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EPR 4 ROS

Applications in biology and medicine

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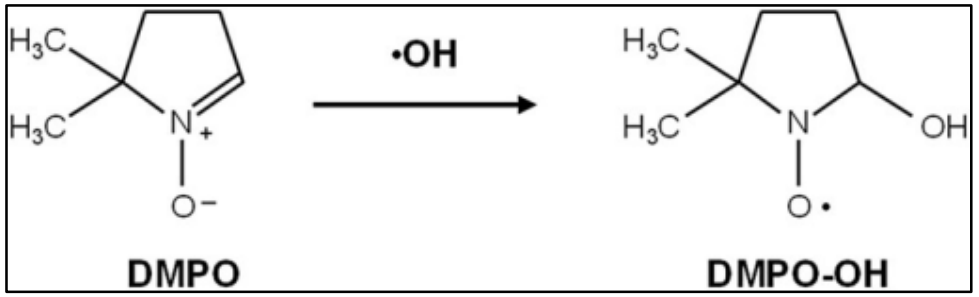
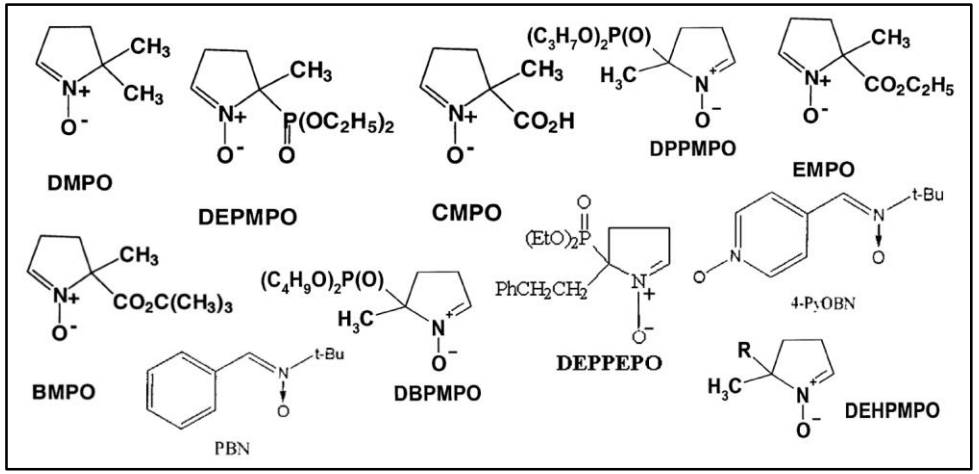
1. EPR in plant research

- It is a great pleasure to study plant systems.
- **Dogma:** Plant plasma membranes are known to produce $^{\bullet}O_2^-$, while the production of $^{\bullet}OH$ occurs only in the cell wall. We used **EPR spin-trapping method.**
- Spin-trapping is a great metod to detect short-lived radicals.
- Good spin-trap can distinguish different radicals, even if produced in the same system.
- Spin-traps for extra/intra cellular radical production.



Spin-trapping principle



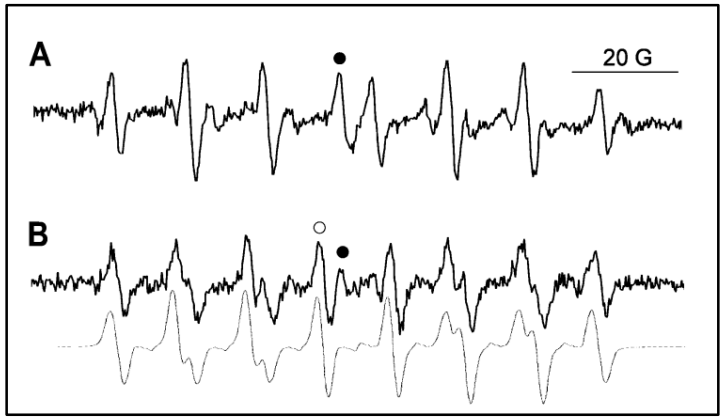
Spin-trapping market

Mojović et. al. Journal of Experimental Botany, 55, 2523–2531 (2004).

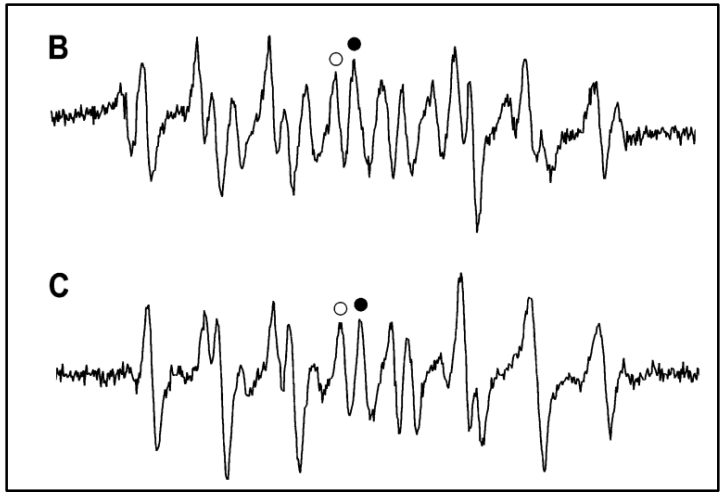
1. EPR in plant research

- Complicated signals, C-C and ST impurities artifacts.
- The best way is to use combined chemical generator systems + computer simulations.
- We showed that isolated plasma membranes from maize roots produce $^{\bullet}OH$ besides $^{\bullet}O_2^-$.
- $^{\bullet}O_2^-$ production goes through an O_2 and DPI sensitive, NADH-dependent mechanism.
- Difficult to use for *in vivo* measurements – fast adduct reduction. Still waiting for the ultimate one.

Mojović et. al. Journal of Experimental Botany, 55, 2523–2531 (2004).



