Benchtop ESR Spectrometer MiniScope

Applications

General Examples

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Contents List

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1. Spin Trapping of Vascular Nitric Oxide using Colloid Fe(II)-Diethyldithiocarbamate

The spin trapping of nitric oxide (NO) with iron complexes is well known. The special feature of the following method is the preparation of Fe(II)-Diethyldithio-carbamate ($Fe(II)(DETC)_2$) in a colloid modification.

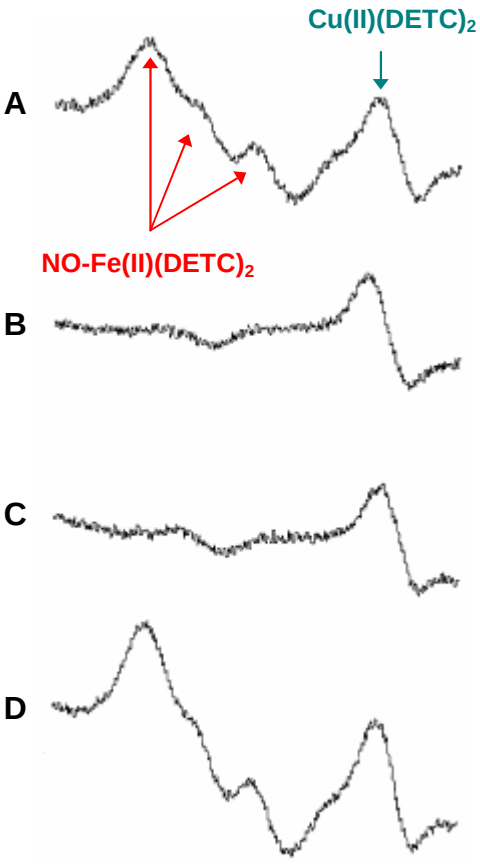

Fig. 1 ESR spectra of strips of rabbit aorta or vena cava (1 cm^2) incubated with 250 µM colloid $Fe(DETC)_2$:
A) Intact aorta,
B) Aorta treated with L-NAME,
C) Endothelium denuded aorta,
D) Intact vena cava.

It has been developed by Dr. Andrei Kleschyov in the group of Prof. Dr. Thomas Münzel (Department of Internal Medicine, Division of Cardiology, University Hospital Eppendorf, Hamburg, Germany) [11].

Advantages of Colloid $Fe(II)(DETC)_2$

- High efficiency of NO spin trapping,
- Basal as well as stimulated NO production can be quantified in aorta and vena cava,
- The colloid $Fe(II)(DETC)_2$ is lipophilic and “watersoluble”,
- $Cu(DETC)_2$ signal doesn’t dominate over the NO- $Fe(II)(DETC)_2$ signal,
- No inhibition of vascular SOD activity,
- The NO detection is not affected by moderately increased levels of extracellular superoxide and nitrite.

Detailed information about this method can be found in the following article:

Kleschyov A. L., Mollnau H., Oelze M., Meinertz T., Huang Y., Harrison D. G., Münzel T., *Biochem. Biophys. Res. Commun.* **275**, 672-677 (2000).

Kleschyov, A. L., Münzel, T., *Methods Enzymol.* **359**, 42-51 (2002).

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2. Detection and Quantification of Oxygen Radicals in Whole Blood

2.1. Introduction

The following assay is based on the spin trapping technique in combination with ESR measurements.

The facts you have to consider if you want to measure radicals in whole blood are the following.

Antioxidants present in Whole Blood

Enzymes

- Superoxide dismutase (cytoplasm),
- Catalase (cytoplasm),
- Glutathione peroxidase (cytoplasm).

Antioxidants

- Tocopherol (vitamin E, membrane),
- Beta-Carotines (membrane),
- Glutathione (merely cytoplasm),
- Ascorbic acid (vitamin C, blood plasma),
- Uric acid (blood plasma),
- All transition metal centres being able to quench radicals by a one electron reduction or oxidation.

Main Requirement for the Measurement of Radicals in Whole Blood

- The blood cells remain intact to avoid release of antioxidative enzymes into the blood plasma.

Therefore it is wise to collect the blood samples on ice analysing them the same day. For freezing and storing of blood samples they have to be incubated with the spin trap for a certain time, frozen and stored. After collection of a number of samples they can be measured at liquid nitrogen temperature in the frozen state without any thawing, because thawing of blood samples leads to a distinct haemolysis.

Sample Collection in Dependence of the Radical Generating System

- In the case you have modified or stimulated cells in blood to generate radicals you can collect the samples mix them with a spin trap, incubate them and record an ESR spectrum.
- If the radical generation in your system is due to an external modification e. g. radiation, it is most important to collect the samples at the time the radical generation is taking place and to have the spin trap being present in the syringes you collecting the samples with. By this protocol you make sure, that the radicals are trapped immediately during the blood collection. Samples will be collected on ice, incubated for a certain time and the ESR spectra will be recorded.

As short living radicals are highly reactive they have to be stabilised for ESR spectroscopic analysis. This job is done by spin traps which react with radicals and lead to stabilised product radicals with a half-life time being long enough to make them detectable by ESR.

- Commonly DMPO is used as the most prominent spin trap. But DMPO and even DEPMPO, a DMPO derivative with improved properties, used in whole blood gives no ESR signal at all.

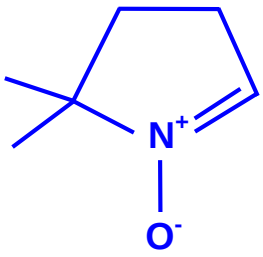

Fig. 2 DMPO (5,5-Dimethyl-1-pyrroline-N-oxide)

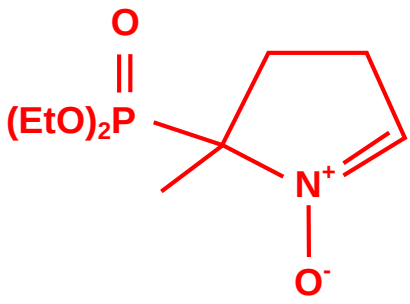

Fig. 3 DEPMPO (5-Diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide)

The reason for this is that the radical adducts are not stable enough to survive further oxidation or reduction dependent on the chemical environment.

- Therefore a new generation of spin traps is used. These hydroxylamines (Fig. 4, example TEMPONE-H) are trapping all kinds of radicals. A hydrogen atom of the hydroxylamine function is abstracted by the radical and a stable nitroxyl radical is generated. The stability is achieved by the complete alkylation in β-position to the nitroxyl radical function leading to a sterical hindrance. These kind of spin traps possess an kinetic specificity. That means some radicals are trapped faster than others.

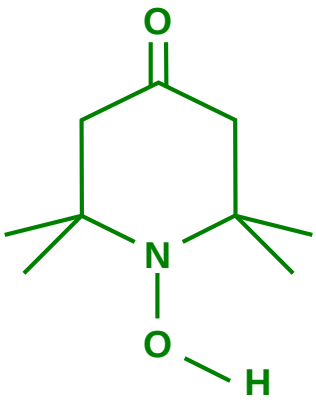

Fig. 4 TEMPONE-H (1-Hydroxy-2,2,6,6-tetra-methyl-4-oxo-piperidine)

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2.2. Method

For a better understanding of the method of detection and quantification of super-oxde and hydroxyl radicals in whole blood it is necessary to explain the chemistry and some properties of these hydroxyl-amine spin traps.

Reactivity of Hydroxylamine Spin Traps

- When radicals are trapped the hydroxylamine function is converted into a nitroxyl radical. This nitroxyl radical can be reduced back to the hydroxylamine or oxidised to an oxoammonium compound. The nitroxyl radical is ESR detectable. The hydroxylamine and the oxoammonium compound are ESR silent (Fig. 5).

Autoxidation

- The nitroxyl radicals are generated by autoxidation and by the trapping of radicals. The velocity of the autoxidation depends on the spin trap you are using. That means your aqueous spin trap solution contains both hydroxylamine and nitroxyl radical.

This is the reason you have to measure a basic radical content in your spin trapping solution before and after your experiments.

As the autoxidation is a reaction of zero order, you can calculate your basic signal for the timepoint of your measurement and subtract it from the signal measured to get your real signal.

The spin trap solutions being used for this method contain both hydroxylamine and nitroxyl radicals in approximately the same concentration.

