip. This EPR-based transcutaneous oximetry method would not require or generate localized heating of the skin. Some of the key advantages of SPOT chip oximetry include: (i) Totally noninvasive measurement, (ii) Excellent temporal response; (iii) High sensitivity at low oxygen levels; (iv) No heating of the skin is required; (v) Repeatability for long period; (vi) Non-perturbing - does not consume oxygen; (vii) Translatable for routine clinical use. Initial measurements in human subjects have demonstrated that SPOT chip can measure transcutaneous pO₂ under ambient conditions. The method has the potential to be used in the clinic where routine monitoring of transcutaneous pO₂ is required for treating, for example, peripheral vascular diseases and wound healing.

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Supplemental Oxygen Protects Heart against Acute Myocardial Infarction

M. Lakshmi Kuppusamy, Shan K. Naidu, Mahmood Khan, Brian K. Rivera, and Periannan Kuppusamy*

Dartmouth-Hitchcock Medical Center, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756; Dorothy M. Davis Heart and Lung Research Institute, Ohio State University, Columbus, OH 43210

*email: kuppu@dartmouth.edu

Myocardial infarction (MI), which occurs often due to acute ischemia followed by reflow, is associated with irreversible loss (death) of cardiomyocyte due to apoptosis potentiated by p53 signaling. If left untreated, MI will lead to progressive loss of cardiomyocytes, deterioration of cardiac function, and congestive heart failure. While supplemental oxygen therapy has long been in practice to treat acute MI, mostly showing beneficial effects but not always, there has not been a clear scientific basis for the observed effects. Using EPR oximetry, we studied the effect of brief periods of exposure to supplemental oxygen (SO) administered by inhalation of 21-100% oxygen for brief periods (15-90 min), daily for one week, using a rat model of MI. Myocardial oxygen tension (pO₂), cardiac function (ejection fraction, and fractional shortening), and pro-survival/apoptotic signaling molecules were measured. SO resulted in a significant reduction of infarct size and improvement of cardiac function. An optimal condition of 30-min SO of 95% oxygen+5% CO₂ under normobaric condition was established for best cardioprotection. Interestingly, the proapoptotic p53, which is known to transcriptionally regulate members of the NOS family, was consistently overexpressed in the MI+SO hearts. We hypothesized that p53 plays an oxygen-dependent dual role in generating apoptotic signals in the MI hearts and NOS3-mediated survival signals in the MI+SO hearts. A 23-bp p53 response element (p53RE) was identified in NOS3 promoter and p53 was found to directly bind and transcriptionally regulate NOS3 promoter in the MI+SO hearts. p53 from the MI heart did not bind to NOS3-p53RE and showed affinity towards apoptotic BAX-p53RE. p53 from MI+SO heart showed affinity towards NOS3-p53RE. Analysis of p53 post-translational modifications showed differential acetylation of p53 core-domain at p53(Lys118) residue in MI and MI+SO hearts. p53 was acetylated at Lys118 in the MI heart and oxygenation revoked this modification. This de-acetylation functioned as the molecular switch in determining the affinity of p53 towards BAX-p53RE or NOS3-p53RE and generation of p53 pro-apoptotic and pro-survival forms. In summary, supplemental oxygenation de-acetylates p53 at the Lys118 residue and alters p53 DNA-binding affinity towards NOS3-p53RE from BAX-p53RE thus generates pro-survival form of p53 in the heart. Cardioprotection and cardiac-tissue regeneration might be achieved by generating p53-prosurvival form through inhibition of p53 core-domain post-translational modifications.


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**In vivo** electron paramagnetic resonance oximetry and applications in the brain: Brain oxygen and antioxidant imbalance in drug abuse

John Weaver,1,2 Yirong Yang,1,2 Rong Pan,1,2 Jia Liang,1,2 Rebecca Purvis,2,3 Gerald Rosen4,5,6,7 and Ke Jian Liu1,2

1Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM; 2COBRE Biomedical Research and Integrative Neuroscience Center, Health Sciences Center, University of New Mexico, Albuquerque, NM; 3Department of Neurology, University of New Mexico, Albuquerque, NM; 4Department of Physiology, University of Maryland School of Medicine, Baltimore, MD; 5Center for Biomedical Engineering and Technology, University of Maryland, Baltimore, MD; 6Center for EPR Imaging In Vivo Physiology, University of Maryland, Baltimore, MD; 7Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD

Email: jmweaver@salud.unm.edu

Oxygen (O₂) is central to life. However, while we need O₂ to live, high or low concentrations of O₂ in the human body (particularly the brain) are potentially toxic and may directly impact normal function or stability. Thus, it is hypothesized that changes in brain O₂ concentration (pO₂) play a key role in various brain disorders or diseases. Specifically, alterations in brain pO₂ by methamphetamine (METH) may directly impact homeostasis and lead to METH-induced oxidative stress neurotoxicity. While METH neurotoxicity has been studied for several decades, there are few studies investigating METH-induced alterations in brain pO₂ and electron paramagnetic resonance (EPR) offers unique methodology for studying a number of important biological parameters related to O₂, including but not limited to changes in tissue pO₂ and the detection of markers for antioxidant imbalance. Accordingly, continuous advancements have enabled us to utilize EPR to measure brain pO₂ and antioxidant imbalance in a METH abuse animal model. Innovative aspects of these findings include: 1) the application of EPR to measure changes in brain pO₂ and antioxidant imbalances in animals, including the ability to use EPR to map brain pO₂; and 2) novel evidence of the potential relationship between METH-induced hypoxia and neurotoxicity. Taken together, advancements and improved EPR technology will offer a unique and innovative means for determining the role of brain pO₂ and antioxidant imbalances in various brain disorders or diseases. This work was supported in part by grants from the National Institutes of Health [P30GM103400, R01AG031725, R21DA023473 and 8UL1TR000041] and by Dedicated Health Research Funds of the University of New Mexico School of Medicine.
Trityl radicals in perfluorocarbon emulsions as stable, sensitive, and biocompatible oximetry probes

Ilirian Dhimitruka, Yasmin Alsayed Alzarie, Craig Hemann, Alexander Samouilov, and Jay L. Zweier.