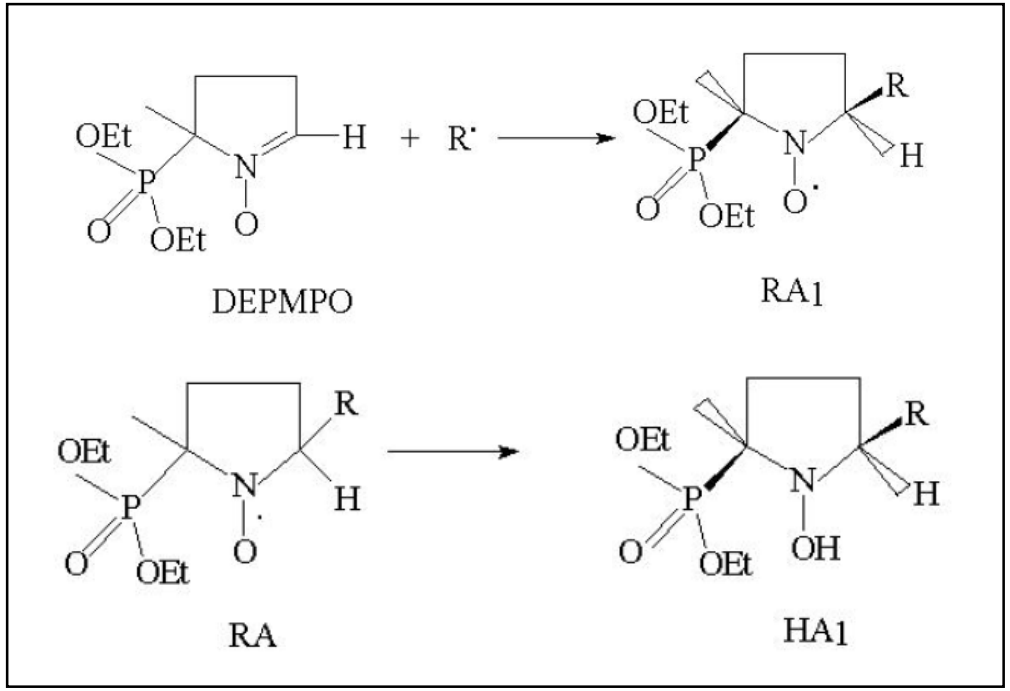
Generator systems & Simulations



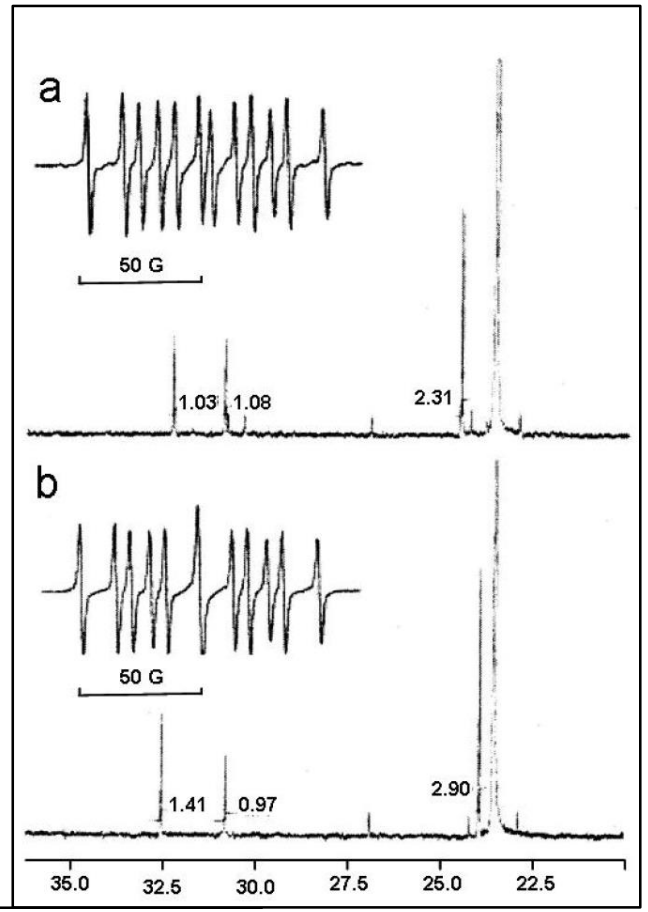
Plant membranes & Simulations

2. NMR spin-trapping

- While waiting, NMR spin-trapping has been proposed.



Spin-trap ↔ Spin-adduct ↔ Hydroxylamine



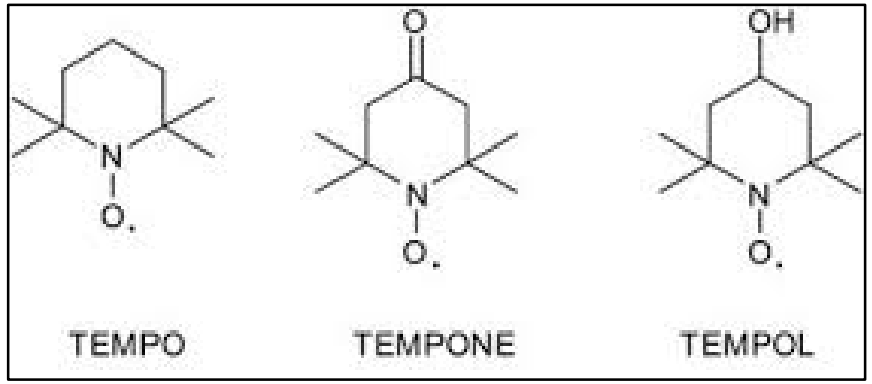
- EPR visible but less stable spin-adduct.
- NMR visible, more stable HA moiety.
- We have peaks at 24.54 ppm, and additional peaks at 30.83 and 32.52 ppm.
- A very small 27.05 ppm peek from DEPMPO/•OH product.

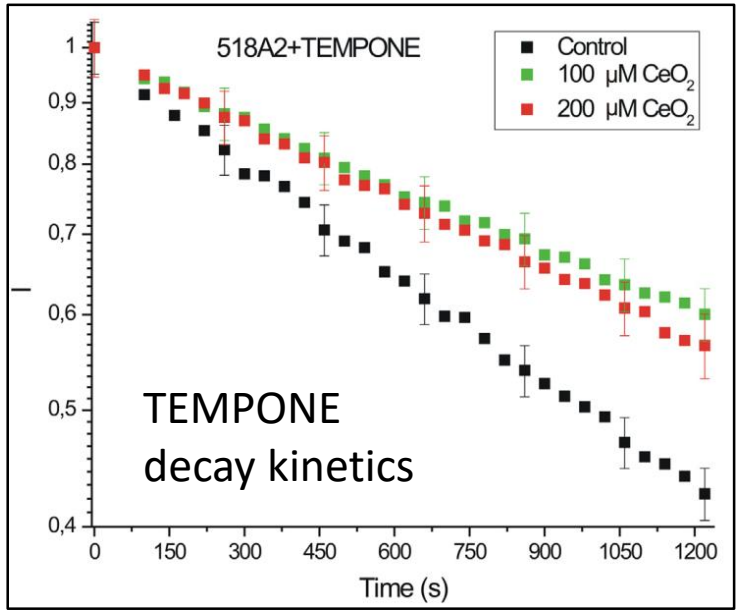
^{31}P NMR specra of DEPMPO/CH_3 (a) and DEPMPO/CH_2OH (b) diamagnetic HA

Beliner et. al. Current Topics in Biophysics, 26, 21-27 (2002).

3. Cancer cell research

- To examine the effect of drugs (cerium oxide nanoparticles CONP) on free radicals produced from melanoma 518A2 and colorectal adenocarcinoma HT-29 cell lines.
- **EPR spin-probing** method using TEMPONE.
- TEMPONE reduction kinetics is directly proportional to the quantity of free radicals produced from the cells.
- Results show the potential of new drugs to damage antioxidant capacity of cancer cells.
- CONP show low inhibitory potential to healthy human cells.





Pesic et. al. Chemico-Biological Interactions 232, 85–93 (2015).

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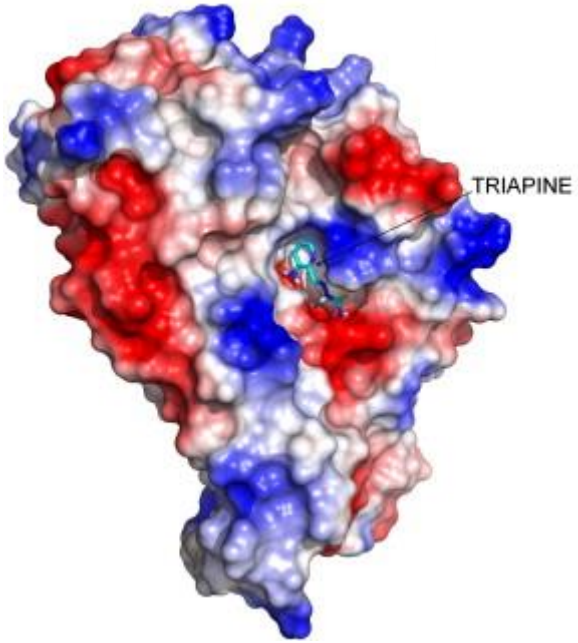
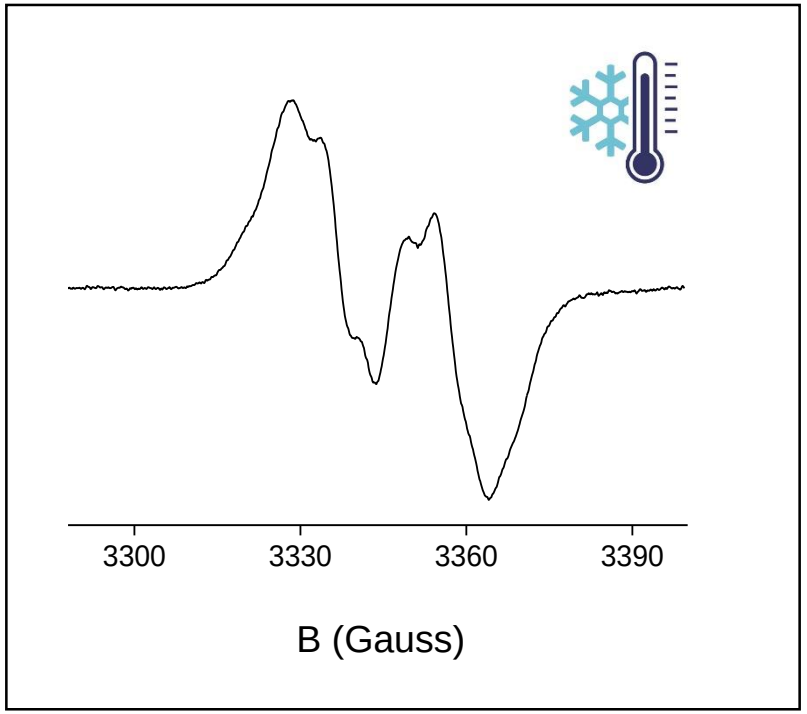
4. Testing efficacy of new potential anticancer drugs