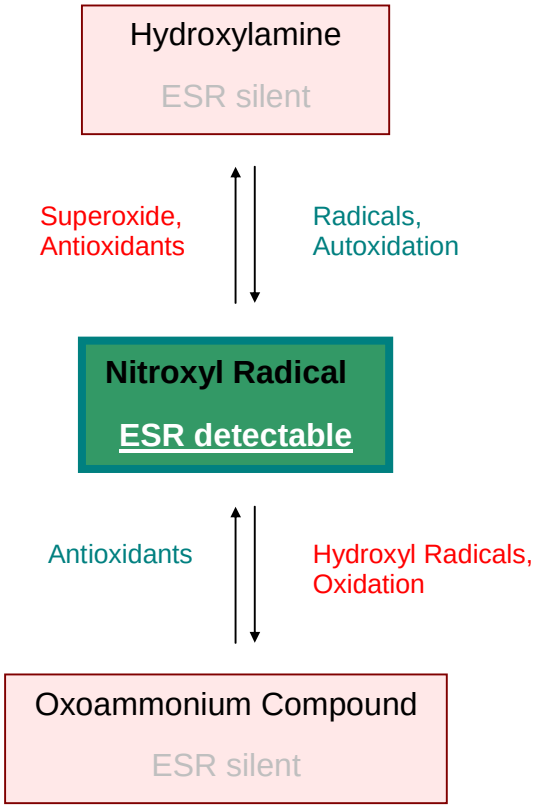

Fig. 5 Reactivity of hydroxylamine spin traps.

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2.2.1. Extracellular Superoxide

The detection and quantification of extracellular superoxide requires two spin trapping experiments with aliquots of a blood sample.

- First the radicals generated during 2 hours are trapped with a spin trap solution containing both TEMPONE-H and TEMPONE.
- In the second experiment superoxide dismutase is added to the spin trap solution and the radicals are trapped in the same way.

Superoxide dismutase stays outside the blood cells and is not penetrating into them. This is the reason why only extracellular superoxide can be detected and quantified by this method.

In the first experiment superoxide preferably reduces TEMPONE back to TEMPONE-H. When superoxide dismutase is added to the spin trap solution superoxide reacts much faster with SOD than with TEMPONE. This means in this case TEMPONE is not reduced back to ESR silent TEMPONE-H and the ESR signal intensity increases. The difference of the radical content determined in these two experiments is the superoxide content as illustrated in Fig. 6.

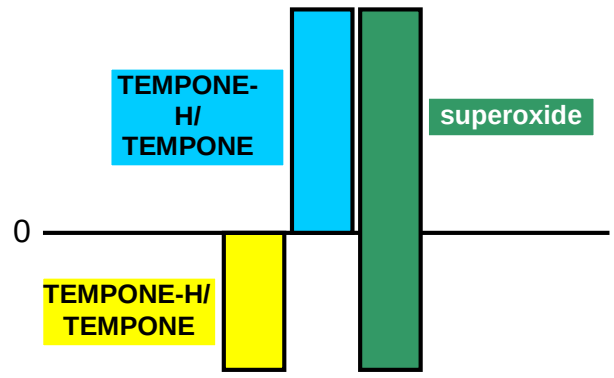

Fig. 6 Calculation of the superoxide content (green) from the spin trapping experiments using TEMPONE-H/TEMPONE (yellow) and TEMPONE-H/TEMPONE + SOD (blue).

2.2.2. Hydroxyl Radicals

In a mixture of TEMPONE-H and TEMPONE as a spin trapping solution hydroxyl radicals preferably react with TEMPONE by oxidising it to an oxo-ammonium compound. Former spin trapping experiments with DMPO have shown, that DMSO is a very effective scavenger of hydroxyl radicals generating methyl or hydroxymethyl radicals.

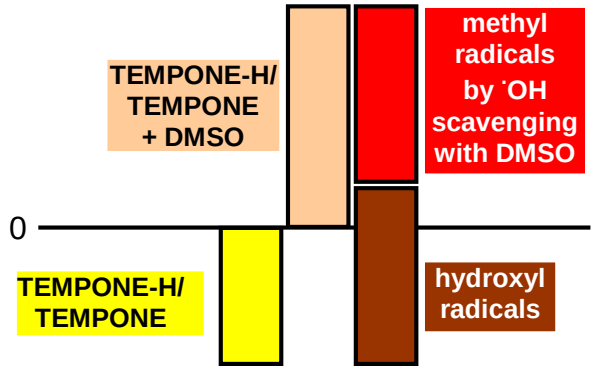

Fig. 7 Calculation of the hydroxyl radical content (dark red) from spin trapping experiments using TEMPONE-H/TEMPONE (yellow) and TEMPONE-H/TEMPONE + DMSO (light brown).

As hydroxyl radicals oxidise TEMPONE and methyl radicals generate TEMPONE by oxidising TEMPONE-H these reactivities can be used to detect and quantify hydroxyl radicals. The difference of two spin trapping experiments using TEMPONE-H/TEMPONE and TEMPONE-H/TEMPONE + DMSO divided by two is the hydroxyl radical content as illustated in Fig. 7.

2.2.3. Intracellular Hydrogen Peroxide

The measurement of intracellular hydrogen peroxide is based on the conversion of hydrogen peroxide into hydroxyl radicals.

This is achieved by the addition of deferoxamine to the spin trap solution TEMPONE-H/TEMPONE + DMSO. The deferoxamine penetrates into the blood cells, chelating iron and copper. But in contrast to iron which is chelated completely in the case of copper prefering the coordination number four there is always enough space for reaction with hydrogen peroxide generating hydroxyl radicals by the Fenton reaction.

These hydroxyl radicals can be detected by performing the spin trapping experiment using TEMPONE-H/TEMPONE + DMSO one time with and without deferoxamine. The hydrogen peroxide content corresponds to the hydroxyl radical content determined as described before.

2.3. Application Examples

2.3.1. Xanthine/Xanthine Oxidase

The addition of xanthine/xanthine oxidase as a source of superoxide to aliquotes of the same blood sample lead to an increase in quantified superoxide. By this test it was proved that the method is working correctly (Fig. 8).

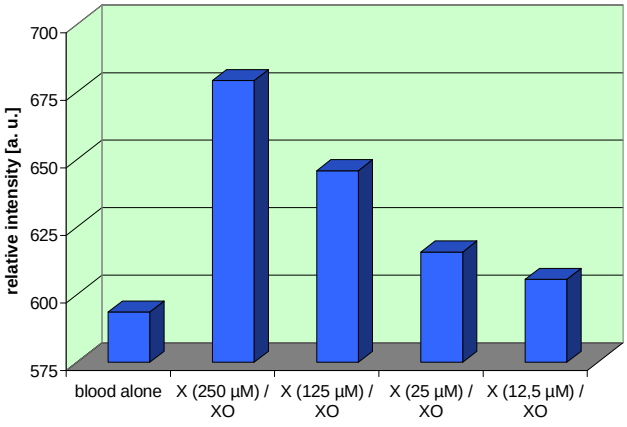

Fig. 8 Superoxide content in whole blood alone and with addition of xanthine/xanthine oxidase (X (concentration) / XO).

2.3.2. Ischemia/Reperfusion

Ischemia/Reperfusion is the classical case of superoxide generation by xanthine oxidase derived from xanthine dehydrogenase and xanthine derived from the degradation of ADP. The following data in Fig. 9 show the relative extracellular superoxide content of pig whole blood during ischemia by aortic bending and subsequent reperfusion. Isoprostane measurements (data not shown) confirmed the results of the ESR measurements.

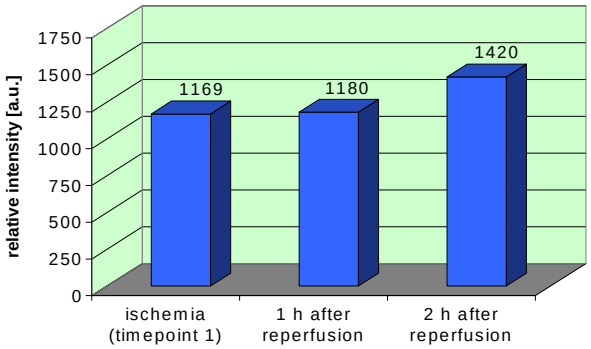

Fig. 9 Relative extracellular superoxide content determined in pig whole blood during ischemia by aortic bending and subsequent reperfusion.

