New Trityl Probes and their Use for Measurement and Imaging of Cellular Oxygenation and Redox State

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Outline

- History of trityl radicals
- Trityl properties and use for EPRI and OMRI
- Examples of trityl applications
- New trityl probes
  - Esterified trityls as intracellular oxygen probes
  - Dendritic trityls for enhanced stability
  - Trityl-nitroxide biradicals as redox probes
  - Trityl-biradicals as thiol probes
What is trityl radical?

Triphenylmethyl radical

Tetrathiatriarylmethyl radical (TAM)


1990’s Nycomed
TAM (tetraphia-TriarylMethyl Radical)-
a novel single line EPR label for
high resolution EPR imaging

water soluble, stable, single sharp line – ideal for EPRI

NYCOMED INNOVATION (AMERSHAM HEALTH, GEMS)

MRM, 37, 479-483, 1997
Why are trityl radicals needed?

<table>
<thead>
<tr>
<th>Trityl</th>
<th>Nitroxide</th>
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<tbody>
<tr>
<td>Sharp EPR Singlet</td>
<td>Moderately broad EPR triplet;</td>
</tr>
<tr>
<td>Biostability: relatively stable - hours</td>
<td>Biostability: easily reduced</td>
</tr>
<tr>
<td>EPR resolution: high, LW &lt; 100 mG</td>
<td>EPR resolution: relatively low</td>
</tr>
<tr>
<td>Oxygen sensitivity: High</td>
<td>Oxygen sensitivity: relatively low;</td>
</tr>
<tr>
<td>Main uses for EPR, EPR oximetry and Overhauser-enhanced MRI.</td>
<td>Multiple use as redox status, pH and ROS probes as well as spin labeling agents and antioxidant, etc</td>
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</table>
Trityl Radical Probes Greatly Facilitate EPRI and OMRI (PEDRI)

- For EPRI- much greater sensitivity, no hyperfine artifacts, no need for hyperfine correction, lower gradient needed for a given image resolution
- For OMRI much less EPR power required, much less sample heating
- Thus, these probes greatly facilitate these techniques
TAM Probes Enable High EPR Image Resolution and High Fidelity Images – *enable microscopy on macroscopic samples*

Right, EPRI showing resolution of 100 μm capillaries containing OX063; Left, 3D EPRI of a phantom.

TAM Probes Greatly Facilitate EPR / NMR Image Registration

Photo, MRI and EPRI of Three Markers Filled with 0.5 mM TAM in PBS

MRM, 47, 571-578, 2002
EPR / NMR Image Registration

Photo, MRI and EPRI of Four Micro Centrifuge Tubes with Two Filled with Water and two filled with 0.5 mM TAM

*MRM, 47, 571-578, 2002*
Co-Registration of EPR Images of a Living Mouse

(load with 0.6 cc of 100 mM 3-CP IV)

MRM, 47, 571-578, 2002
Hybrid Instrument for EPR/NMR Coimaging

• Is there a way to do EPR and NMR imaging without moving the animal?

• If so, can we have a system in which the both sets of images are intrinsically registered?

• Since NMR and EPR based MRI both use a magnetic field system and field gradients, can we use the same magnet and gradients for both and thus have an intrinsically common coordinate frame?
Hybrid EPR/NMR Co-imaging system

CW EPR at 1.1 GHz,  
~ 400 G

Proton MRI at 16.2 MHz,  
~3800 G

Same magnet and gradient system

I. Iron core; II. Main magnet coils; III. Gradient coil assembly; IV. Movable support frame; V. Resonators assembly

block diagram of the co-imaging system.

M. Modulation coils; E. EPRI Resonator; N. Proton NMR Resonator
Diagram of the assembly with movable EPR and NMR resonators

I. Side walls of the assembly with tongues; II. Support tube for sample holder; III. EPR Resonator (inside sliding case); IV. Sample holder (shown with “sample” in place); V. NMR Resonator (inside sliding case); VI. Sample holder support tube.