- Ribonucleotide reductase (RNR) as a target for anticancer drugs (Tyrosyl radical).
- Interaction with Triapine results in the labilization of the diferric centre in the R2 protein (Triapine molecules act as iron chelators).
- Formation of the iron(II)-Triapine complex, promotes further reactions with molecular oxygen, which give rise to reactive oxygen species (ROS) and thereby damage the RNR enzyme.
- RNR inhibition = Tyrosyl radical reduction in R2 subunit of RNR.
- In short, if no Tyrosil radical is present, the drug is efficient.



R2 subunit of RNR

Human R2 RNR Tyrosyl radical @30K



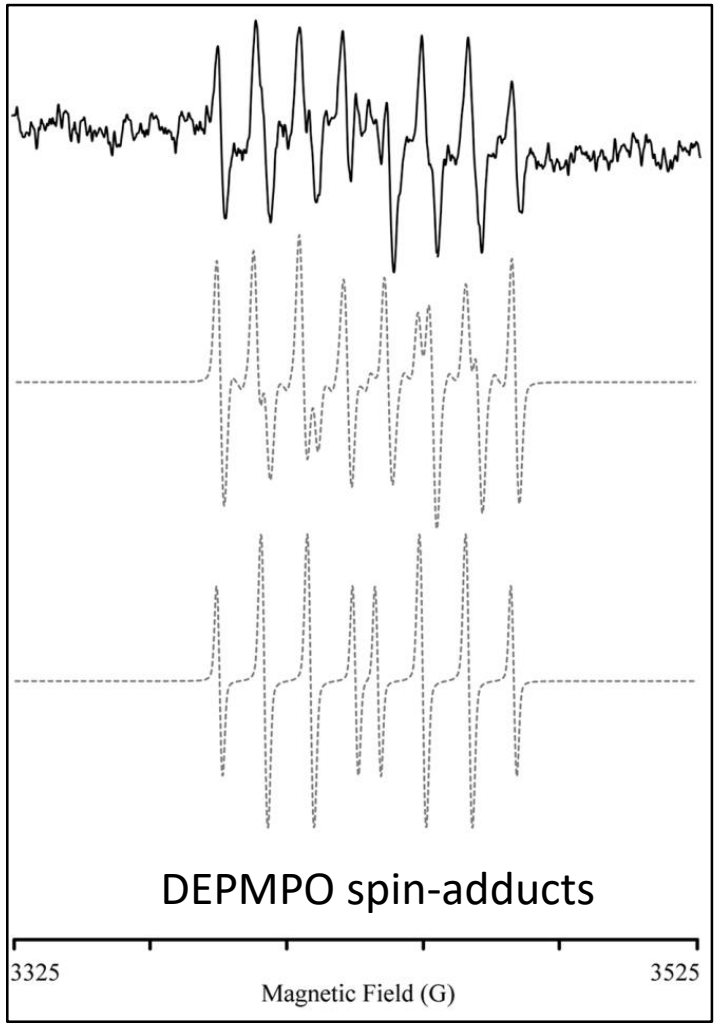
Popovic-Bijelic et.al. J. Inorg. Biochem.105,1422-1431 (2011).

5. ROS and micro-animals

- Anhydrobiosis is an adaptive strategy that allows withstanding almost complete body water loss.
- The loss of water during anhydrobiosis leads to oxidative stress.
- To date, the metabolism of free radicals in tardigrades remained unclear.

Tardigrade *(Paramacrobiotus richtersi)*

- We showed that hydrated specimens of the tardigrade produce pure $^{\bullet}O_2^-$ radical.

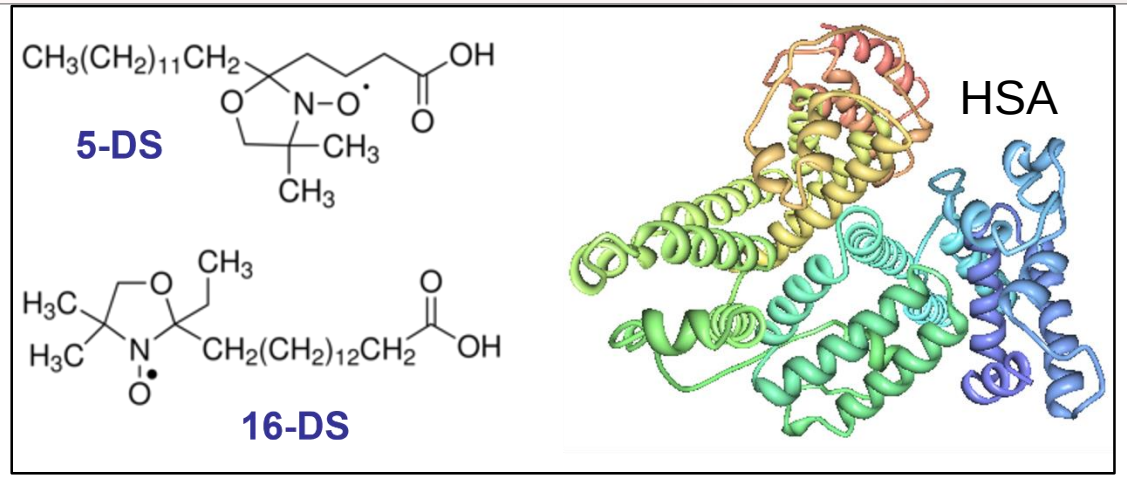


DEPMPO spin-adducts

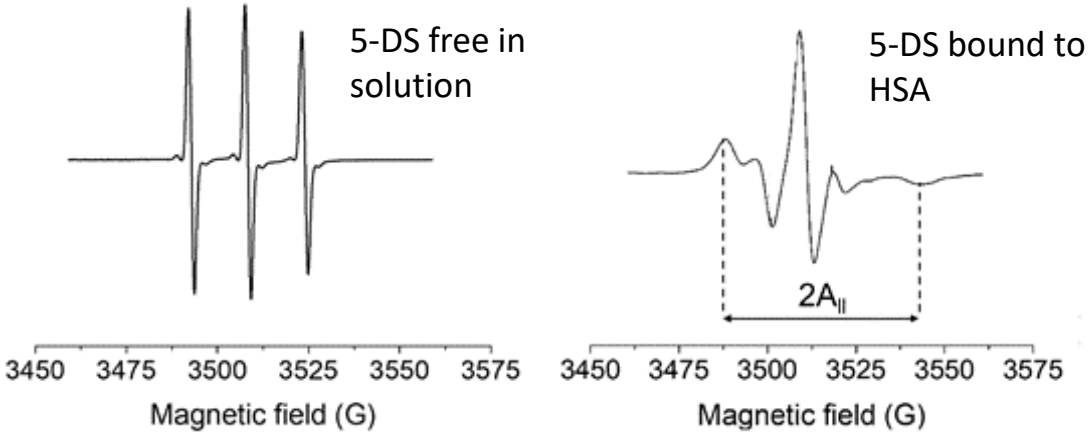
Mojovic et. al. Physiol Biochem Zool. 88, 451-454 (2015).

6. Spin-labeling of HSA as a biomarker technique

- Proteins secreted from cancer cells, bind to HSA modifying its structure.
- HSA changes its binding capacity for fatty acids.
- These conformational changes can be studied by the **EPR spin-labeling method.**
- Spin-labeled fatty acids binds to the HSA.
- Anisotropic rotational motion effects.
- From the EPR spectra we can resolve the amount of bounded FA and potential oxidative damage.