The relative hydroxyl radical content in pig whole blood in the same experiment shown in Fig. 9 was monitored (Fig. 10). There are several explanations for the decreased hydroxyl radical content 1h after reperfusion. One is the increase of cellular metabolism in reperfusion. The second an increased expression of antioxidative enzymes like catalase and peroxidases degrading hydrogen peroxide the precursor of hydroxyl radicals.

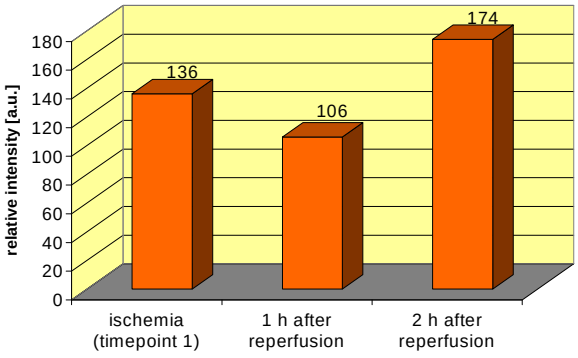

Fig. 10 Relative hydroxyl radical content in pig whole blood determined by ESR. Same experiment like in Fig. 9.

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The intracellular hydrogen peroxide content is monitored in pig whole blood by the method described before. The experiment was the same as in Fig. 9 and Fig. 10. The data match perfectly with the superoxide and hydroxyl radical content determined by ESR. One hour after reperfusion the superoxide level was increased but cells are able to convert superoxide to hydrogen peroxide. The hydrogen peroxide is easily degraded by the activated enzymes. Two hours after reperfusion the oxidative stress is exceeding the antioxidative capacity and leads to a complete break down of the biological system.

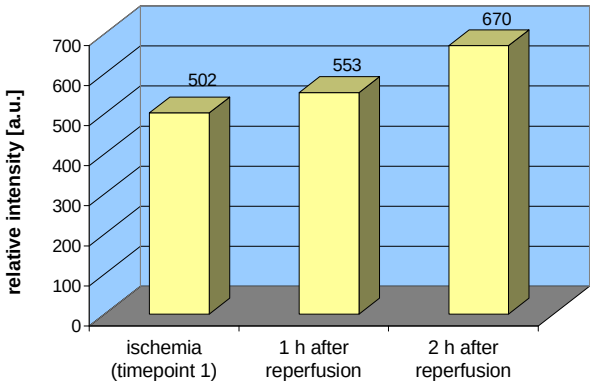

Fig. 11 Relative intracellular hydrogen peroxide content determined in pig whole blood. Experimental setting where the same as in the experiment shown in Fig. 9 and Fig. 10.

2.3.3. Superoxide and smoking

The superoxide content in whole blood was monitored before and during smoking a cigarette. The results are shown in Fig. 12. During smoking the superoxide level is increased by 9.5 %.

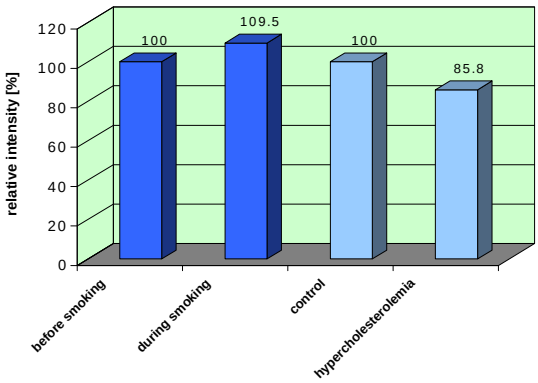

Fig. 12 Superoxide level in whole blood before and during smoking and in the blood of a control and a person suffering from hypercholesterolemia.

2.3.4. Superoxide and Hypercholesterolemia

The superoxide level in whole blood of a healthy person and a patient suffering from hypercholesterolemia was determined. Surprisingly the superoxide level in the blood of the letter person was 14.2 % lower than the one of the healthy person serving as a control Fig. 12. This may be due to superoxide scavenging properties of cholesterol or due to activation of the generation of other superoxide scavenging compounds.

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2.3.5. Hydroxyl Radicals and Therapeutic Irradiation

A test person was irradiated with visible light of a very high intensity. During irradiation the hydroxyl radical level measured in whole blood was increased by 53.7 % (Fig. 13). When the experiment was repeated after one week and drinking green tea every day the effect of hydroxyl radical generation during irradiation was compensated.

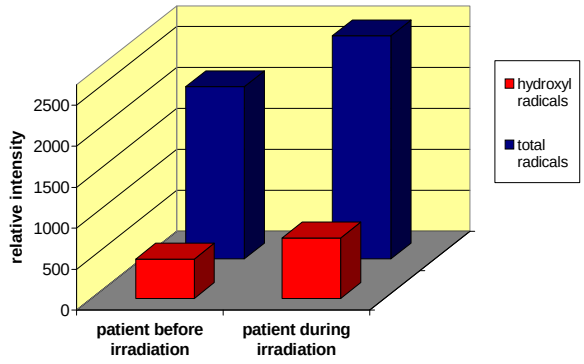

Fig. 13 Hydroxyl and total radical content in whole blood of a patient before and during irradiation.

2.4. Closing Remark

In principle it is important to consider the environment of the radical and possible reactions with antioxidants being competitive to the reaction of the radicals with the spin trap molecules. Keep in mind that trapping radicals is a dynamic process and reproducibility is only achieved if the sample preparation and measurement parameters are controlled exactly [7].

For further information about the method please contact Jörg Müller PhD, Sales and Application, Magnettech GmbH.

Literature: 1) Ashton, T. et al., *Eur. J. Appl. Physiol.*, **77(6)**, 498-502 (1998).
2) Dikalov, S. et al., *Biochem. Biophys. Res. Commun.*, **248**, 211-215 (1998).

3. Irradiation, Dosimetry, Oxymetry, Membrane Fluidity

Structural elucidation studies are a classical field of application, however, ESR spectroscopy contributes in a different way to many areas such as biophysics wherever radicals may play a chemical role or may be used as labels or probes. Since this spectroscopic method is only able to „see“ the unpaired electron, and nothing else, it is an unique way to detect and measure radicals directly. Minimum requirement is the presence of a sufficiently high concentration of radicals, i.e. in the high nanomolar range, which will survive the scan time.

Depending on the specific problem to be solved different methods of measuring radicals are available which are presented here with the help of practical examples:

- Radicals induced by radiation can be detected directly as long as they have a chance to survive the scan period. Since radicals derived from dry alanine powder persist for very long periods this material can be used efficiently for dosimetry.
- Radical existing only for a short period may be trapped by so-called spin traps. The unpaired electron is preserved for measurement in an adduct compound of the spin trap.
- Certain radicals stable for a long time period, so-called spin labels, can be used as probes for the fluidity or viscosity of the phase environmental to the unpaired electron.
- Since the line width in a spectrum of a spin label depends on the content of molecular oxygen within the sample this effect can be used to quantify oxygen (oximetry).

In the absence of water radicals induced by radiation will survive for months, even years. The existence of radicals in dried food (e.g. spices) can proof radiation treatment. Another practical application of measuring radiation induced radicals is alanine dosimetry. Fig. 14shows an ESR spectrum of irradiated alanine. Dry alanine powder in the form of tablets or films are used as dosimeters [8].

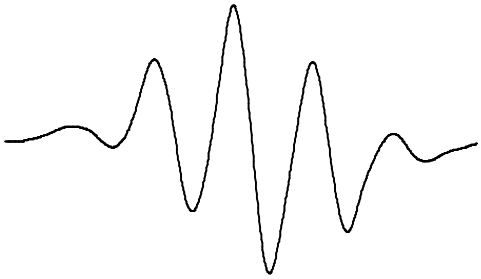

Fig. 14 ESR-spectrum of an irradiated alanine powder sample. (*MiniScope MS100*: Scan time 11 s, scan 15 mT, 9.4 GHz, modulation amplitude 1 mT)

Since radicals are the agents making oils and fats rancid antioxidative substances are of broad interest as protective additives within the food and cosmetic industry. The efficiency of such additives are tested also by ESR spectroscopy after incubation with reactive radicals [6].