Department of Internal Medicine, Davis Heart & Lung Research Institute, College of Medicine, The Ohio State University, Columbus Ohio 43210, USA

jay.zweier@osumc.edu

EPR oximetry with the use of trityl radicals can enable sensitive O$_2$ measurement in biological cells and tissues.$^{1,2}$ However, in vitro cellular and in vivo biological applications are limited by rapid trityl probe degradation or biological clearance and the need to enhance probe O$_2$ sensitivity. We have synthesized novel perfluorocarbon (PFC) emulsions, ~200 nm droplet size, containing esterified perchlorinated triphenyl methyl (PTM) radicals dispersed in physiological aqueous buffers.$^3$ These formulations exhibit excellent EPR signal stability, over 20-fold greater than free PTM probes, with high oxygen sensitivity ~17 mG/mmHg enabling pO$_2$ measurement in the hypoxic region, in aqueous solutions or cell suspensions with sensitivity > 0.5 mmHg. We used these formulations to follow cellular respiration, and the resulting oxygen depletion. Thus, PFC-PTM probes hold great promise to enable combined O$_2$ delivery and sensing as needed to restore or enhance tissue oxygenation in disease.

References


Concurrent treatment of triple-negative breast tumor xenografts with paclitaxel and oxygen supplementation leads to a predictable spike in oxygen therapy, priming the tumor for radiation.

Jesse Mast, Periannan Kuppusamy

Geisel School of Medicine at Dartmouth, Norris Cotton Cancer Center
Jessica.M.Mast.GR@Dartmouth.edu

Locally advanced solid tumors are characterized with dysfunctional vasculature and severe hypoxia, leading to difficulties in delivering therapeutic agents and a decrease in the efficacy of radiation therapy. Hyperoxygenation, either by respiratory administration of enriched oxygen gas or by other pharmacological modalities has been shown to enhance radiation therapy in both animal models of tumor as well as in some clinical cases. The beneficial effect of hyperoxygenation is often utilized in combination with chemotherapy to increase the therapeutic efficacy. A recent clinical study reported that the use of paclitaxel, a common microtubule stabilizing drug, in breast tumors decreased interstitial pressure and increased tumor oxygenation (Taghian et al., 2005). However, it was not known whether this increase in tumor oxygenation by paclitaxel treatment would be therapeutically significant for enhancing radiation therapy. The objective of this study was to predictably manipulate oxygen in a xenograft tumor, and use radiation at a time when oxygen is high. Triple-negative MDA-MD-231 breast tumor xenografts were grown orthotopically in Nod-scid-gamma (NSG) mice. The animals were treated with paclitaxel, 100% oxygen, or both, and LiNe-BuO OxyChips were implanted into the tumors, and oxygen was measured longitudinally using L-band EPR oximetry. Concurrent treatment of MDA tumors with paclitaxel led to a reliable spike in oxygen 48 h after injection of paclitaxel 2 wk after beginning treatment (p = 0.001). X-band EPR oximetry was used to measure the oxygen consumption rate of MDA-MB-231 cells treated with escalating doses of paclitaxel in atmospheric conditions, 1% oxygen, or carbogen for 24 hours. Preliminary data indicates that cells treated with 2uM paclitaxel in carbogen have decreased OCR when compared to cells grown in 1% oxygen and treated with 2uM paclitaxel. Radiation doses were delivered to MDA-MB-231 xenograft tumors at the time of peak oxygenation induced by paclitaxel administration. The results showed a therapeutic advantage of paclitaxel, oxygen, or a combination of both when combined with radiation therapy. An advantage of both oxygen and paclitaxel over one or the other when combined with radiation was not detected. Details of this study and implications for cancer treatment will be presented.


Poster 27
Hemodynamic effects of glutathione-liganded binuclear dinitrosyl iron complex: evidence for nitroxyl generation and modulation by plasma albumin

Taiming Liu¹, Meijuan Zhang¹, Michael H. Terry², Hobe Schroeder³, Sean Wilson³, Gordon Power³, Qian Li⁴, Trent E. Tipple⁴, Dan Borchardt⁵, Arlin Blood¹,³
¹Division of Neonatology, Department of Pediatrics, and ²Department of Respiratory Care, and ³Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda, CA 92354
⁴Neonatal Redox Biology Laboratory, Division of Neonatology, University of Alabama at Birmingham, Birmingham, AL 35294
⁵Department of Chemistry, University of California, Riverside, CA, 92521

Email: tliu@llu.edu; taiming1203@gmail.com

Abstract (235 words): Glutathione-liganded binuclear dinitrosyl iron complex (glut-BDNIC) has been proposed to be a donor of nitric oxide (NO). This study was undertaken to investigate the systemic hemodynamic effects, pharmacokinetics, and mechanisms of vasoactivity of glut-BDNIC. Using isolated ovine mesenteric arterial rings the relaxation caused by glut-BDNIC was eliminated by ODQ (soluble guanylate cyclase inhibitor) and DTT (HNO scavenger). CPTIO, which scavenges both HNO and NO⁻, partially attenuated relaxation by glut-BDNIC and an HNO donor, but more fully attenuated dilation by an NO⁻ donor. SOD, which converts HNO to NO⁻, facilitated the attenuating effects of CPTIO on glut-BDNIC- and HNO donor-mediated relaxation in a similar manner. Electron paramagnetic resonance identified BDNIC as a HNO donor. In contrast to S-nitroso-glutathione (GSNO), which was vasodilative both in vitro and in vivo, low doses of BDNIC lost hypotensive effects in both rats and sheep. Wire myography showed that plasma albumin contributed to this loss of hypotensive effects, possibly by a reaction involving the cysteine-thiol residue. High doses of glut-BDNIC caused long-lasting hypotension in rats. In vivo, glut-BDNIC was rapidly and completely converted into mononuclear DNIC-like metabolites with a half-life of ~44 min. Plasma albumin played an important role in the conversion and binding of BDNIC. This study suggests that glut-BDNIC is an HNO donor and generates little, if any, NO⁻ or NO⁺. Plasma albumin lessens but prolongs glut-BDNIC-mediated vasodilation, possibly by stabilizing NO moieties in the Fe(NO)₂ cores.
Immune complex augments NO production by pro-inflammatory macrophages

Murugesan Velayutham¹, Arturo J. Cardounel¹, and Shanmugam Nagarajan¹,²

¹University of Pittsburgh School of Medicine, 450 Technology Drive, Pittsburgh, PA 15219, USA.
²University of North Carolina, 160 N. Medical Drive, Chapel Hill, NC 27599, USA.