Spin-labes have nitroxide groups attached at different positions on the fatty acid chain



Pavicevic et. al. J. Phys. Chem. B, 10898−10905, 118 (2014).

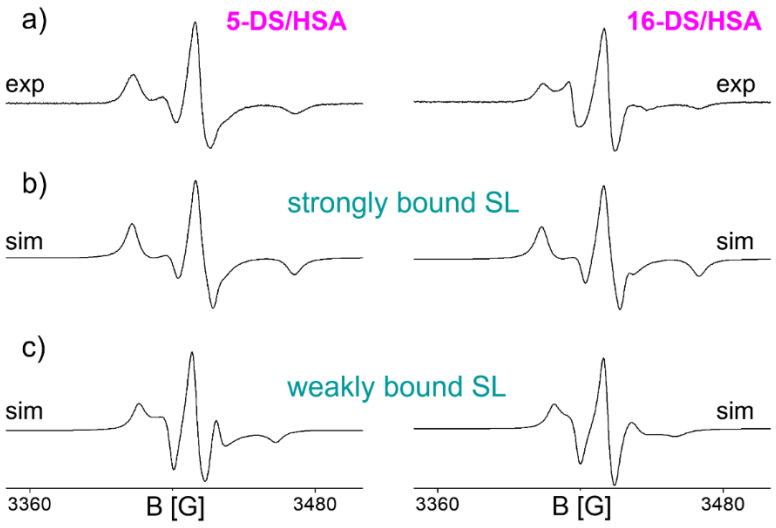
6. Spin-labeling of HSA as a biomarker for LABC

- EPR spectra of 5-DS and 16-DS spin labels (SLs) bound to HSA from healthy individuals (control) and LABC patients.
- Spectra have to be simulated and decomposed.
- Components: strongly (SB) and weakly (WB) bound SLs.
- The SB/WB ratio is significantly different and could use as a biomarker for LABC.
- We showed that 5-DS is more sensitive to LABC-induced HSA conformational changes.
- Problem: HSA can bind up to seven equivalents of fatty acids, making it difficult to determine which parts of the molecule undergo conformational changes.

The strongly and weakly bound EPR spectral component contributions to the simulated spectra of 5-DS/HSA and 16-DS/HSA complexes.

	Strongly bound (SB)	Weakly bound (WB)	Ratio SB/WB
5-DS / control-HSA	0.79 ± 0.08*	0.15 ± 0.02	5.3 ± 0.5
16-DS / control-HSA	0.63 ± 0.06	0.33 ± 0.03	1.9 ± 0.2
5-DS / LABC-HSA	0.49 ± 0.05	0.44 ± 0.04	1.1 ± 0.1
16-DS / LABC-HSA	0.43 ± 0.04	0.39 ± 0.04	1.1 ± 0.1

*The mean value of 15 simulations. The goodness of fits for all simulations was <5.

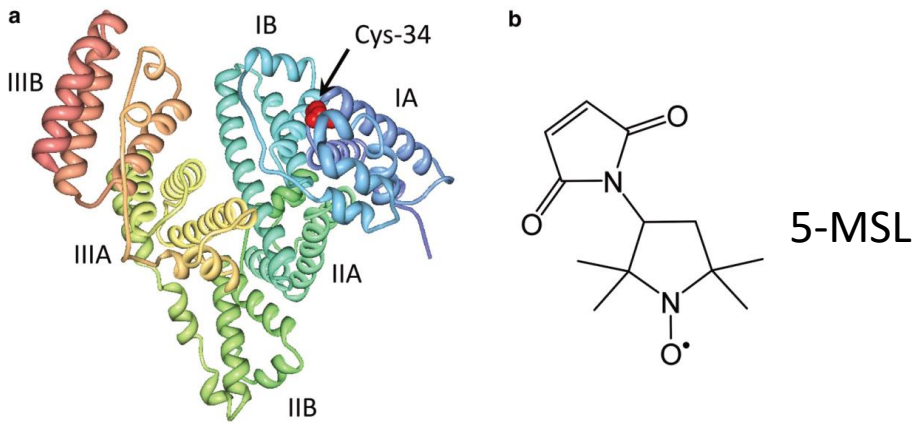


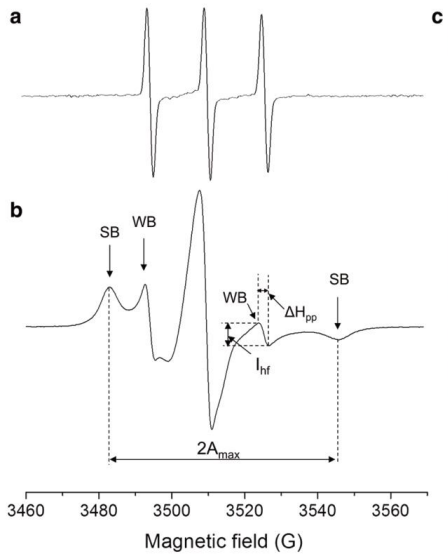
Experimental EPR spectra of 5-DS and 16-DS bound to HSA and the simulated main components corresponding to b) the strongly, and c) the weakly bound spin label.

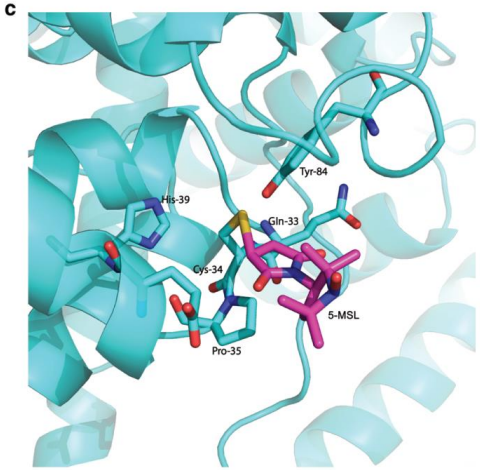
Pavicevic et. al. J. Phys. Chem. B, 10898–10905, 118 (2014).

6. Spin-labeling of HSA for drug pharmacodynamics

- To obtain information from a specific site on a protein, spin labels that bind to free cysteine residues are used.
- Such a label is 3-maleimido proxyl (5-MSL).
- Rigidly bound maleimido-nitroxides are more sensitive to the motion of the whole protein rather than local changes.
- 5-MSL successfully reported not only marked alterations in the albumin structure, such as unfolding and denaturation, but also subtle changes occurring because of fatty acid and drug binding as well as the influence of ROS.
- The results are essential for a better understanding of albumin-drug binding mechanisms and drug pharmacodynamics.



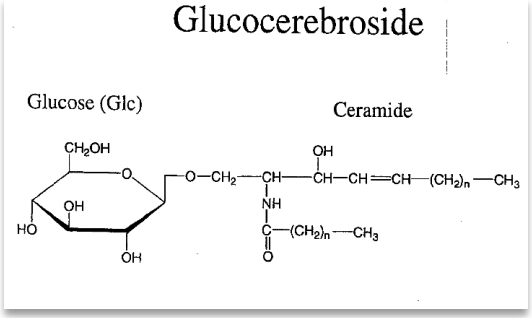


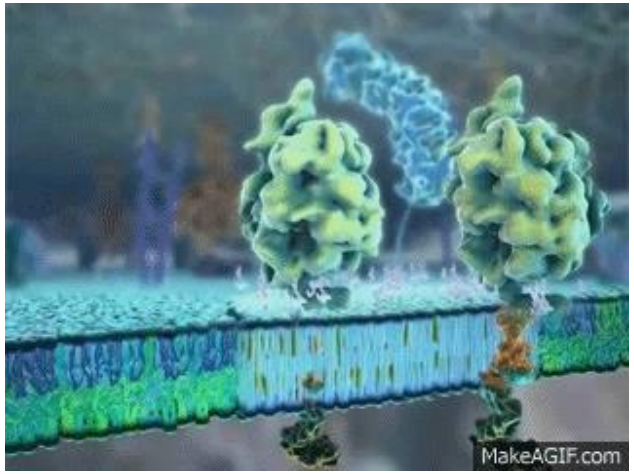


Pavicevic et. al. Eur Biophys J. 773–787, 46 (2017).