Specific applications of ESR spectroscopy are shown in more detail on the following pages.

All spectra shown have been recorded using the ESR spectrometer *MiniScope MS100 and MS200*.

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4. Biochemical Generation of Radicals: Xanthine / Xanthine Oxidase

Radicals may occur within enzymatic oxidations as e.g. in the biological degradation of purines (see scheme below). The enzyme involved is the xanthine oxidase which catalyses the reactions from hypoxanthine to xanthine and from the latter to uric acid. In both steps molecular oxygen is reduced forming the superoxide anion ($O_2^{\bullet -}$) in the first instance.

purines (adenosine, guanine)
↓ ↓
↓
hypoxanthine
← H_2O, O_2
↓ → H_2O_2
↓
xanthine
← H_2O, O_2
→ H_2O_2
uric acid

The generation of radicals can be studied by the help of spin traps alternatively to the use of fluorescent dyes. The spin trap DMPO (see page 5) has the advantage to allow identification of original radicals by some characteristics of the spectra. However, oxygen adducts may be so reactive that they continue to react with the spin trap to form further products which could make ESR spectra sometimes rather complicated. A simple incubation of hypoxanthine (1 mM in phosphate buffered saline, pH 7,4) with xanthine oxidase (0,1 U/ml) results in a mixture of products with a complex ESR spectrum. The components of such a spectrum can be separated by using spectral simulation programs which are commonly available even for PCs. Fig. 15 shows the result of fitting a simulated spectrum to experimental data. Some of the components are displayed separately [7].

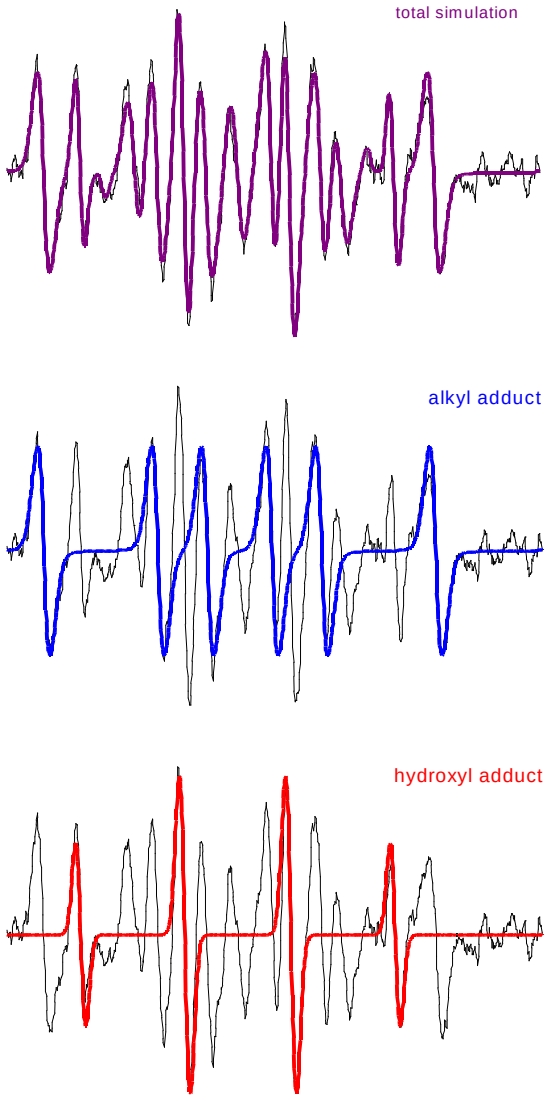

Fig. 15 ESR-spectrum of DMPO-reaction products overlaid with simulated spectra (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

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5. Detection of Intracellular Generation of Radicals

Currently the generation of free radicals within the living cell is of increasing interest, however, only indirect detection methods are widely applied such as the use of fluorescent dyes or measuring cytochrome-c reduction activity. Since electron spin resonance spectroscopy is the only method to study radicals directly and specifically it provides a reliable alternative method to those already commonly used in many laboratories. Living cells can be studied when they are cultured on suitable small slides which can be mounted onto special holders in order to place them in the resonator.

Fig. 16 shows an example of living cells, lens epithelial cells adherent on coated slides which have been incubated with DMPO for two hours (upper panel) compared with free floating cells (lower panel). The adherent cells show a different composition of DMPO reaction products. The size of the support for confluent cells was 0,5 x 2 cm and were mounted on a flat holder with a groove of 0.3 mm filled with buffered cultural medium which prevented squeezing of cells. Cells in suspension were measured in glass capillary tubes.

It will need more detailed studies to localise the site of radical generation within the cell and/or where radicals are trapped. The complexity of the spectra yielded in this kind of experiments may also indicate secondary reaction even involving the spin trap itself. Environmental factors such as the pH may also have an influence (see below: artefacts in spin trapping).

Currently intracellular radical generation is detected indirectly by fluorescence spectroscopy with suitable dyes. Electron spin resonance spectroscopy provides an alternative method which additionally offers a specific detection of certain radical species [5].

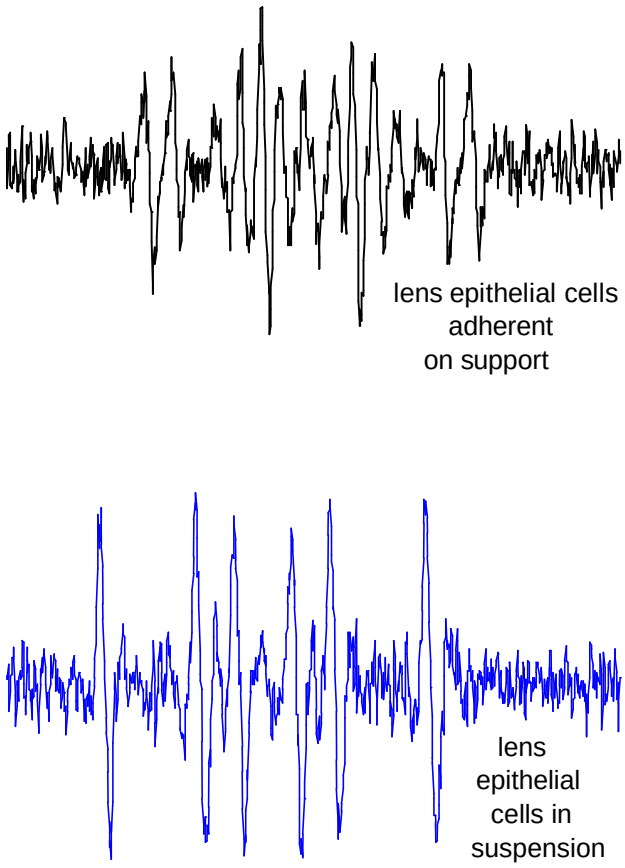

Fig. 16 ESR-spectra of DMPO-adducts formed in a culture of lens epithelial cells. While cells in suspension only show the alkyl adduct (lower panel) those growing on a support have obviously produced additionally hydroxyl radicals which were able to react with the spin trap. (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

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6. Radicals used in Photo-dynamic Therapy

Radicals may not only cause harm but may be useful tools, e.g. in the application of photodynamic therapy (PDT) of cancer diseases. They are purposely generated by the irradiation of photosensitiser localised in the malign tissue with laser light. Photosensitisers are dyes leading to the generation of singlet oxygen and/or hydroxyl radicals while being irradiated. During this treatment the patient is only allowed to stay in rooms with a light of the complementary colour. Only the tumour incorporating the photosensitiser should be exposed to the laser light.

In principle ESR spectroscopy is a valuable tool to study the generation of radicals. This kind of research may also contribute to the selection of suitable photosensitisers and elaborating effective radiation schemes. Radical generation has been studied *in vitro* by using the *MiniScope MS100* spectrometer. Solutions of a photosensitiser have been exposed to laser light in a flat cell cuvette inside the resonator.