Email: velayuthamm@upmc.edu/mvelayutham@hotmail.com

Atherosclerosis is a chronic inflammatory disease, and a leading cause of morbidity and mortality in the world. Oxidized low-density lipoprotein, oxLDL, uptake by macrophages resulting in the formation of foam cells in the arterial intima is a hallmark of early event in the development of atherosclerosis. Accelerated atherosclerosis is common in patients with autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). The molecular mechanisms that contribute to early onset of atherosclerosis in patients with SLE or RA are not well defined. The level of circulating immune complexes is increased in SLE and RA. We hypothesize that immune complex will prime macrophage to become pro-inflammatory. We tested this hypothesis using bone marrow derived macrophages exposed to immune complexes and determined the NO generation and inflammatory cytokine response. Quantitative RT-PCR analyses showed LPS induced Nos2 expression. Interestingly, immune complexes augmented LPS-induced Nos2 expression. The electron paramagnetic resonance (EPR) spin trapping study using Fe-(MGD)₂ shows that NO productions by LPS-activated macrophages were increased. The NO production of LPS-activated macrophages was further increased in the presence of immune complexes. Notably, immune complexes also augmented mRNA and protein expression of pro-inflammatory cytokines including MCP-1 and RANTES. Our findings suggest that the pro-inflammatory responses augmented by immune complexes contribute to the progression of accelerated atherosclerosis in patients with autoimmune diseases.

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Dual effect of nitric oxide on phenoloxidase-mediated melanization

Urikhan Sanzhaeva1,*, Yana Vorontsova2, Yuriy Glazachev1, Irina Slepneva1

1Institute of Chemical Kinetics and Combustion, Siberian Branch of the Russian Academy of Sciences, Institutskaya Str. 3, Novosibirsk 630090, Russia
2Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences, Frunze Str. 11, Novosibirsk, 630091, Russia

Urikhan.Sanzhaeva@gmail.com

Melanization is the process of formation of natural polymer melanin, which is widespread in living organisms. The most important function of this process is the protective activity. In invertebrates, the melanization underlies the immune response. The penetration of pathogen into an organism initiates the melanization cascade which finally results in the formation of a melanin capsule around the pathogen. Phenoloxidase (PO) is the key enzyme of the melanization process. The active site of PO contains a coupled binuclear copper similar to the active site of haemocyanin, the arthropod’s oxygen carrier protein.

Nitric oxide (NO) is considered an important signaling molecule, playing a role in a variety of biological processes. Some investigations report that NO manifests a high affinity for copper-containing enzymes and proteins, such as hemocyanin, laccase, and cytochrome c.

The aim of our work was to understand how interaction of NO with PO affects its enzyme activity and melanization velocity.

The study has demonstrated a dual effect of nitric oxide on phenoloxidase (PO)-mediated DOPA oxidation and melanization process [1]. NO generated at low rates proportionally increased in PO-mediated DOPA oxidation. Competitive PO inhibitor, phenylthiourea, resulted in significant inhibition of DOPA oxidation in this system. Further analysis using fluorescent and EPR methods demonstrated that the effect of NO on DOPA oxidation is explained by oxidation of NO to NO2 at the active site of PO followed by oxidation of DOPA by NO2. On the contrary, the bolus addition of NO gas solution resulted in a significant decrease in observed PO activity. Similar dose-dependent effect of NO was observed for the insect’s haemocytes quantified as percentage of melanized cells after treatment with nitric oxide. In conclusion, the results of the study suggest that NO may have a significant regulatory role on melanization process in invertebrates as well as in human and result in protective or damaging effects.


*Current institution - West Virginia University, 1 Medical Center Drive, Morgantown, WV 26506, United States
Effects of ozone preconditioning on isolated hearts from diabetic rats followed by EPR

Stephany N Arellano Ahumada¹, Sindy J Olvera Vazquez¹, Yasmi Reyes Ortega², Robert D Kross³, Daniel Ramírez Rosales¹, Cleva Villanueva López⁴

²Instituto de Ciencias, Centro de Química, BUAP, Edif 103 H, Complejo de Ciencias, CU, Col. San Manuel, Puebla, C.P. 72570, México.
³Krosslink Laboratories, Bellmore, USA

torchynsnat@yahoo.com.mx

Obesity and diabetes are important health issues worldwide. Oxidative preconditioning has been used to decrease oxidative damage in diabetes. Repeated Ozone (O₃) exposure is used to produce preconditioning. O₃ exposure gives up systemic oxidative stress. Decreased nitric oxide (NO) production has been related to the development of diabetic angiopathy. The spin trapping iron-diethyldithiocarbamate (Fe-DETC) is used to produce mononitrosyl iron complexes (MNIC, NO adducts) detected by spectroscopy technique EPR. The goal of this study was to assess the effects of O₃ preconditioning on NO production by measuring MNIC on the hearts of type I diabetic rats (streptozotocin model).

Preconditioning was produced by exposing the animals to 0.5 ppm of O₃ (4 h a day for 8 weeks). At the end of the experiments DETC was administered to diabetic rats with or without preconditioning. Animals were sacrificed by anesthesia overdose. The hearts were isolated to carry out EPR spectra at 77 K. It was not possible to distinguish the characteristic MNIC EPR spectra in any sample. However, it was detected a mixed signal corresponding to Cu–DETC and MNIC spectra. A weak NO adduct signal was detected when the Cu–DETC signal was subtracted. The NO adduct signal was significantly higher (around 24 times) in diabetic rats subjected to O₃ preconditioning compared to the animals that did not receive such treatment. It is concluded that O₃ preconditioning increases heart NO production in diabetic animals, which could be dangerous since large quantities of NO have been related to heart failure in other pathological conditions.
EPR Investigation on Structured Fluids

Fengdan Zhao, Alexander M. Brugh, Marelic Danilczuk, Malcolm D. E. Forbes

Center for Photochemical Sciences, Bowling Green State University, Bowling Green, OH 43403

Abstract:

Structured fluids, complex mixtures containing interacting dispersed materials, using silica-based aerosils have been explored by electron paramagnetic resonance (EPR) in order to better understand fluid dynamics on the micro scale. Simulations conducted on steady state EPR spectra of solutions using the spin probe TEMPONE revealed that the rotational correlation time is very sensitive to the loading of aerosils material. In addition to measurements in the static state, flow experiments were carried out to probe fluid properties under stressed conditions. Using benzophenone as a photosensitizer, transient radicals were created and observed by time resolved EPR to analyze translational diffusion in these fluids.

Acknowledgments:

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Reference:


Synthesis of Metal Organic Frameworks from Push-pull phenylene isophthalates

Authors: Mayokun J. Ayodele, Matthias Zeller and Jeremy Klosterman.
E-mail: mayokua@bgsu.edu

Solid-state lighting devices based on organic chromophores offer a potential escape from an overreliance on inorganic devices based on rare-earth metals.\textsuperscript{1,2} However, close-packing of organic chromophores leads to poor solid-state emission due to non-emissive excimers and H-aggregates. We use metal organic frameworks (MOFs), robust, porous crystalline materials that assemble from organic linkers and metal ions, to control interligand interactions and achieve enhanced solid-state emission. Here we report synthetic progress towards bisisophthalate acid ligands with a substituted phenylene core and preliminary structures of copper-based MOFs showing well spaced ligands in the targeted nbo-topology. The absorption and emission profiles of the isostructural push-pull dye ligands can be tuned by adding methoxy donors on the central phenylene ring to tailor the photophysical properties of the bulk MOF crystals. The CuMOF were also characterized with EPR.