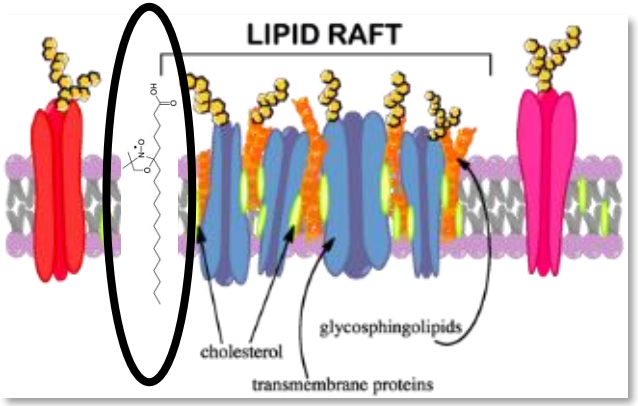
7. SL of PBMC in diagnosis and therapy of GD

- Impaired function of β-glucocerebrosidase, which results in accumulation of GlcCer (membranes).
- Sphingolipids, together with cholesterol, phospholipids and GlcCer form rafts.
- Increased amounts of GlcCer cause raft growth, and alteration of the membrane fluidity.
- **So, use spin labeling** (could also be used to detect lipid peroxidation caused by ROS).
- The goal was to measure PBMC membrane order parameter (S).
- Fast method for diagnosis and following up therapy.





5-DS



Pavicevic et. al. Biological Chemistry , 447-452, 399 (2017).

8. Using EPR to study neural tissue in ALS

- The reason for the occurrence and the mechanism of progression of ALS - still unknown.
- No confident biomarkers for early stage of the disease.
- It is assumed that developing of ALS is linked to the cell malfunctions caused by **disturbed metabolism of reactive oxygen and nitrogen species** which lead to motor neuron cell damage.

Lou Gehrig

- For years it has been speculated that there is an increase of free brain iron in a number of neurodegenerative disorders.
- Accordingly, **$^{\bullet}$OH radicals**, which are generated via Fenton reaction, are suspected as main culprits responsible for the neuron damage.
- So, the crucial factor for the free radical formation is **not the amount of iron but its redox activity**.
- EPR provides experimental approach which could expose the existence of metal oxidation state and form.

Ignjatović, et.al. Amyotroph Lateral Scler. 13, 357-362 (2012).

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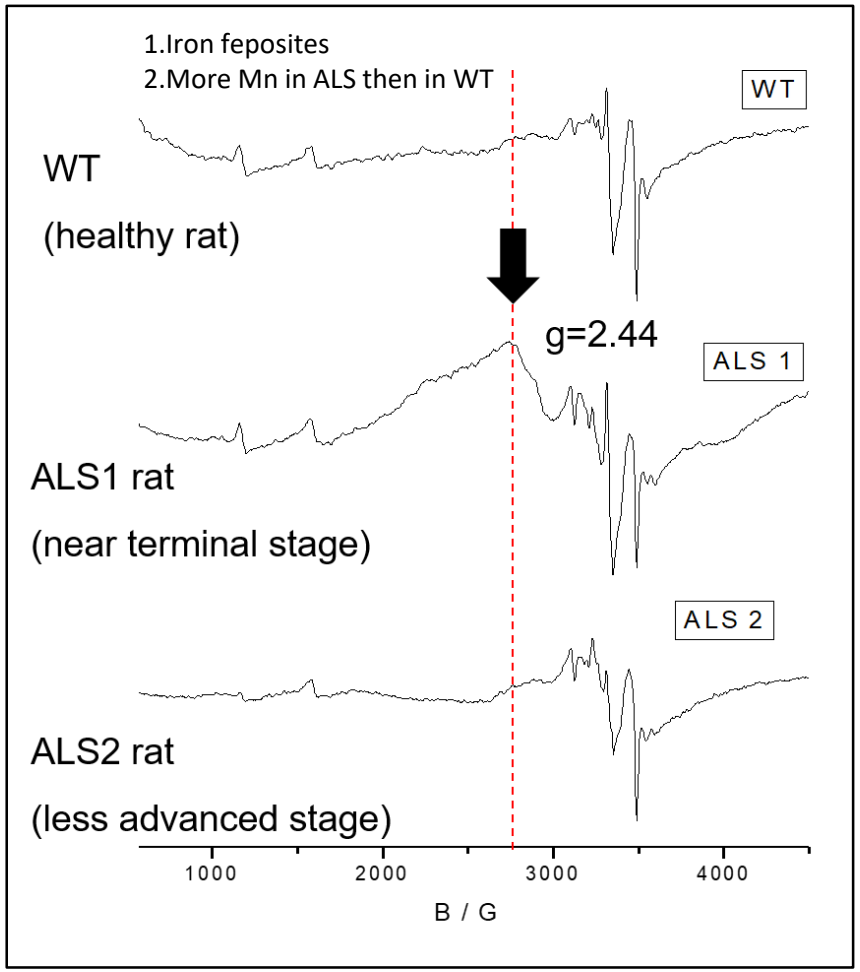
8. Using EPR to study neural tissue in ALS

- Whole intact neural tissues were directly inserted into the EPR quartz tubes.
- Samples: Brainstem (BS), spinal cord (SC), cortex (CTX), hippocampus (HC) were frozen and recorded on temperatures 4-77K.
- **EPR detected:** Cytochrom c, free mononuclear unspecifically bounded Fe, Fe-S clusters from ETC complexes and Mn from MnSOD were detected.
- SC showed **new signal from ALS rats** compared to WT.

TG rats of Sprague-Dawley breed with a larger number of copies of the human SOD1 gene and inserted G93A mutation.

- Changes: More iron-oxide aggregates and MnSOD.
- Theory: Deposites of redox active iron and when Cu,ZnSOD does not act properly, MnSOD takes over.

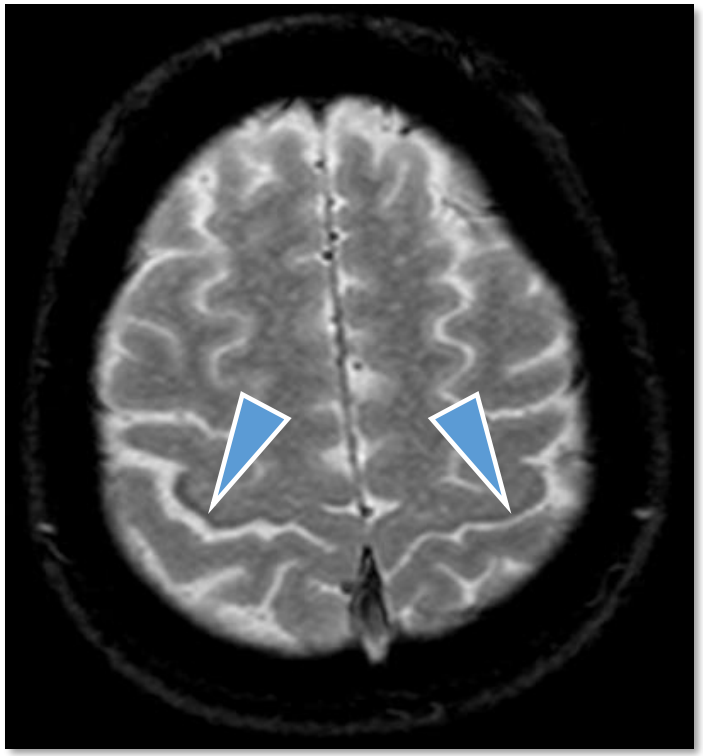


SC for WT, ALS1, ALS2 at 20K

Popović-Bijelić et. al. Free Radic Biol Med. 96, 313-322 (2016).

9. Using EPR to study BBB permeability in ALS

- Our MRI studies showed the presence of iron deposits in the motor cortex of ALS patients, indicating the potential leakage of iron to CSF through compromised BBB.
- But, this assumption **has to be confirmed**.
- Also, some info about **total redox status in brain tissue** would be usefull.
- The idea was to **use in vivo L-band EPR spectroscopy** and a selection of spin-probes.