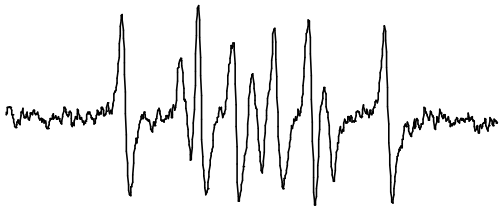
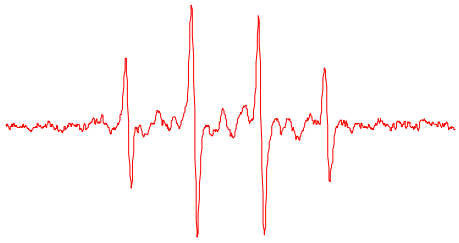
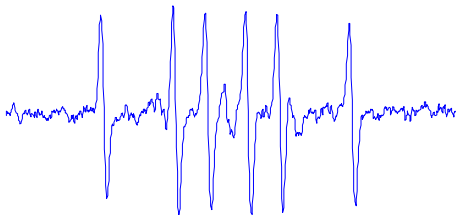


Fig. 17 ESR-spectrum of DMPO adducts after radiation of a porphyrine solution with laser light (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz.

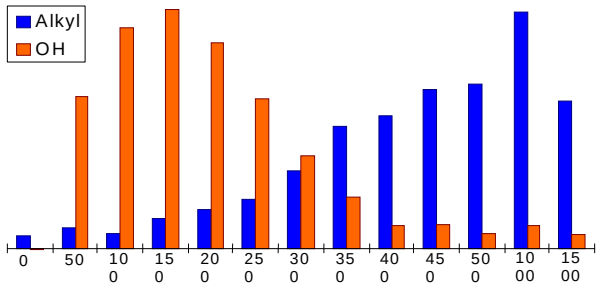
Fig. 17 and Fig. 18 show the result of such experiments with porphyrine (Photosan 3) and methylene blue (at oxygen saturation), respectively. The latter dye obviously produced hydroxyl radicals in the first instance. After continued light exposure this kind of adduct was decreased and a spectrum of the alkyl type appears, the time course is shown in the lower panel of Fig. 18. The porphyrine reacts differently, the alkyl adduct spectrum occurred immediately. Obviously the generation of singlet oxygen leads to secondary alkyl radicals being trapped by the spin trap DMPO [2].



after 100 flashes



after 1000 flashes



Different DMPO adducts depending on light exposure (consecutive flashes of 50 mJ, 10 Hz).

Fig. 18 ESR-spectra of DMPO after multiple laser flashes into a methylene blue solution, oxygen saturated (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

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7. Radiation-Induced Radicals: By-Products of Sterilisation with γ-Rays

Since the pharmaceutical development and production includes more and more drug formulations with a retarded or controlled release of the effective substance there will be a growing demand for biodegradable polymers. If these products are destined for parental use they have to be sterilised. Heat and steam cannot be applied because they may certainly destroy these substances, leaving only γ -radiation as an appropriate procedure. However, this method of sterilisation could have an effect on the drug itself. The generation of radicals during this kind of treatment has been studied with the *MiniScope MS100*.

Three different test preparation of biodegradable polymers have been investigated, one with a spin label (TEMPOL), another one with a spin trap (PBN) and a third one with alanine which is converted to a radical stable for a long time. In the first preparation any radical induced by the γ -radiation will neutralise the spin label and thus decrease its ESR signal by pairing electrons (Fig. 19), in the second one the newly generated radicals will be trapped and the spectrum of the spin trap will appear (Fig. 20). In the third case simply the ESR spectrum of the new born radical will show up (Fig. 21). Since all these experiments have been performed in a dry matrix the nitroxyl radicals (Fig. 19 and Fig. 20) show the typical anisotropic ESR spectra of immobilised molecules.

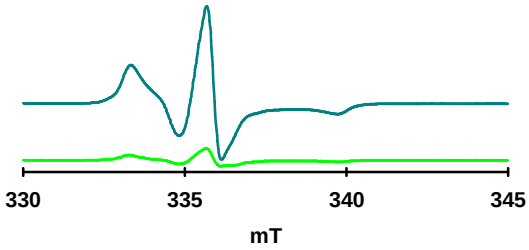

Fig. 19 ESR-spectra of a spin label (TEMPOL) incorporated in to a biologically degradable polyester (PLGA) before and after γ -radiation (25 kGy, *MiniScope MS100*: Scan time 60 s scan 15 mT, 9.4 GHz). Radiation induced radicals cause spin pairing with the spin label (TEMPOL) and thus decrease the ESR-signal.

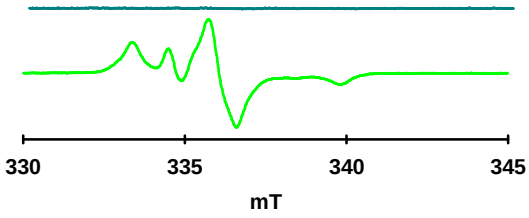

Fig. 20 ESR-spectra of a spin-trap (PBN) in polyester (PLGA) before and after irradiation (experimental parameters as in Fig. 19).

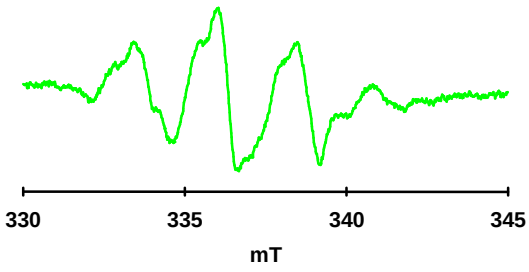

Fig. 21 ESR-spectrum of a polyester (PLGA) containing alanine after irradiation (experimental parameters as in Fig. 19).

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8. Effects of Heavy Metal Ions on Living Tissues

Heavy metals are rather efficient in catalysing chemical reaction with radicals at least as intermediates. This may in part explain their toxicity. Plant roots e.g. produce different DMPO adducts if there have been exposed to cadmium (1 µM, 1 week) or simply were grown in normal aqueous culture solution. Fig. 22 shows the result of the experiment, Fig. 23 that of the control.

While the control spectrum is dominated by a three line signal derived from a double substituted DMPO molecule the cadmium exposure resulted in an increase of the part of the monosubstituted adduct with six lines. After incubation with DMPO the culturing medium (Fig. 24) shows an ESR spectrum different from those obtained from the roots, the three line component of the double substituted compound is replaced by another one typical for the hydroxyl adduct. Radicals must react differently in the plant tissue and in free solution [9].

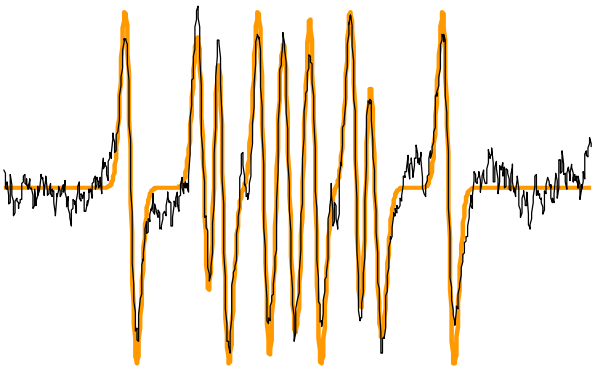

Fig. 22 ESR-spectrum of DMPO-adducts from plant roots exposed to cadmium, overlaid with a fitted simulation (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

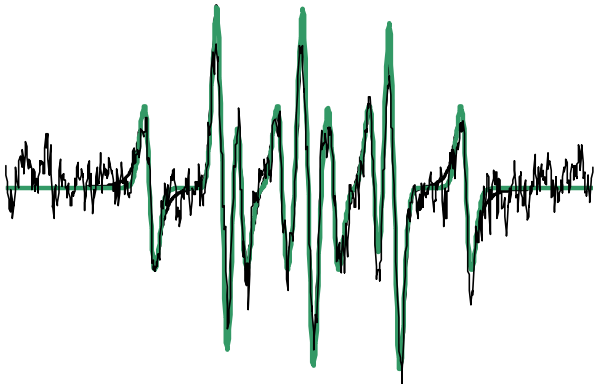

Fig. 23 ESR-spectrum of control roots, fitted simulation superimposed (parameter settings see Fig. 22).

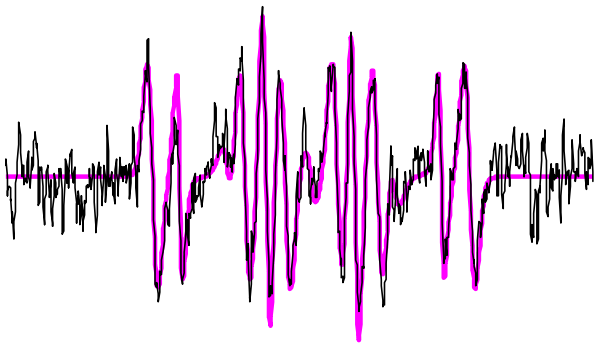

Fig. 24 ESR-spectrum of culturing medium for plant roots, DMPO added (50 mM), together with fitted simulation (parameter settings see Fig. 22).