Reference:
Exciting A Younger Generation of EPR Spectroscopists. What You Can Do.

Reef (Philip D., II) Morse, Director,
Steppingstone MAagnetic Resonance Training Center, 30250 Grand River Ave., Farmington Hills, MI, 48336

The Steppingstone MAgetic Resonance Training (SMART) Center provides a template for providing a challenging, welcoming, and authentic scientific experience for middle and high school students using magnetic resonance instrumentation. With 9 years of experience with students ages 10 to 18, the SMART Center is a resource for those willing to take on this important educational function. We can provide mentoring guides, ideas, help in getting started, how to market, pricing structures and other materials.

http://smart-center.steppingstoneschool.org/
Advanced ESR in Tooth Enamel for Retrospective Dosimetry

Lotem Buchbinder,1,2 Hanan Datz2 and Aharon Blank1

1 Schullich Faculty of Chemistry, MR Lab, Technion - Israel institute of technology, Haifa, Israel
2 Radiation Safety Division, Soreq Nuclear Research Center, Yavne, Israel
lotem@technion.ac.il

The study examine radiation-induced paramagnetic defects in the enamel layer of the human tooth using advanced electron spin resonance (ESR) methods, with the ultimate goal of applying these methods in retrospective biodosimetry (RBD). Conducting large-scale triage after major radiological events, such as radiation accidents, nuclear-reactor accidents, or radiological terrorist attacks, requires fast, accurate, and non-invasive methods of dose estimation.1,2 The main challenge for RBD is to quantify exposures to ionizing radiation with doses in the range of 0.5–6 Gy, based only on monitoring their effects on the human body. A well-known method with the potential for meeting this challenge is ESR, which can be used to detect and measure radiation-induced defects—radicals—in the enamel layer of the teeth. The concentration of these defects in teeth is known to be linearly correlated with the dose in the applicable range.3 Despite its great potential, and its proven results when applied to extracted teeth, ESR is still struggling to provide accurate in-vivo readings. This is mainly because all available ESR-based methods rely on quantitative signal measurements of the teeth in order to derive the concentration of radicals, and hence the dose received.4,5 However, such quantitative measurements have an inherent limitation for in-vivo studies, in that the volume of the measured enamel cannot be known a priori; thus it is difficult to correlate well the measured signal with the concentration of radicals, and thus to estimate the dose.

This work concentrate on using advanced pulse ESR techniques in order to measure relaxation times ($T_1$, $T_2$) to better understand the spectroscopic and physical characteristics of these paramagnetic defects, their distribution in the enamel layer, their interaction with the enamel layer, and the interactions between them. All these parameters measured as a function of radiation dose to caratrize the relation between the dose and these parameters. Our hypothesis is that this type of advanced ESR data can be correlated well with the concentration of defects, and thus enable the development of new markers for in-vivo estimation of spin concentration without the need for quantitative measurements of both the ESR signal and enamel volume. The development of a new method of this kind for in-vivo RBD will greatly improve our ability to measure relevant doses (0.5–6 Gy) with greater accuracy and speed, which is critical for medical triage following radiological events.

Reference
In-vivo tooth dosimetry using a deployable L band EPR spectrometer

Minoru Miyake1, Yasuhiro Nakai1, Ichiro Yamaguchi2, Hiroshi Hirata3, Naoki Kunugita2 and Harold M. Swartz4

1 Department of Oral and Maxillofacial Surgery, Faculty of Medicine, Kagawa University, JAPAN
2 Department of Environmental Health, NIPH (National Institute of Public Health), JAPAN
3 EPR group in the Division of Bioengineering and Bioinformatics, Hokkaido University, JAPAN
4 Dartmouth EPR Center, Department of Radiology, The Geisel School of Medicine at Dartmouth, USA

E-mail: dentmm@kms.ac.jp

EPR tooth dosimetry using L-band (1.0-1.2GHz) has several desirable characteristics for screening and providing guidance such as triage following a mass-exposure incident because of recent progress in the instrumental capabilities of in vivo EPR. To test the utility of the developments and help to determine practical factors in the use of EPR dosimetry we carried out in vivo EPR tooth dosimetry at different sites as part of our investigation of the exposure dose of residents near the site of the incident at Fukushima.

MATERIALS AND METHODS
All measurements are performed using surface loop resonators that have been specifically designed for EPR measurements of the upper incisor teeth. 40 volunteers were studied. The measurements were made at both in our lab and in the vicinity of Fukushima. These measurements were made using the in vivo EPR tooth dosimeter developed by the EPR Center for the Study of Viable Systems, Geisel School of Medicine at Dartmouth.

RESULTS
The EPR signals from the upper incisor of volunteers were successfully measured at the two types of sites. These signals included not only RI’S (Radiation Induced Signals) but also background signals. Using post-measurement questionnaires, we ascertained that the volunteers comfortable during the measurements and appreciated the fact that they were able to obtain immediate feedback on the results. Analysis of the noise levels of the spectra indicated that the quality of the spectra was moderately impacted by the environment. Preliminary analysis indicates that a major component of this variation was the impact of the commercial source of electrical power. We also found that important sources of noise were due to improper positioning of the loop of the resonator on the surface of the tooth and motion during the measurement, resulting in reduction of the S/N ratio of the signals.

DISCUSSION AND CONCLUSIONS
This trial demonstrated that the EPR tooth dosimeter is capable of being transportable to a distant site and successfully make estimates of radiation exposure. The potential application of tooth EPR dosimetry may include estimating long-term risks/consequences of the residences after a radiation event such as the Fukushima Power Plant Disaster. We did identify a need for further improvements to immobilize the resonator positioning system and the adjustment of the loop location in order to make the system suitable for use by non-expert operators.