Phantom details
20 mm long clusters capillary tubes containing 1mM TAM
   A, 1.3 mm ID capillary tubes
   B, 0.9 mm ID capillary tubes
   C, 0.5 mm ID capillary tubes
D, 2 tubes of 6 mm ID with TAM surrounding voids from packed 0.4 mm OD rods tubes
E, 1 tube of 6 mm ID with TAM surrounding voids from packed 0.3 mm OD rods
The central 3 large tubes were arranged into a triangular shaped pattern, and all were packed into an 18 mm ID plastic cylinder.
“Fusing” of the proton MR, EPR images from TAM phantom and live mouse fed with paramagnetic charcoal.
3D renderings of the proton MR, EPR and fused images from a live mouse fed with paramagnetic charcoal.

Layout of the dual mode EPR/NMR resonator assembly

A, 19 mm wide capacitive element
B, 7 mm wide transmission line element
C, Connection plates
D, Resonator body
E, Through hole
F, Slot, 1.15 mm depth
G, Coaxial cable, 1 mm diameter
H, 22 mm cylindrical bore for the sample
I, Conductive short element (EPR side)
K, 800 pF capacitor connection, and NMR feed connection (MRI side)
L, 0.2 pF capacitor connection to coaxial cable (EPR side)

TAM Probes Enable High Quality Measurements in Physiological Samples and Living Tissues

Isolated rat heart perfused with 1 mM TAM

Top row NMR, middle EPR, bottom the superimposed images for each view. Additionally, D shows a 3D surface rendering of the MRI, EPRI signal matrix and the corresponding superimposed matrix.

Accelerating EPR Imaging Using Spinning Magnetic Field Gradient

- Hardware Implementation at 300 MHz & L-band

- Controllable field up to 0.117 T
- Homogeneity of 3 ppm over a SVD 10 cm

<table>
<thead>
<tr>
<th></th>
<th>R (Ω)</th>
<th>I (mH)</th>
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<tbody>
<tr>
<td>X</td>
<td>0.143</td>
<td>1.32</td>
</tr>
<tr>
<td>Y</td>
<td>0.166</td>
<td>1.55</td>
</tr>
<tr>
<td>Z</td>
<td>0.266</td>
<td>0.76</td>
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Accelerating EPR Imaging Using Spinning Magnetic Field Gradient

- 2-D Imaging Results of TAM at L-band

<table>
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<tr>
<th>Regular</th>
<th>Spinning</th>
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<tbody>
<tr>
<td>4x1, 11 s</td>
<td>24 Hz, 411x1, 5 s</td>
</tr>
<tr>
<td>8x1, 22 s</td>
<td>12 Hz, 821x1, 11 s</td>
</tr>
<tr>
<td>12x1, 33 s</td>
<td>6 Hz, 1639x1, 21 s</td>
</tr>
<tr>
<td>16x1, 44 s</td>
<td>3 Hz, 3277x1, 42 s</td>
</tr>
</tbody>
</table>

Imaging parameters
- CF = 387.3 G, SW = 16 G, Linewidth = 0.2 G, DA = 1024 (128), FOV = 40 x 40 mm²
- MA = 0.2 G, TM = 2.6 s, TC = 10 ms (0.64 ms), Image size = 128 x 128, Gradient = 4 G/cm
Accelerating EPR Imaging Using Spinning Magnetic Field Gradient

- 3-D Imaging Results of TAM at L-band

### Imaging parameters

- CF = 387.3 G, SW = 16 G, Linewidth = 0.2 G, DA = 1024 (128), FOV = 40x40x40 mm³
- MA = 0.2 G, TM = 2.6 s, TC = 10 ms (0.64 ms), Image size = 64x64x64, Gradient = 4 G/cm
OMRI of the \textit{in vivo} distribution and clearance of a triaryl methyl (TAM) radical in mice

Time-dependent EPR on and off PEDRI images of a living mouse intravenously injected with TAM PBS solution: (a) EPR off, (b–o) 3000 mW EPR irradiation power.