Iron deposits in a brain of ALS patient

Ignjatović, et.al. Journal of Magnetic Resonance Imaging, 38, 1472–1479, (2013).

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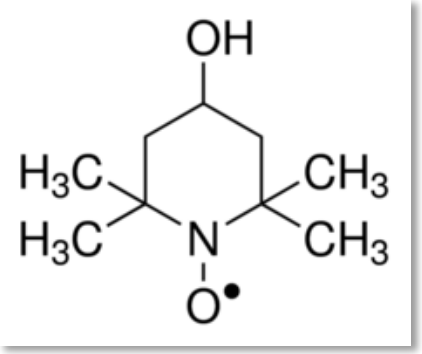
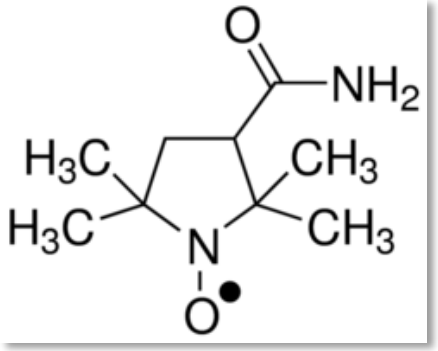
9. Using EPR to study BBB permeability in ALS model

- We have tested 3 spin-probes (TEMPOL, 3CP, 3CxP).
  - TEMPOL (can pass CM and BBB)
  - 3CP (can pass CM, but not BBB)
  - 3CxP (can not pass CM or BBB)

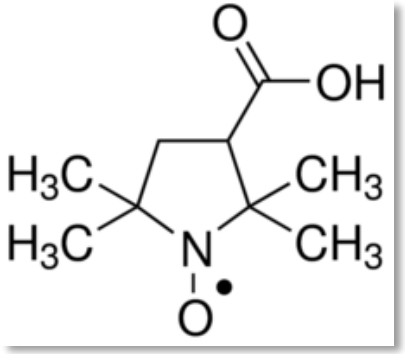
Same EPR signal



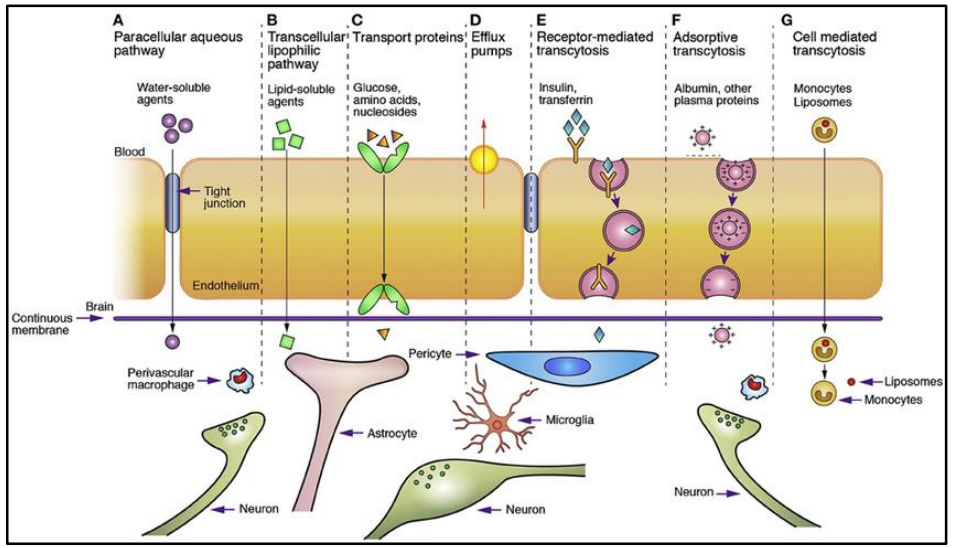
4-Hydroxy-TEMPO (TEMPOL)



3-Carbamoyl-PROXYL (3CP)



3-Carboxy-PROXYL (3CxP)



Transport pathways across blood brain barrier (Chen and Liu,2012.)

Stamenkovic, et.al. Free Radical Biology and Medicine, 258-269, 108 (2017).

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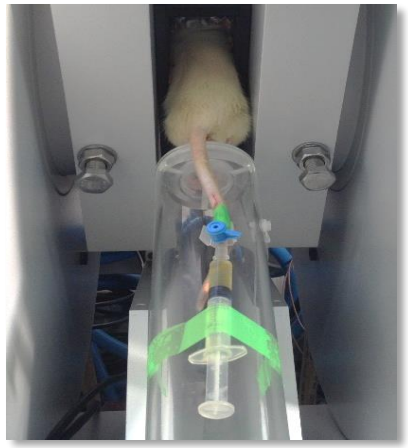
9. Using EPR to study BBB permeability in ALS model

- Rats were anesthetized by ketamine-xylazine.
- The tail vein was cannulated to inject spin probe solution (2 µmol/g b.wt.).
- The head of the rat was inserted into L-band BLGR36 resonator.
- Immediately after injection of spin-probe, EPR spectra were recorded.
- EPR signal of spin-probes decays under the influence of reactive oxygen species which readily reduce aminoxyl radical.



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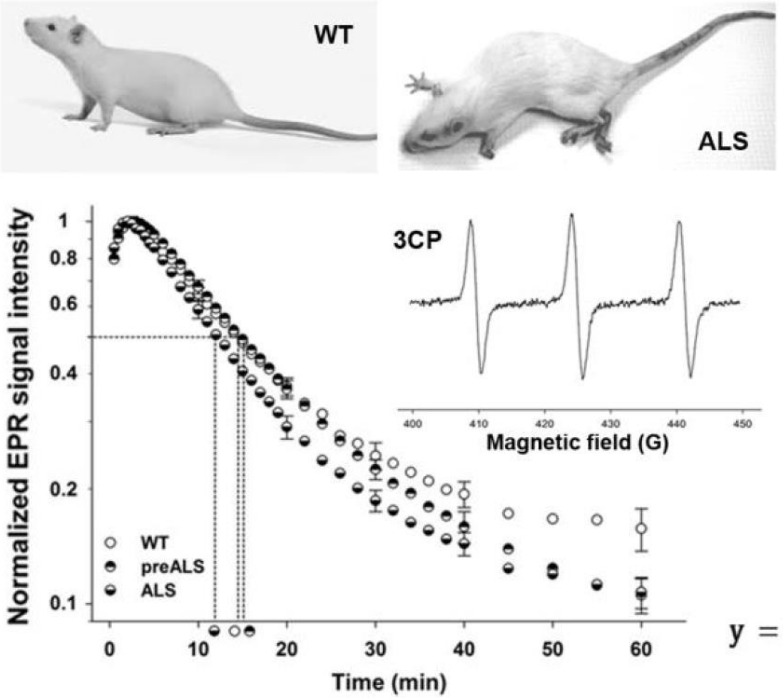
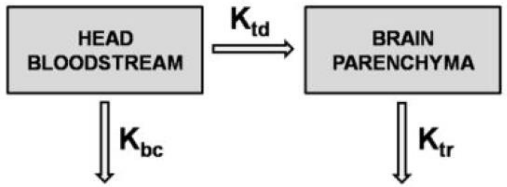
9. Using EPR to study BBB permeability in ALS model





$$y = \left(1 + \frac{k_{td}}{k_{tr} - k_{td} - k_{bc}}\right) e^{(-(k_{bc}+k_{td})x)} - \frac{k_{td}}{k_{tr} - k_{td} - k_{bc}} e^{(-k_{tr}x)}$$

k_{bc} the rate constant of bloodstream clearance, k_{td} the rate constant of tissue distribution, and k_{tr} the rate constant of tissue reduction

- 3 different kinetics could be observed.
- Symptomatic ALS rats reduce 3CP by fastest rate.
- Two-compartment model using 3 factors that contribute to the changes in the spin probe signal intensity:
  - (i) bloodstream clearance
  - (ii) tissue distribution
  - (iii) reduction in the tissue

- This *in vivo* study revealed a disrupted BBB and changed oxidative status in the brain tissue of the transgenic SOD1G93A rat model of ALS.