REFERENCE

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High-frequency EPR of surface impurities on Nanodiamond

Zaili Peng¹, Susumu Takahashi¹,²
¹Department of Chemistry, University of Southern California
²Department of Physics, University of Southern California
zailpeng@usc.edu

Diamond is a fascinating material, hosting nitrogen-vacancy (NV) defect centers with unique magnetic and optical properties. There have been many reports that suggest the existence of paramagnetic impurities near surface of various kinds of diamonds. Electron paramagnetic resonance (EPR) investigation of mechanically crushed nanodiamonds (NDs) as well as detonation NDs revealed g~2 like signals that are attributed to structural defects and dangling bonds near the diamond surface. In this presentation, we investigate paramagnetic impurities in various sizes of NDs using high-frequency (HF) continuous wave (cw) and pulsed EPR spectroscopy [1]. Strong size dependence on the linewidth of HF cw EPR spectra reveals the existence of paramagnetic impurities in the vicinity of the diamond surface. We also study the size dependence of the spin-lattice and spin-spin relaxation times (T1 and T2) of single substitutional nitrogen defects in NDs Significant deviations from the temperature dependence of the phonon-assisted T1 process were observed in the ND samples, and were attributed to the contribution from the surface impurities.

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Regularization-free and Model-free Determination of Distance Distributions for Pulsed Dipolar Spectroscopy

Madhur Srivastava and Jack H. Freed

1Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY, USA - 14853
2National Biomedical Center for Advanced ESR Technology (ACERT), Cornell University, Ithaca, NY, USA - 14853
3Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY, USA - 14853

Presenting Author: ms2736@cornell.edu

Pulsed Dipolar Spectroscopy (PDS) is a powerful method for studying the structure and function of biological systems. In PDS, a dipolar signal is acquired from the interaction between a pair of spin labels, from which the distance distribution between them, P(r) may be obtained. The P(r) can be in the range of 1 to 10 nm. However, due to the ill-posed nature of the inversion of the dipolar signal to yield the P(r), one must resort to regularization or model fitting methods to obtain reasonable results. The method of Tikhonov regularization (TIKR) is commonly used, but it relies heavily on the choice of regularization parameter (λ) that yields a compromise between good resolution and stability of the P(r). However, this procedure is still vulnerable to the appearance of spurious peaks and negative values for P(r) as well as effects of noise in the dipolar signal. Model fitting methods, on the other hand, require a priori model functions to estimate P(r), which may not accurately represent the actual distance distributions. This is especially true if the P(r) is multimodal. We developed a new and objective approach to this matter which yields accurate distance distributions with high resolution and without any spurious peaks or negative P(r)s. The dipolar signal is first denoised using our recently developed WavPDS method based on wavelet transforms. This denoised signal is then directly converted into the P(r) by our new Singular Value Decomposition (SVD) based method. Its effectiveness is illustrated below and compared with TIKR plus the Maximum Entropy Method (MEM), also on the denoised dipolar signal.

Unimodal Sample:
Initial SNR 3.8
Denoised SNR 488

TIKR

TIKR + MEM

New SVD Method

Bimodal Sample:
Initial SNR 11
Denoised SNR 1046

TIKR

TIKR + MEM

New SVD Method

*Blue: References for TIKR+MEM and for New SVD Method

Segmented-Loop Resonators for Surface EPR Oximetry Measurements


EPR Center for the Study of Viable Systems, Geisel School of Medicine at Dartmouth, 1 Medical Center Drive, Lebanon, NH 03756

Sergey.V.Petryakov@dartmouth.edu

The design of the resonator is crucial for the use of EPR in vivo to achieve sufficient sensitivity and reliability of data from anatomical sites, where involuntary and physiological movements (such as cardiac and respiratory) are expected during a given measurement. Conventional surface L-band resonators are rigidly attached to a static mount, which does not necessarily accommodate well to adapting to physiological motion, and are, therefore, susceptible to motion and/or pressure artifacts. We have developed a new class of lightweight resonators that use soft and flexible coaxial cables that can be attached to the measurement site via clinically accessible fixation rings. This "flexible resonator", therefore, can move with the object without causing any artifacts from movement or pressure. In addition, the new resonators have multiple segments which can contour to a given surface. Resonators that utilize multiple segments/gaps establish an extremely homogeneous field B1 which is necessary for measuring surface and deep tissues. Two versions of the flexible resonator are presented.

1. Resonators with mechanical (trimmer) capacitive coupling

The advantage of a symmetrical multi-segmented resonator with a mechanical capacitive coupling is that it does not need a balun transformer (Hirata et al. J. Magn. Reson. 190: 124-134, 2008)). The coupling technique is based on the partial reflection of the electromagnetic wave in the cable from the variable capacitor, located on some odd integer quarter-wavelength (1/4, 3/4, 5/4, 7/4, etc.) from the resonant circuit. The change in the frequency of the resonator can be achieved by partially reflecting the electromagnetic wave in the cable from an additional variable capacitor located at some distance from the sensor loop, between the loop(s), and the coupling capacitor. This construction yields the design to be quite compact, as there are only the cable, loop(s), and trimmer capacitor in this resonator design. Although the insertion loss in one flexible coaxial cable is higher than that of a rigid coaxial cable (however, rigid coaxial cable can also be used), its flexibility allows for a fuller accommodation to physiological motions, and therefore allows us to collect more data on more anatomical locations with minimal operator input and minimal patient discomfort. The mechanism of electrical (capacitive) coupling, which we use in a flexible resonator, provides a very strong coupling in lossy environments, such as measurements within the mouth, or other in vivo cavities. We have successfully used this flexible resonator to measure oxygen in many anatomical sites, including the intraoral, thoracic, neck and head, where physiological movement is expected. Physiological movement is no longer a problem during measurement with a flexible resonator moving with the object of measurement. This is a very important advantage for practical measurements.

2. Resonators with electronic tuning and coupling

The design of a flexible resonator with adjustable voltage settings and communication capabilities is described in the poster. This added functionality and associated feedback circuitry will function to ensure that the resonator is always critically connected regardless of the sample. This goes towards enabling fully automated operation of the resonator without the need for interaction with the user. The connecting and tuning elements will be controlled by a feedback loop, which is already present in traditional bridges (e.g., Magneffect L-band bridge). Electrical adjustment and communication functionality are possible due to the recent availability of very linear, non-magnetic, electrically controlled variable capacitors (STPTIC) from STMicroelectronics, which allowed us to create practical resonators for in vivo EPR oximetry.

Acknowledgements

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Instrumentation for EPR imaging using fast acquisition.