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9. Bioinorganic Transition Metal Compounds

Many but not all transition metal ions are bearing unpaired electrons. The ones with unpaired electrons are ESR-detectable.

In many cases still the detection provides information about the ligand field (high spin or low spin) or the oxidation state of the ion.
For example an ESR-silent complex of Fe^{2+} possesses a d^6-low spin electron configuration, were all electrons are paired. An Fe^{2+} complex showing an ESR spectrum has a high spin configuration. As Fe^{2+} low spin, Cu^{+} is also ESR silent, because of its d^{10}-configuration, where all d-orbitals are fully occupied and all d-electrons are paired. In contrast Cu^{2+} bears one unpaired electron resulting in an one line spectrum.

Beside this ESR provides an ideal tool to compare the coordination geometry of active metal centres of enzymes and their low molecular weight model complexes. An example is shown below, displaying the ESR-spectra of the active Cu^{2+} centre of $Cu(II)_2Zn_2$ superoxide dismutase and Cu(II)PuPy a superoxide dismutase mimic.

These spectra are recorded at 77 K in frozen solutions of ethyleneglycol : water 1:1. The hyperfine splitting of these anisotropic signals provide information if the coordination geometry is tetragonal planar or tetrahedrally distorted.

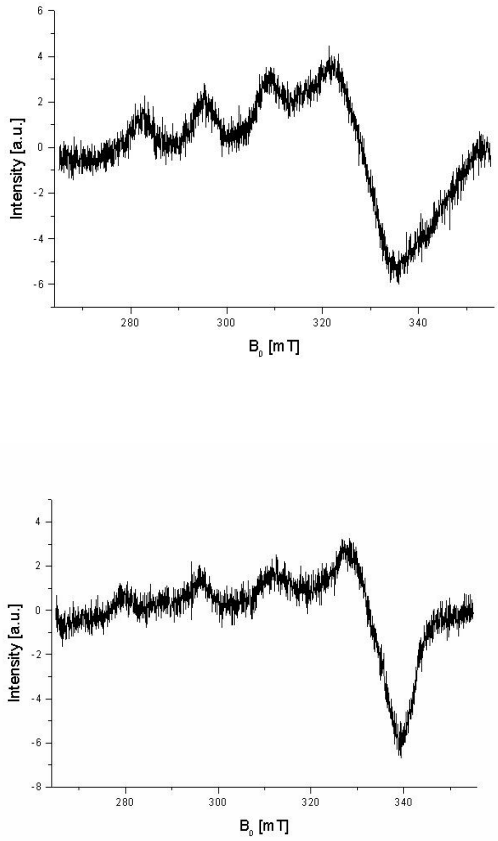

Fig. 25 ESR spectrum of $Cu(II)_2Zn_2$ superoxide dismutase (top) and Cu(II)PuPy (bottom).

Most of the enzymes and model complexes examined are copper and iron bearing compounds. But electron spin resonance (ESR) spectroscopy is useful for all paramagnetic transition metal centres with unpaired electrons in general.

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10. Spin Labels: Probes in Micro-Environments

„Stable“ radicals, i.e. compounds which preserve their unpaired electron for a long time (even months) are used as spin labels in ESR spectroscopy.
Hydrocarbons with a nitroxyl group are widely used for this purpose, they show the typical three line ESR signal which is explained in detail on page 10. However, the magnetic field of the nitrogen is asymmetric as shown in Fig. 26. The hyperfine splitting in the z-axis e.g. is definitely larger than those in the xy-plane. The reason for a symmetric (isotropic) three line spectrum of a nitroxyl radical in solution is simply that the molecule is moving free in all directions of the space so only the average of all possible hyperfine splitting constants contribute to the final appearance. In other terms, the rotation of the molecule is faster than the relaxation of the single electrons.
On the other hand, if the rotation of the radical molecule is retarded, e.g. by freezing or by high viscosity, then the spectrum becomes asymmetric (anisotropic) as expected from the nitrogen orbitals (see Fig. 27).

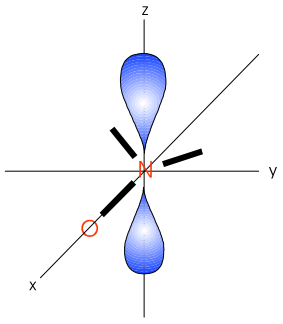

Fig. 26 Asymmetric distribution of the orbital of the unpaired electron according to the axis in space at the nitrogen atom of the spin label.

In practice long chained nitroxyl compounds such as substituted stearic acids are used for studying the fluidity of lipid membranes. The rigidity of such a spin label incorporated into a membrane layer determines the degree of asymmetry of the anisotropic ESR spectrum. With increasing fluidity the spin label can move more freely in its surrounding phase. This makes the resulting ESR spectrum more symmetrical so the grade of anisotropy is a direct measure for the fluidity of the membrane layer in which the spin label is located [3].

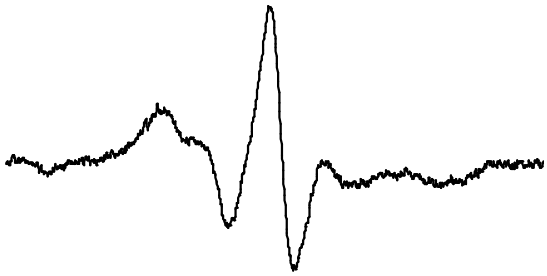

Fig. 27 Anisotropic ESR-spectrum of a spin labelled fatty acid incorporated into liposomes. (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

11. Coupling to Separation Techniques (HPLC)

Many chemical reactions normally end up in a mixture of compounds, some of them may be stable radicals. The existence of such fractions can be proven after analytical separation, in many cases by the help of chromatographic methods (HPLC). Since radicals are specifically detected by ESR spectroscopy Magnettech has developed a coupling device to link the *MiniScope MS100* as a radical detector to any chromatographic device. A particular flat cell cuvette combined with a valve unit allow measuring in a bypass during continuous flow of the HPLC (Fig. 28). In detail the arrangement enables one to keep the chromatographic flow running while a fraction could be kept without flow in the resonator for longer times exceeding the duration of the passage of a separated peak. No stopping of the whole flow is necessary to scan a spectrum if the interested fraction is eluting in a shorter time.

The valve at the end of the connecting tubings opens the flow through the resonator by closing the shunt. Whenever a spectrum is taken from a fraction of interest, the shunt is opened and the passage through the resonator is closed [18]. Inserting just a T-connector between the chromatographic device and the cuvette in the resonator keeps the dead volume low and avoids possible turbulence at this site. An additional reaction chamber or radiation window can be installed between this T-connector and the flow through cuvette if applicable.

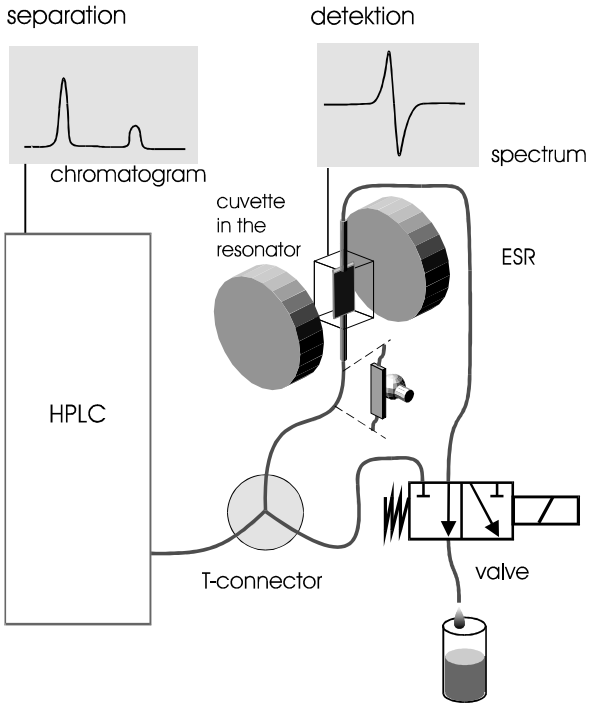

Fig. 28 Combination of a HPLC-device with the *MiniScope MS100* as a radical detector.