Stamenkovic, et.al. Free Radical Biology and Medicine, 258-269, 108 (2017).

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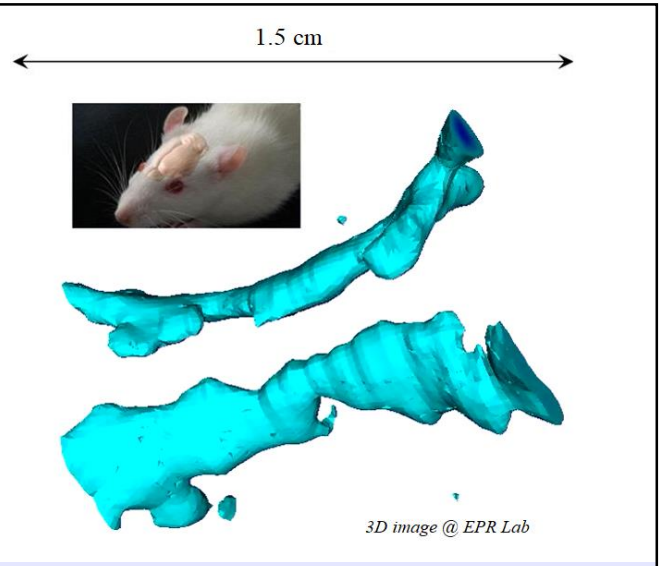
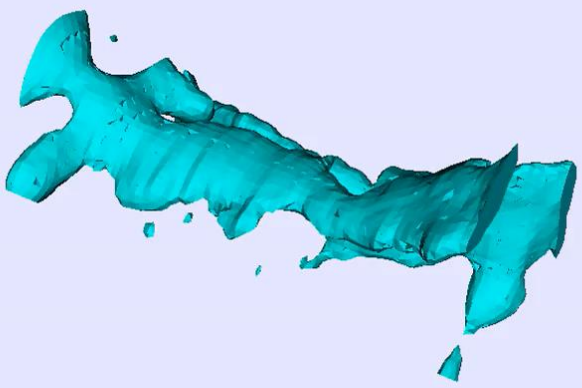
9. Using EPR to study BBB permeability in ALS model



3D EPR image of ALS mice brain

3D EPR image



BBB impermeable 3CxP in brain tissue of ALS mice

- 3 different kinetics could be observed.
- Symptomatic ALS rats reduce 3CP by fastest rate.
- Two-compartment model using 3 factors that contribute to the changes in the spin probe signal intensity:
  - (i) bloodstream clearance
  - (ii) tissue distribution
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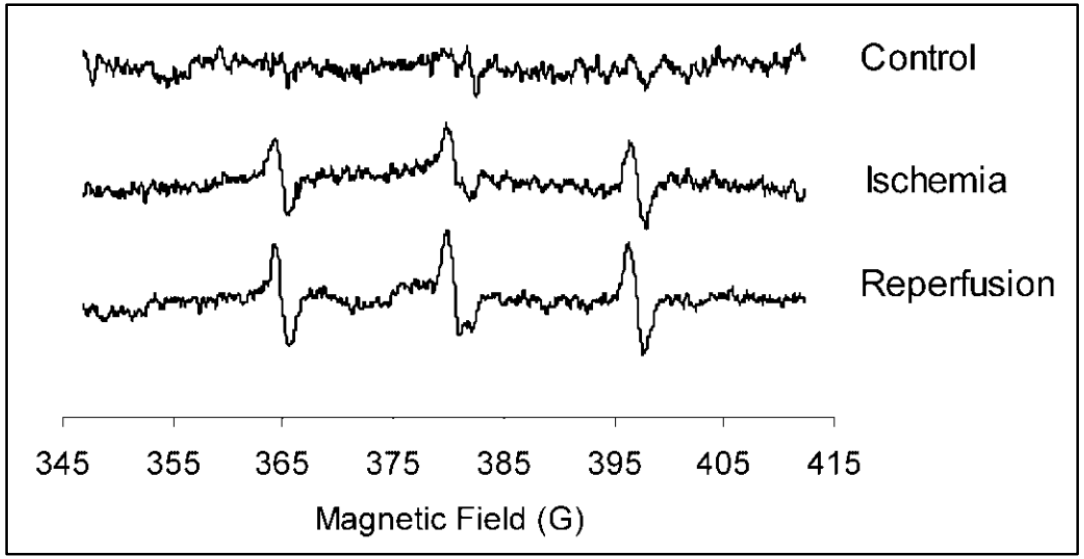
- This *in vivo* study revealed a disrupted BBB and changed oxidative status in the brain tissue of the transgenic SOD1G93A rat model of ALS.

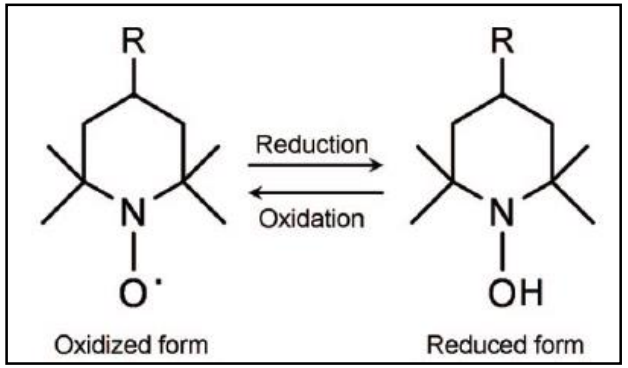
Stamenkovic, et.al. Free Radical Biology and Medicine, 258-269, 108 (2017).

10. EPR for detecting ROS *in vivo*

- Difficult task since ROS are short-lived.
- We have to use **spin-trapping agents**.
- But spin-adducts are also short-lived *in vivo* and they are easily reduced to HA.
- The solution could be to inject HA and trace it´s reoxidaton by free radicals.
- Experiment: i.p. injection of hydroxylamine CP-H.
- In the middle cerebral artery occlusion (MCAO) rat, the EPR signal of oxidized CP-H was increased after MCAO, indicating that ROS generated by cerebral ischemia were able to react with HA to produce EPR-detectable nitroxide.
- Upon reperfusion, the oxidized CP-H signal appeared to increase further, suggesting an increased ROS formation after reperfusion.



EPR measurement of oxidative stress in brain during cerebral ischemia and reperfusion

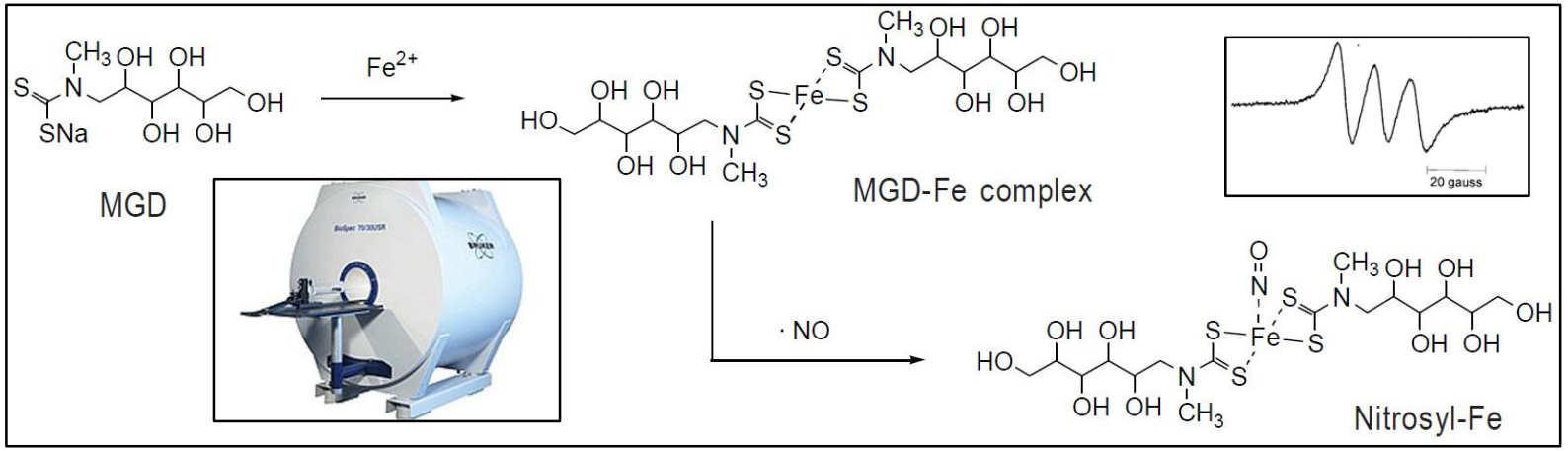


Reoxidation of the hydroxylamine to the aminoxyl radical

11. Imaging of NO• radicals *in vivo*

- Is it possible to perform EPR imaging of short-lived radicals *in vivo*?
- **Problem:** Trapped radicals always have hyperfine multiplets and usually more than one spin-adduct.
- **Possible solution:** To use MRI and paramagnetic properties of obtained spin-adducts as **MRI contrast agents**.
- **Example:** Detecting distribution of NO• *in vivo,* induced by lipopolysaccharide (LPS) septic shock in rat.