A. Samouilov, R. Ahmad, J.L Zweier.

The Ohio State University, United States

Alex.Samouilov@osumc.edu

Electron paramagnetic resonance Imaging (EPRI) allows quantitative imaging of many physiological parameters, which provides important information about ischemic syndromes, cancer and other pathologies. For continuous wave EPR, broad application of imaging is limited by speed of acquisition. This is a major limitation for imaging dynamic processes in vivo including tissue redox, where measurements are based on the kinetics of the probe reduction. Here, we present a CW EPR imaging system optimized for the fast scan acquisition suitable for spatial and spectral-spatial measurements. The system is built around an open core spherical solenoid high inductance magnet capable of creating a field of 0.1 T, permitting EPRI in L and S bands. The key for fast acquisition is a separate sweep coil of the main magnetic field: a water-cooled cylindrical coil constructed by Resonance Research Inc. The coil is capable of providing a field sweep of ±3 mT with rise time of 1 ms. The sweep coil has 396 μH inductance and 84 mΩ resistance. Inhomogeneity of the field in a 50 mm diameter spherical volume is less than 0.01%. X and Y cylindrical fast field gradients are multilayer coil sets made from 1.5 mm thick sheet copper and formed around a fiberglass tube with inner diameter of 138 mm. The Z gradient is a 66-turn symmetrically distributed solenoid. The gradient coils have low inductance and low resistance (X coil: 1320 μH/143 mΩ; Y coil: 1550 μH/166 mΩ; Z coil: 760 μH/266 mΩ) and are capable of generating a sustained gradient of 250 mT/m along the X, Y, and Z, and 500 mT/m at 25% duty cycle with linearity of about ±1% in a spherical volume of 80 mm and ±5% in a spherical volume of 140 mm. The rise time from 10–90% is 1 ms. Phantom imaging studies indicate that this instrument supports fast acquisition, and, with a combination of STAR (FASTAR) [1], enables high-fidelity recovery of a volumetric image series, with each volumetric image employing less than 10 s of scan time. This development will enhance the capability of EPR to study fast dynamic processes that cannot be investigated using existing EPR imaging techniques.


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Construction of Double-tuned Resonator for Overhauser MRI

Chihiro Tamura, Tatsuya Naganuma, Kazuhiro Ichikawa

Innovation center for Medical Redox Navigation, Kyushu University, Japan
1-1, Maidashi, Higashi-ku, Fukuoka-shi, Fukuoka, Japan 812-8582

Japan Redox Ltd, Japan
4-29-49-805 Chiyo Hakata-ku, Fukuoka-shi, Fukuoka, Japan, 812-0044

Faculty of Pharmaceutical Sciences, Nagasaki International University, Japan
Huis Ten Bosch, Sasebo, Nagasaki, Japan 859-3298
camura@redoxnavi.med.kyushu-u.ac.jp

Functional MRI using free radical compounds as molecular probes [1] has increasing interests in biomedical researches such as partial oxygen measurements in tumor [2], redox status in acute oxidative diseases [3]. Overhauser enhance OMRI (OMRI) is one of free radical imaging technologies and OMRI resonator consists of ESR excitation coil, NMR transmission and receives coils. This triple configuration needs large resonator area in the OMRI magnet and wastes limited-size of effective volumes for animal placement in OMRI magnet. In this study, a $^1$H-e$^-$ double tuned resonator using single coil element and multiple-tuned circuits was developed for field-cycling OMRI (FC OMRI) application.

FC OMRI system is frequently used for ensuring microwave penetration for e$^-$ excitation into small animal at low magnetic field strength and for better NMR signal to noise ratio at high magnetic field strength. For use of mouse experiments in open-type FC OMRI magnets, which we have been developing, a solenoidal coil element (40 mm i.d., 5 mm width, 0.02mm thickness) was used. A double tuned circuit was prepared for two types of OMRI resonators, i.e., 13.1/132MHz and 12/376MHz for 0.3/0.005 Tesla and 0.3/0.013 Tesla FC OMRI system, respectively. Non-magnetic capacitors (American Technical Ceramics, Johanson Manufacturing) were used. The electric properties were examined using a $^1$H/$^{13}$C double tuned resonator (64.9MHz /16.32MHz ) as reference.

$^1$H-e$^-$ dual-tuned OMRI resonators were prepared for 0.3/0.005 Tesla and 0.3/0.013 Tesla FC OMRI systems. Unloaded Q values for $^1$H and e$^-$ were 70/70.1 and 90/30 for 13.1/132MHz and 12/376MHz systems, respectively. Transmission properties $S_{12}$ for $^1$H and e$^-$ were -46/-21 dB and -38/-24 dB for 13.1/132MHz and 12/376MHz systems, respectively. A reference system, $^1$H/$^{13}$C double tuned resonator (64.9MHz /16.32MHz ) had Q value of 65 and $S_{12}$: -12.1/-49.7 dB. Both resonators showed practically good Q values and electrical properties for animal experiments, though the resonant frequencies were 10 times and 30 times different for $^1$H and e$^-$. The resonators will be applied for FC OMRI measurements of mouse disease models.

**In Vivo EPR Spectrometer/Imager: The New Beginning**

Mark Tseytlin\textsuperscript{a,b,c}, Andrey Bobko\textsuperscript{a,c}, Oxana Tseytlin\textsuperscript{a,c}, Boris Epel\textsuperscript{d}, Timothy Eubank\textsuperscript{e,c}, Valery Khramtsov\textsuperscript{a,c}, Benoit Driesschaert\textsuperscript{e,c}, Martin Poncelet\textsuperscript{e,c}, Eiad Kazkaz\textsuperscript{b}, Priyaankadevi Guggilapu\textsuperscript{b}, Kaylee Flohr\textsuperscript{f}, Artem Gorodetskii,\textsuperscript{a,c} Marieta Gencheva\textsuperscript{a,c}, Kayla Steinberger\textsuperscript{e}, Emily Ellis\textsuperscript{e}, Matt Duespohl\textsuperscript{e}, Christopher Cuff\textsuperscript{e}, Hussien Alahmad\textsuperscript{g}, Alexander Stolin\textsuperscript{h}, Raymond R. Raylman\textsuperscript{h}.

(a) Biochemistry Department, West Virginia University, Morgantown, WV 26506, USA  
(b) Lane Department of Computer Science and Electrical Engineering, West Virginia University, Morgantown, WV 26506, USA  
(c) In Vivo Multifunctional Magnetic Resonance center at Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506, USA  
(d) Center for EPR Imaging In Vivo Physiology, University of Chicago, IL 60637, USA  
(e) Department of Microbiology, Immunology & Cell Biology, West Virginia University School of Medicine, Morgantown, WV 26506, USA  
(f) Department of Chemical and Biomedical Engineering, West Virginia University, Morgantown, WV 26506, USA  
(g) Department Industrial & Management Systems Engineering, West Virginia University, Morgantown, WV 26506, USA  
(h) Department of Radiology, West Virginia University, Morgantown, WV 26506, USA

mark.tseytlin@hsc.wvu.edu

A rapid scan electron paramagnetic resonance (1, 2) (RS EPR) imaging system operating at a frequency range of 650-850 MHz has been custom designed and built in our laboratory. The design is tailored for pre-clinical investigation of \textit{in vivo} tissue measurements of small rodents. An ultra-low noise 750 MHz frequency source drives the clock of an arbitrary waveform generator (AWG) with a 120 MHz bandwidth. Clock frequency is mixed with the output of AWG to produce the required EPR frequencies and generate sweeps for resonator tuning. In-house designed and 3D-printed rapid scan coils produce scans up to 100 kHz with ~40 G peak-to-peak amplitude sufficient for imaging soluble trityl probe and particulate LiPc derivatives. EPR imaging with nitroxides is possible with measure of a single hyperfine line. A more detailed description of the system and recent \textit{in vivo} experiments will be presented. On the software side, a second-generation RS EPR full-scan algorithm has been developed (2) that overcomes the limitations of the first-generation half-scan algorithm.