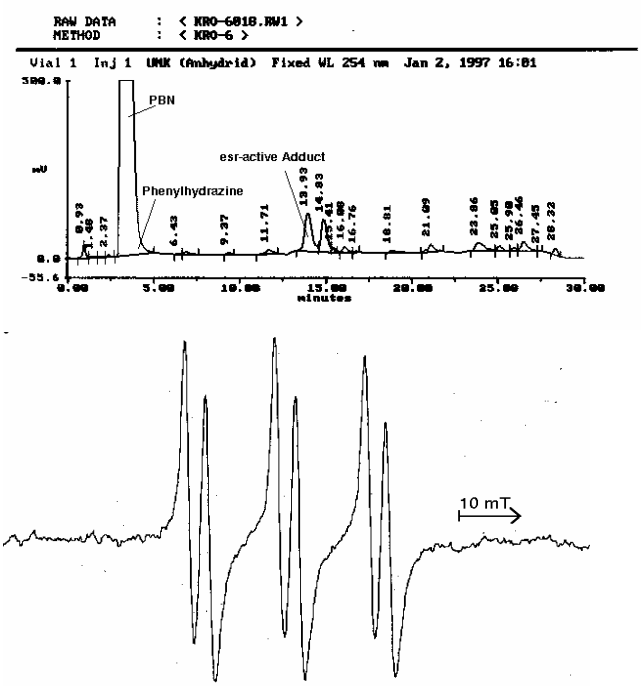

Fig. 29 HPLC chromatogram (upper panel) and ESR-spectrum of the radical containing fraction, recorded on-line (lower panel). (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

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12. Application in Petrol Industry

Chemical polymerisation may have a reaction mechanism involving radicals. Well known in the common laboratory practice is the preparation of polyacrylamide gels for gel electrophoresis. The polymerisation of acrylamide is initiated either by addition of peroxodisulfate or by riboflavin and light. In both cases free radicals are generated which trigger the chain reaction. On the other hand, existing free radicals are able to interfere with or even interrupt such a process by electron pairing. In cases where polymerisation reactions are completely undesired, the addition of stable free radicals may serve as a chemical to inhibit such reactions.

As a consequence, the monitoring of the content of free radicals which may have been added as in-process inhibitors could be a valuable control instrument. The most common stable free radicals used for this purpose are nitroxyl compounds having a very characteristic ESR spectrum entailing three lines when dissolved in a liquid (Fig. 30).

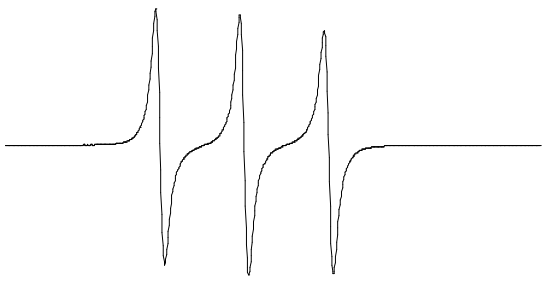

Fig. 30 Spectrum of a stable free radical entailing the NO group of a nitroxyl compound (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

In a flow through system with an appropriate cuvette it is even possible to set up an on-line monitoring system.

This example shows that the *MiniScope MS100* can be used for monitoring of free radicals used for in-process controlling.

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13.Principles in ESR

The unpaired electron either aligns with an external magnetic field or may be directed into the opposite direction. These two alignments are represented by two energy levels, the lower one as the parallel and the higher one as the antiparallel alignment (*Zeeman*-splitting, Fig. 31). The necessary outer magnetic field is provided by the magnet shown in Fig. 1. The energy needed for the transition from the lower to the higher level is within the microwave range and is radiated from the microwave bridge into the resonator. The sample containing the unpaired electrons is placed in the cavity of the resonator. Whenever such an unpaired electron absorbs energy by swapping direction a loss of energy is registered by the receiver diode of the microwave bridge.

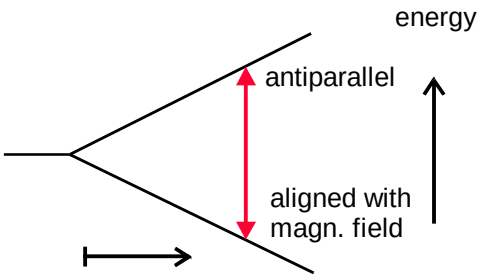

Fig. 31 Energy levels of a single electron in a magnetic field. The difference between these levels increases proportional with the magnetic field strength.

Two ways are conceivable for scanning an absorption spectrum: Either to sweep the microwave at a constant magnetic field or, vice verse, to sweep the magnetic field. Since it is technical much more feasible to realise the second way the outer magnetic field is commonly swept at a constant microwave frequency. Due to the amplification technique the absorption signal is recorded as the first derivative.

In the case the unpaired electron is located in an orbital of an atom with an uncertain number of protons, neutrons or both there is an additional magnetic field generated by the resulting nuclear spin. This magnetic field is added to the external magnetic field. Consequently the electron is also influenced by this additional field. The point of transition from the low to the high energy level is shifted accordingly to the magnetic field caused by the nuclear spin.

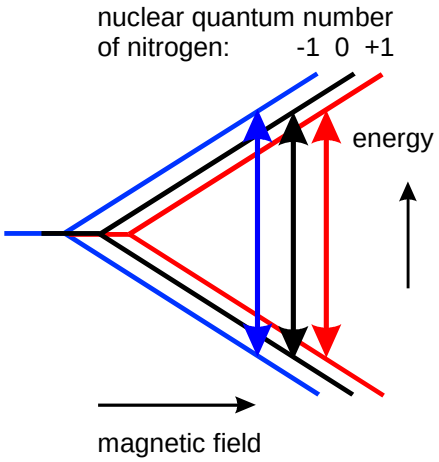

Fig. 32 Hyperfine splitting by the nuclear spin of nitrogen.

The nitrogen isotope N^{14}, with 99,64 % mostly abundant in nature, has the nuclear spin quantum numbers -1, 0 and +1. As a consequence the magnetic field seen by an unpaired electron in the vicinity of nitrogen is shifted either to a lower value (-1), left unchanged (0) or increased (+1). Microwave energy can be absorbed at three different levels, the corresponding ESR spectrum shows a *hyperfine structure* of three lines.

In the case of nitrogen the spectral absorption line corresponding to the Zeeman-effect (see above) is splitted into three hyperfine lines of equal size. If a proton with its spin quantum numbers of -½ and +½ is located in the β-position to the nitrogen then it exerts an influence on the unpaired electron and each of the three hyperfine lines caused by the nitrogen nuclear spins is splitted again into two lines. As a consequence the whole spectrum in this case consists of six hyperfine lines (3 x 2 = 6).

Once absorbed the microwave energy will be returned to the environment with time, i.e. the unpaired electron directed against the magnetic field will realign with that field. Principally two ways are possible to return energy to the environment, by interactions either with adjacent electrons or with the molecular lattice. Both relaxation processes are characterised by their own velocities. Since the relaxation velocity within the molecular lattice cannot be changed without altering the whole molecule the spin to spin distance has a significant influence on the corresponding velocity. This distance can be increased by dilution with an appropriate solvent so far that the spin-lattice relaxation becomes dominant. In this case the hyperfine splitting into three lines, caused by the adjacent nitrogen nucleus, is only seen in highly diluted samples. At higher concentrations these structures are blurred by a faster spin - spin relaxation.

14.Spin Trapping of Radicals

Commonly radicals are very reactive so they are rather short living. Only some radicals such as nitroxyl compounds stay unchanged for a longer time, even for months or years. However, life of many radicals is too short to scan a spectrum. The way how to solve this problem is to form a new compound with the highly reactive radical. Suitable reagents are nitrones or nitroxides. They adopt radical character by taking over the unpaired electron after reaction with the original radical. Since those N-oxide compounds are able to catch and to preserve the unpaired electron they are widely called spin traps. A commonly used spin trap is DMPO (Fig. 33).