- Spin-adduct is primarily verified by EPR *in vivo* and *ex vivo.*
- Radical distribution is visualized in MRI due to the decrease in proton spin-lattice (T1) and spin-spin (T2) relaxation times, caused by EPR active $(MGD)_2$-Fe(II)-NO.



NO• is complexed with the Fe(II)-chelate spin trap (N-methyl-D-glucamine dithiocarbamate - MGD) obtaining EPR active (MGD)2-Fe(II)-NO

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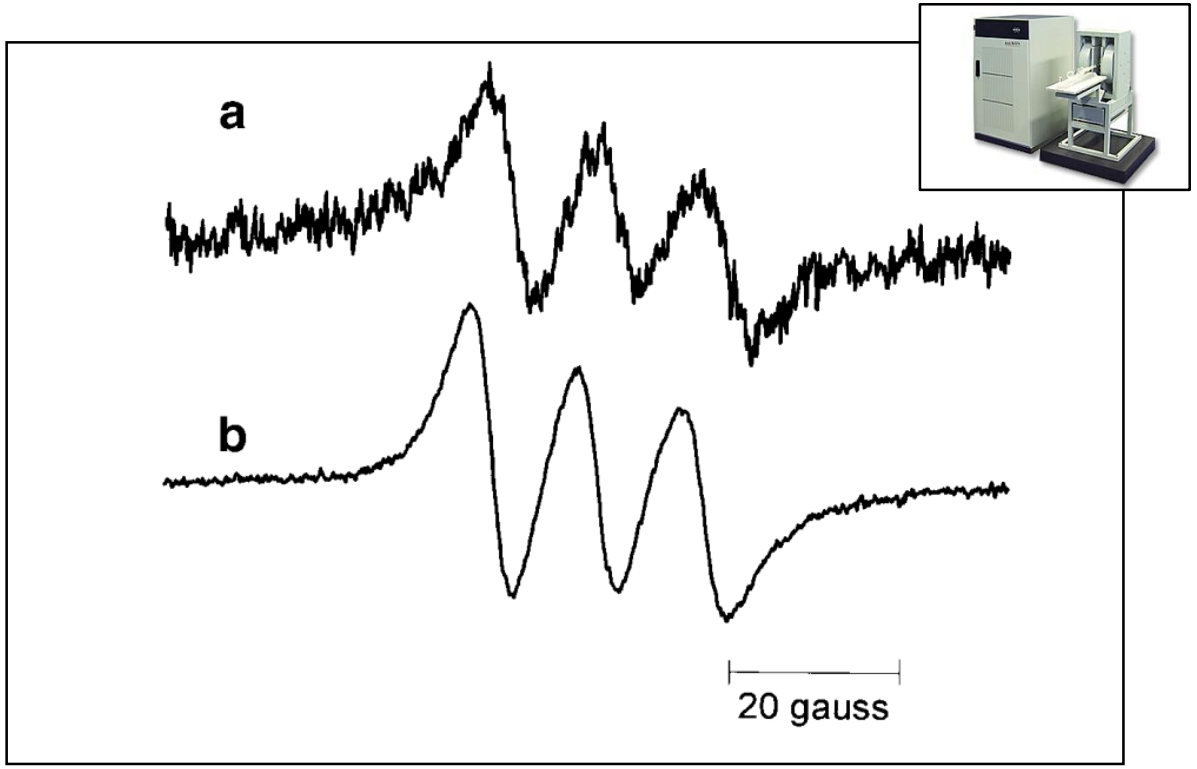
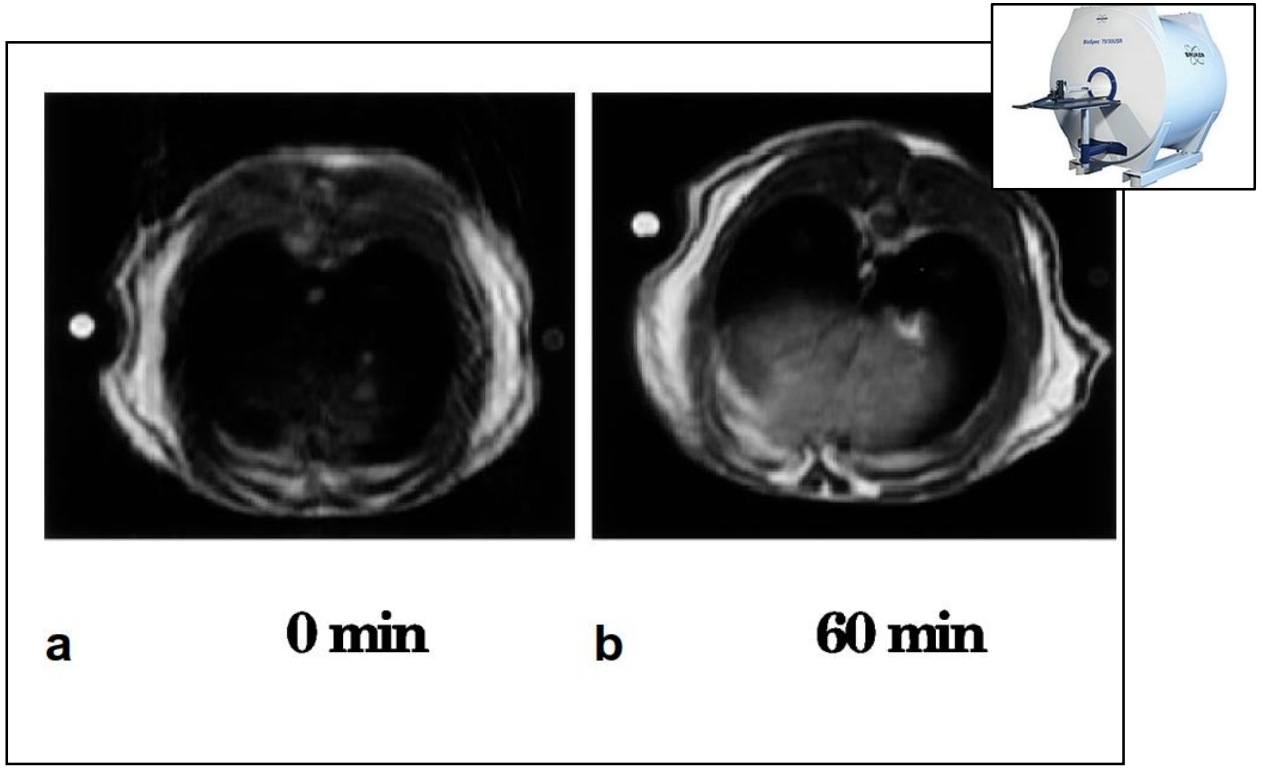
11. Imaging of NO• radicals *in vivo* using EPR + MRI



EPR spectra of $(MGD)_2$-Fe(II)-NO in LPS-treated animal.
a: In vivo L-band EPR spectrum in liver area after 2 hours.
b: Ex-vivo X-band EPR spectrum of the excised liver recorded after 1 hour.



Transverse T1-weighted MR images in the axial plane of the liver area.
a: Control before MGD complex injection;
b: 60 min after the injection of MGD complex.

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Thank you