The profiles of the time-dependent enhancement factor in different organs of the living mouse loaded with TAM saline solution. 

a: The contouring of the PEDRI image composed from the addition of all the image slices from Fig. 3. 

b: Graph of the clearance of TAM in different organs of the living mouse. 

c: Maximum enhancement factor at different organs in the mouse. In c the enhancement factor in the ROIs of the image presented in a was measured in each pixel and the average values are shown with SD bars. SD values shown are for at least 30 pixels.
Need For Trityl Probe Development

• Original molecules like OX63 do not enter cells
• Need to target the probes to enter cells and subcellular compartments
• Need to maintain O$_2$ sensitivity
• Need to enhance stability and minimize toxicity
• Need to impart other functional properties, such as redox sensing, pH sensing, thiol sensing
Esterified Trityl Radicals for Intracellular Targeting

The need for intracellular measurements
Synthesis of Esterified Trityl Radicals

CAT-03 (86%) → CF₃COOH

CH₃I KOH → MT-03 (93%)

BrCH₂OCOCH₃ K₂CO₃ → AMT-03 (39%)

BF₃·Et₂O SnCl₂ → BT-03 (93.4%)

Methyl-ester

Acetoxymethoxy-ester (3)

Acetoxymethoxy-ester (2)
Oxygen sensitivity of Esterified trityl radicals

CT-03  R = CH₃, R₁ = R₂ = R₃ = Na
Ox063  R = (CH₂)₂OH, R₁ = R₂ = R₃ = Na
MT-03  R = CH₃, R₁ = R₂ = R₃ = CH₃
BT-03  R = CH₃, R₁ = R₂ = R₃ = tert-Butyl
AMT-03 R = CH₃, R₁ = R₂ = R₃ = CH₂OC(O)CH₃
AMT-02 R = CH₃, R₁ = R₂ = CH₂OC(O)CH₃, R₃ = H

Intracellular EPR oximetry using esterified trityl radicals

Hydrolysis of AMT-02 by PLE

(A) UV-vis spectra of 10 μM AMT-02 in PBS with 5% DMSO incubated over 0-60 min with PLE (50 U/mL) at 37 °C; (B) EPR spectra showing hydrolysis of the ester-derivatized trityl radicals (10 μM) with PLE (50 U/mL) at 37 °C in PBS containing 5% DMSO at various incubation times. (a) AMT-02, 0 min; (b) AMT-02, 15 min; (c) AMT-02, 45 min;

Intracellular hydrolysis of AMT-02

(Left) EPR spectra of AMT-02 in BAECs at various incubation times; (Right) Relative EPR signal intensity as a function of incubation time in BAECs.

Measurement of Intracellular Oxygen Consumption Using the Trapped Trityl Radical

(A) Plots of \(O_2\) concentration as a function of time in the presence of BAECs (8×10^6 cells/ml) alone (■), with addition of menadione (25 \(\mu\)M) (●), or KCN (100 \(\mu\)M) (▲) using AMT-02. (B) \(O_2\) consumption rates of the cells. Values are means±SD of three experiments.

Development of highly stable dendritic trityl radicals
Molecular structure of the Trityl PAMAM dendrimers

CT-03

DTR1

DTR2

Synthesis of the Trityl PAMAM dendrimers

Reagents and conditions. (a) (COCl)₂, CH₂Cl₂, 2h; (b) ethylenediamine, CH₂Cl₂, overnight, 47% (two steps); (c) methyl acrylate, MeOH, 3 days, 86%; (d) ethylenediamine, MeOH, 7 days, 78%; (e) methyl acrylate, MeOH, 5 days, 84%; (f) LiOH, MeOH, 3h, quantitative.
Dendritic trityls have high stability

Cyclic voltammograms of CT-03, DTR1 and DTR2 in PBS buffer (0.1 M, pH 7.4) containing 0.1 M NaCl; scan rate, 50 mV/s.