All conference participants are invited to visit our laboratory and observe the instrument in action during the scheduled tour. If there is a conflict of schedule, a visit can be arranged by contacting Dr. Tseytlin.

References:


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Finite Element Method analysis of the implantable resonator (IR)

Maciej Kmiec¹, Wilson Schreiber¹, Sergey Petryakov¹, Rose M. Caston¹, Huagang Hou¹, Benjamin B. Williams¹, Victoria A. Wood¹, Ann B. Flood¹, Periannan Kuppusamy¹, Harold M. Swartz¹

¹Geisel School of Medicine at Dartmouth, EPR Center, 1 Medical Center Drive, Williamson Bldg, Lebanon, NH 03756
maciej.kmiec@dartmouth.edu

Engineering simulation tools enables researchers to virtually design and test operational performance of the EPR implantable resonators, surface resonators and coupling structures. Parametric models of the implantable resonator (IR) can be easily modified and multidimensional analysis can be automatically performed. Output from simulation software tools like ANSYS HFSS allowed us to understand the design space and introduce amendments to the resonator design. Some simulation scenarios may be impossible to reproduce experimentally.

Results can be displayed in multiple data formats or two, three-dimensional images or animations as well as exported to external analysis tools. This allows engineers to visualize and understand the design performance and interactions with other design structures. Electromagnetic safety of the implantable resonator (IR) was tested using FDA approved Finite Element Method without complex experimental testing. Three-dimensional heterogeneous human head model was created and electromagnetic power density absorbed by the tissue and resulting locally in an increase in temperature was calculated according to the IEEE/IEC 62704 standard.

We were able to use engineering simulations in the design process of the implantable resonator and surface resonators used in in-vivo EPR oximetry measurements. Electromagnetic safety of the EPR detectors used externally and internally was also tested with computer simulation tools. Computer simulation made the design models come alive on the screen.

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Acknowledgements
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Fast backprojection-based reconstruction of spectral-spatial EPR images from projections with the constant sweep of a magnetic field

Denis A. Komarov and Hiroshi Hirata*

Division of Bioengineering and Bioinformatics, Graduate School of Information Science and Technology, Hokkaido University, North 14, West 9, Kita-ku, Sapporo, 060-0814, Japan

dkomarov@ist.hokudai.ac.jp

Spectral-spatial EPR imaging with three spatial and one spectral dimensions requires EPR instrumentation that is capable of rapid data acquisition and a time-efficient reconstruction algorithm. In the present work, we propose using a constant field-sweep for scanning the projections of spectral-spatial EPR images. The application of the constant field-sweep simplifies the requirements for EPR imaging instrumentation and ensures high stability, linearity and reproducibility of magnetic field sweep during fast projection scanning. Simultaneously, a fixed data sampling rate of the constant-sweep projections can benefit reconstruction of EPR images allowing to swap the data between the image and its projections without application of a spectral pseudo-angle, which is traditionally used for spectral-spatial EPR imaging. Here we present a procedure for calculating the projections of a spectral-spatial EPR image and for backprojecting the experimental data collected with the constant sweep of a magnetic field. The proposed approach was applied to the reconstruction of a four-dimensional numerical phantom and to actual spectral-spatial EPR measurements. EPR images can be reconstructed three times faster using constant-sweep projections compared to the traditional approach using the pseudo-angle and projections with a scan range that depends on the applied field gradient. Spectral-spatial EPR imaging with a constant field-sweep for data acquisition does not reduce the signal-to-noise ratio or functional resolution of the resultant images and can be applied together with any common backprojection-based reconstruction algorithm.

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Sequestration of Transferrin-Bound Iron as a Marker of Systemic Response to Lung Trauma. 
Assessment with EPR Spectroscopy.
Nikolai V Gorbunov
Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA
Nikolai.V.Gorbunov@gmail.com

**Introduction:** Impact of air blast overpressure waves, i.e., shock waves (SW) on the body can produce shear force-type trauma to soft tissues along with a barotrauma to gas-filled organs, e.g., lung. Such injuries often have no external signs, are difficult to diagnose, and therefore, are frequently underestimated. Blast lung injuries characterized by bilateral traumatic hemorrhage, which can be complicated by neurogenic and circulatory shock. The following pulmonary impairment is accompanied by exaggerated acute phase responses and aseptic inflammation developing during first hours post-exposure that can be ultimately culminated by acute respiratory distress syndrome (ARDS) and lethal outcomes.

**Hypothesis:** EPR analysis of acute phase sequestration of transferrin-bound iron ([Fe$^{3+}$]TRF) can be employed for predictive assessment of the blast lung trauma.

**Rationale:** Plasma [Fe$^{3+}$]TRF cycling is controlled by the acute phase IL-6 – hepsidin axis. Sequestration of the circulatory iron is required for the cell “proliferative responses“ triggered by major trauma; yet it is considered to be a part neuro-immune defense response to trauma. [Fe$^{3+}$]TRF can be quantitatively analyzed in blood and tissue samples with low-temperature EPR techniques. Assessment of [Fe$^{3+}$]TRF in combination with pro-inflammatory cytokines and granulocytes could provide a ground for development of a surrogate marker of trauma. This report represents: (i) analysis of time-dependent dynamics of [Fe$^{3+}$]TRF in blood in a blast lung model; (ii) fluctuation of granulocyte (PMN) counts and expression of CD11b adhesion molecules in PMNs during the first 24 h following trauma.