Table 1. Percentage of CT-03, DTR1 and DTR2 remaining after exposure to various reactive species in PBS buffer.*

<table>
<thead>
<tr>
<th>Trityl</th>
<th>Asc</th>
<th>GSH</th>
<th>ROO•</th>
<th>O₂•</th>
<th>‘CH₃</th>
<th>HO•</th>
<th>H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-03</td>
<td>96.7</td>
<td>94.6</td>
<td>59.7</td>
<td>55.2</td>
<td>86.5</td>
<td>90.5</td>
<td>97.1</td>
</tr>
<tr>
<td>DTR1</td>
<td>99.2</td>
<td>100</td>
<td>99.4</td>
<td>97.8</td>
<td>96.6</td>
<td>99.7</td>
<td>98.8</td>
</tr>
<tr>
<td>DTR2</td>
<td>99.3</td>
<td>99.9</td>
<td>99.7</td>
<td>99.5</td>
<td>99.7</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*EPR spectra were recorded 30 min after the radical production was initiated in the presence of trityl (10 μM) in PBS. The percentage was obtained by dividing spectral intensity of each sample by that of the control consisting of the trityl alone. Each value was the average of three replicate measurements.

Dendritic trityls have improved water solubility

Acidic titration of DTR2 (Left) and CT-03 (Right) as detected by EPR and UV-vis spectroscopies. Spectra were recorded after incremental addition of HCl solution (1 M) to the TAM radical solution (20 μM) in PBS buffer (20 mM).
Oxygen sensitivity

The Linewidth (mG)

Percent Oxygen

DTR1
DTR2
CT-03

DTR2-Cu$^{2+}$ complex as pH probe

pH dependence of the EPR spectra (A) and signal intensity (B) of the complex of DTR2 (10 μM) with Cu$^{2+}$ (25μM).

Novel trityl-nitrooxide biradicals as unique probes for the simultaneous measurement of redox status and oxygenation
Traditional principle of EPR redoximetry

Disadvantages:
1. A spin quenching mechanism;
2. Triplet and moderately broad EPR signal of nitroxide;

The sensitivity for the assessment of redox status can be greatly increased by monitoring the trityl signal enhancement.
Trityl-nitroxide Biradicals for Redox Status and Oxygen

Spin-spin interaction

Signal Amplitude: redox status
Linewidth (LW): oxygen concentration

Molecular structures

TN1

TN2

J = 160 G

J = 52 G

Exp.

Sim.

Synthesis of trityl-nitrooxide biradicals

\[ \text{TN1} \]

\[ \text{TN2} \]
Assessment of biradicals as redox probes using ascorbate

EPR spectra obtained by the reaction of TN1 with Asc in various incubation times

Plot of EPR signal Intensity as function of time
Oxygen sensitivity of TN1-H
Oxygen consumption and redox status in fresh rat liver homogenate

(A) Time-dependent EPR spectra obtained by incubating TN1 (50 mM) with fresh rat liver homogenate (1.8 mg protein per mL) in the presence of succinate (50 mM) in a sealed capillary. (B) Variations of EPR signal double integration (circle) and O2 concentration (triangle) with time in the presence (unfilled) or absence (filled) of succinate.

<table>
<thead>
<tr>
<th>Succinate</th>
<th>Initial reduction rate (μmol min⁻¹ mg⁻¹ protein)</th>
<th>Oxygen consumption (nmol min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.5±0.03</td>
<td>1.2±0.03</td>
</tr>
<tr>
<td>+</td>
<td>1.6±0.04</td>
<td>2.0±0.03</td>
</tr>
</tbody>
</table>
Advantages and disadvantages

**Advantages:**
- EPR signal intensity enhancement after bioreduction of biradicals;
- Strong single sharp triplet signal is monitored instead of broad triplet signal of nitroxide;
- The resulting trityl monoradicals have enhanced oxygen sensitivity.