**Design, Measurements, and Main Results:** SWs were generated in a laboratory shock-tube. CVF Sprague-Dawley rats were exposed to SWs at peak overpressure of either ~90 or 120 kPa producing various degree lung injury. Exposure to SW led to a significant decrease in the amount of blood [Fe$^{3+}$]TRF. This effect was correlated with the extent of lung injury and developed gradually during the first 24 h. Thus, [Fe$^{3+}$]TRF sequestration occurred as early as 1 h post-impact. At 3 h, the steady state concentration of [Fe$^{3+}$]TRF in blood samples decreased from 19.7±0.6 µM (sham) to 7.5±1.3 µM (trauma). The levels of [Fe$^{3+}$]TRF remained decreased over the entire period of observations. At 3 h post-impact PMN count was 5-fold than in sham-treated animals. These effects were accompanied by an increase in expression of CD11b in PMNs. A massive increase in levels of IL-1, IL-6, MCP-1, and MIP-2 was observed in BAL fluid and blood plasma during 24 h post-exposure.

**Conclusion:** Alterations in EPR signal of the blood [Fe$^{3+}$]TRF reflected acute phase sequestration of the circulatory iron, can serve as surrogate biomarker of systemic aseptic response, and therefore, were proposed for assessment of severity of blast lung trauma.

**Acknowledgments:** This work was sponsored by the Department of the Army Peer Reviewed Medical Research Program #PR033201. The experiments were designed and conducted at the Walter Reed Army Institute of Research, Silver Spring, MD, USA. The WRAIR Institutional Animal Care and Use Committee reviewed and approved all animal procedures.
Modeling peptide-spin label conjugates for targeted imaging of tumors

Violetta Burns, Blake Mertz

C. Eugene Bennett Department of Chemistry, 217 Clark Hall, West Virginia University, Morgantown, WV, 26506 USA

blake.mertz@mail.wvu.edu

The pH-Low Insertion Peptide (pHLIP) has the ability to bind, fold, and insert unidirectionally into cell membranes under acidic conditions [1]. This functionality makes pHLIP a promising tool for applications in targeted drug delivery and diagnostic imaging, due to the fact that several disorders are associated with acidosis (e.g., cancer, ischemia, arthritis). Electron paramagnetic resonance (EPR) spectroscopy has recently been utilized as a technique sensitive to changes in extracellular pH [2,3]. In particular, triarylmethyl radical (TAM) probes have been effective in the measurement of oxygen levels in cells due to their high solubility, non-toxicity, and high sensitivity to oxygen. In addition, TAM probes have high resistance towards oxidizing and reducing agents as well as enhancement of signal-to-noise ratio. We hypothesize that conjugation between pHLIP and TAM probes would lead to enhanced targeting and significant amplification of EPR signals. An essential first step in validating this hypothesis is to determine if the peptide-spin label construct restricts the conformation of TAM when pHLIP is inserted into the cell membrane. In this study, a p1-TAM-D probe was attached to the N-terminus of a pHLIP peptide inserted into a 1-palmitoyl-2-oleoyl-sn-phosphatidyl-choline (POPC) lipid bilayer. Its stability was modeled using molecular dynamics (MD) simulations. Our results show that insertion into the cell membrane does not hinder movement of the spin label, providing compelling evidence for further testing of the pHLIP-TAM conjugate in in vivo studies.

References


Acknowledgments
EPR and Photoconductivity of Mn Doped ZnS Nanocrystals at various Temperature

1Atul K. Gupta, 2R. Kripal, 3K.K. Tiwari
1,2EPR Laboratory, Department of Physics, University of Allahabad, India-211002
3Allahabad Degree college University of Allahabad, India-211009
atulkumar.physics@rediffmail.com; ram_kripal2001@rediffmail.com;

Abstract:

X-ray diffraction (XRD), Ultraviolet-visible (UV-vis), Photoluminescence (PL) and Electron paramagnetic resonance (EPR) spectroscopy techniques are used for structural analysis of Mn doped ZnS nanoparticles at different temperatures. XRD analysis confirms the nanostructure of the sample with 5-15 nm of average crystallite size. As temperature increases size of the particles are also increases. UV-vis absorption spectra show blue shift as compared to bulk ZnS. EPR shows the existence of Mn$^{2+}$ with different local structures in ZnS nanoparticles. The values of spectroscopic splitting factor (g) and hyperfine interaction constant (A) decrease as Mn$^{2+}$ concentration increases in ZnS nanoparticles as well as increase in temperature. The photoconductivity behavior of Mn doped ZnS nanoparticles are also studied at different temperatures. It is observed that on increasing the temperature of the samples, photoconductivity increases.
ABSTRACT

Altered metabolism of N-acetyl-aspartyl-glutamate in the cingulated cortices in autism spectrum disorders—A magnetic resonance spectroscopy study

Jiménez-Espinoza C.1,2, Marcano F. 2, Padilla N4, Ádén U4,5, González-Mora J.L.1,2,3

1Physiology Department, School of medicine. Basic Medical Sciences Department. Health Sciences Campus. 2Neurochemistry and Neuroimaging laboratory. 3Magnetic Resonance Center IMETISA, Univ. Hospital, Universidad de La Laguna, Tenerife, Canary I., Spain. 4Department of Women's and Children's Health. 5Department of Neonatology, Karolinska University Hospital, Stockholm, Sweden.

Background. The neuropeptide N-acetyl-aspartyl-glutamate (NAAG) modulates glutamate release in a cycle reaction together with NAA, which requires the participation of neurons, oligodendrocytes, and astrocytes. Our previous studies using proton-Magnetic Resonance Spectroscopy (1H-MRS) in bilateral anterior (ACC) and posterior cingulate cortex (PCC) have described the altered patterns in adults with ASD.

Aims. To compare NAAG, NAA, and Glu concentrations in the ACC and PCC in adults with ASD and typically developed (control) individuals.

Methods. Single-voxel (1H-MRS) in bilateral ACC and PCC, in 19 adults with a clinical diagnosis of ASD and 41 controls, matched for age, gender. Autism quotients (AQ) were assessed. One-way ANOVA and Bonferroni’s correction were applied.

Results. Significant differences in ACC between ASD and TD, where glutamate was significantly increased (12.10 ± 3.92) mM; (* P = 0.02) and the (NAAG) decreased (0.41 ± 0.27) mM; (*p = 0.02) in the group with ASD, suggesting a metabolic deregulation of the NAAG system in the ACC, contrary to the PCC where no significant differences were observed.

Conclusions. This deregulation of the NAAG and consequently of its entire metabolic pathway (ie, all that it entails, since the amino acid NAA, the excitatory-inhibitory neurotransmitter Glu, and the neuropeptide-neuromodulator NAAG, in addition to the enzymes), appears as a possible cause of the neurochemical imbalance present in autism spectrum disorders, which could be the origin of the hypofunction of the salience networks, the DMN, and the temporal, visual and motor fronto networks reported by other authors, confirming the functional and neurochemical differences between ACC and PCC in ASD, suggesting new therapeutic avenues.

Referens