**Disadvantages:**
- Still fast bioreduction of biradicals containing six-membered ring nitroxide;
- Partially overlapped triplet signal from the trityl-hydroxylamine decreases EPR resolution;
- Cellular impermeability
New trityl-nitrooxide biradicals

TNN14, N=\textsuperscript{14}N
TNN15, N=\textsuperscript{15}N

J = 820 G for TNN14 and TNN15
160 G for TN1
52 G for TN2
Synthesis of trityl-nitrooxide biradicals
TNN14 and TNN15

3-CP

HOSu, EDC, HOBT, DIPEA, DMF, 18h

3-CP-OSu

15NH4Cl, Dioxane, K2CO3, 22h

3-CaP

NaOBr, H2O, 0-70 °c, 3h

15NH2

14NH2

4 Steps

CT-03

15NN or 14NN

BOP, HOBT, DIPEA, DMF, 18h

TNN14 or TNN15
Reaction Kinetics of TNN14 with Ascorbate

EPR spectrum obtained by the reaction of TNN14 with Asc

EPR spectra under anaerobic conditions

\[ k_2 = 0.442 \pm 0.017 \, \text{M}^{-1}\text{S}^{-1} \] for TNN14
\[ 4.14 \pm 0.14 \, \text{M}^{-1}\text{S}^{-1} \] for TN1
\[ 3.48 \pm 0.09 \, \text{M}^{-1}\text{S}^{-1} \] for TN2
Oxygen sensitivity of trityl-hydroxylamine

EPR spectra of TNN14-H (20 μM) and TNN15-H (20 μM) in 10%O₂

Plot of $I_{\text{in}}/I_{\text{out}}$ as function of percent oxygen
Cyclodextrin enhances the stability of TNN14

Plot of EPR signal intensity as a function of time; EPR spectra were recorded after addition of ascorbate (4 mM) to the solution containing TNN14 (50 μM) and cyclodextrins (2 mM) in PBS.

Proposed CD conjugated biradicals
New biradicals with various linkers

TNN14

TN1

CT02-GT

CT02-AT

CT02-VT

CT02-PPT
Experimental and simulated EPR spectra

**TNN14**

- J = 1230 G
- ΔJ = 20 G

**CT02-GT**

- J = 433 G
- ΔJ = 10 G

**CT02-VT**

- J = 91 G
- ΔJ = 7.5 G

**TN1**

- J = 110 G
- ΔJ = 17 G

**CT02-AT**

- J = 105 G
- ΔJ = 20 G

**CT02-PPT**

- J = 41.3 G
- ΔJ = 11.4 G
- J' = 3.65 G
- ΔJ' = 4.1 G
Trityl radical-conjugated disulfide biradicals for the measurement of thiol concentrations
Molecular structures

TSSN

TSST
Room-temperature EPR spectra

Experimental and simulated EPR spectra of (A) TSSN and (B) TSST in PBS (pH 7.4, 20 mM) at room temperature
Reaction of biradicals with GSH

(A) EPR spectra obtained by reaction of TSSN (50 μM) with GSH (2 mM) in PBS (50 mM, pH 7.4) at room temperature; EPR signals of (○) trityl monoradical, (■) nitroxide monoradical and (●) the biradical TSSN were observed; (B) EPR spectra obtained by reaction of TSST (50 μM) with GSH (4 mM) in PBS (50 mM, pH 7.4);
Summary and Conclusions

- Trityl probes have great value for EPRI and PEDRI.
- New trityl-derived probes enhance their stability and cellular targeting.
- These new probes enable use for measurement of oxygen, pH, redox state and thiol levels.
- These and other trityl probes enable major advances for EPRI and PEDRI greatly expanding the power and versatility of these techniques.
- Further work is needed. This field is still in its infancy. We have only just begun to see what is possible.
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