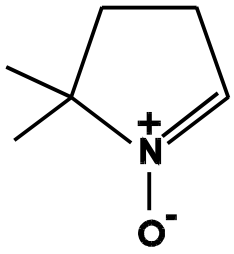

Fig. 33 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO)

Spin traps enable the detection of short living radicals by forming secondary radical adducts which exist for a longer time period so they can be studies extensively by ESR spectroscopy. Fig. 7 shows a methyl adduct of this compound.

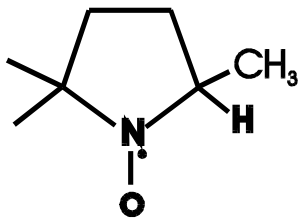

Fig. 34 DMPO methyl adduct

In this case both the nitrogen and the hydrogen in ß-position cause hyperfine splitting which results in a six line spectrum shown in Fig. 35.

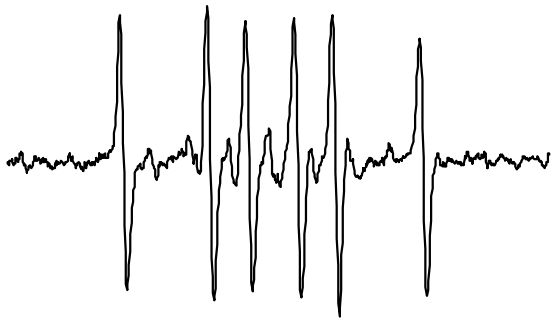

Fig. 35 ESR-spectrum of a DMPO-alkyl adduct which has been produced by massively illuminating a methylene blue solution with laser light for ca. half an hour. (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz)

A particular advantage of the spin trap DMPO is to form characteristic ESR spectra with different radicals. The hyperfine splitting by the hydrogen in ß-position depends on the radical which forms the adduct. In case of the hydroxyl radical the hyperfine splitting is lower than in the previous example leading to a four line spectrum with different heights (see Fig. 36).

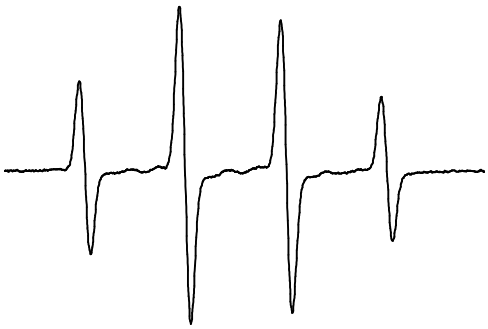

Fig. 36 ESR-spectrum of a DMPO hydroxyl adduct. (100 mM DMPO in phosphate-buffer pH 7.4, Fe(II), H_2O_2 1 %, *MiniScope* MS100: Scan time 60 s, scan 7.5 mT, 9.4 GHz).

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15.Spin Trapping Artefacts

The examples of the application of spin traps, namely DMPO, have shown that reactions with radicals may have more than a single and well defined product. Depending on the milieu a DMPO adduct may continue to react and form different subsequent products. The presence of molecular oxygen (air!) cannot always be neglected. Simple demonstration experiments may show some of the possible pitfalls everyone has to be aware of when studying radicals by ESR spectroscopy.

Hydroxyl radicals form an adduct with DMPO which shows the typical ESR spectrum with two big and two smaller lines at neutral or slightly alkaline pH values (Fig. 37). However, when incubating in an acid environment (pH 4) an additional six line component occurs in the spectrum which is typical for alkyl adducts (Fig. 38). This may be a result of secondary reactions of the adduct. As a consequence all reaction conditions should be controlled as precisely as possible. In addition alternative reaction pathways should be considered.

Many organic solvents and oils are able to solve molecular oxygen more efficiently than water. The solubility of oxygen in some oils may be about eight times higher than in water. This may have significant consequences for ESR studies: on one hand higher concentrations of superoxide radicals may occur, and on the other hand molecular oxygen is a ESR spectral line broadening agent due to its nature as a biradical (Heisenberg's spin exchange).

Fig. 39 shows the result of a very simple experiment where a DMPO sample solved in dioxane was exposed to atmospheric oxygen. The superoxide adduct gave rise to a ESR signal with four major lines which in addition were broadened by the presence of molecular oxygen. When using passive spin labels this line broadening effect of oxygen can serve to quantify its amount in the sample.

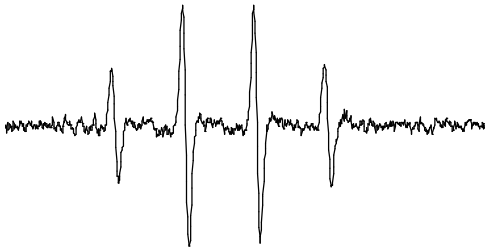

Fig. 37 DMPO, at pH 8 after addition of H_2O_2 (*MiniScope MS100*: Scan time 60 s, scan 10 mT, 9.4 GHz).

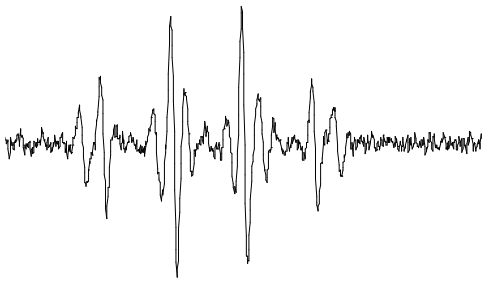

Fig. 38 DMPO, at pH 4 after addition of H_2O_2 (parameters see Fig. 37).

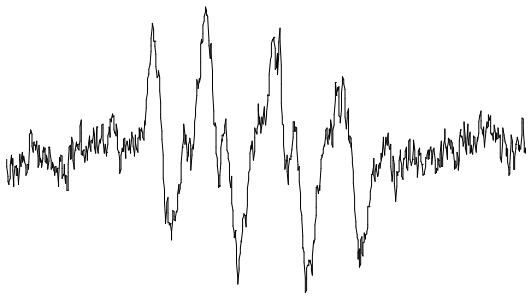

Fig. 39 DMPO solved in dioxane after 1,5 h exposed to atmospheric oxygen (parameters see Fig. 37).

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16. Acknowledgements and Literature

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11. Prof. Dr. Münzel, Medical Hospital, Dept. Cardiology, University Hospital Eppendorf, Hamburg, Germany (vascular NO measurements)
12. Prof. Dr. H. Sies, Institute of Physiological Chemistry I, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany (antioxidants)
13. Prof. Dr. Borm, Medical Institute of Environmental Hygiene, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany (radicals generated by dust)
14. Dr. Zschörnig, Institute of Medical Physics and Biophysics, University Leipzig, Leipzig, Germany (protein binding studies)
15. Dr. Hartwig Schütte, Justus-Liebig-University Gießen, Medical Hospital II, Gießen, Germany (radicals in perfused isolated organs)
16. Prof. Dr. Gerlach, Charité, University Hospital, Humboldt-University Berlin, Berlin, Germany (NO measurements)
17. Dr. Beckert, working group “Time-resolved Spectroscopy”, University Leipzig, Leipzig, Germany
18. Ch. Kroll, Institute of Pharmacy, Humboldt-University Berlin, Berlin, Germany
19. Dr. W. Herrmann, Institute of Pharmacy, Humboldt-University Berlin, Berlin, Germany

20. Prof. Francoise Nepveu, Université Paul Sabertier, Pharmcophores Redox, Phytochemie & Radiobiologie, Toulouse, France (radicals in cells and tissue)
21. Dr. Cos, Dept. of Pharmaceutical Sciences, University of Antwerp (UIA), Antwerp, Belgium (oxygen sensitive spin probes)
22. Dr. Beate Bittner, Hoffmann-LaRoche, Cardiovascular research, Dept. PRPV 93/7.24, Basel, Switzerland (release of drugs from formulations)

Selected literature

Principal:

L. J. Berliner, J. Reuben (ed.), *Biological Magnetic Resonance*, **Vol. 8**, “Spin Labelling, Theory and Applications”, Plenum Press 1989.

Spin labels:

G. A. Morrill et al. (1989), *Arch. Biochem. Biophys.* **269**, 690-694W (1989).
L. Hubbell, H. M. McConnell, *J. Am. Chem. Soc.* **93**, 314-326 (1971).
J. Seelig, *J. Am. Chem. Soc.* **92**, 3881-3887 (1970).

Spin traps:

K. T. Knecht, R. P. Mason, *Arch. Biochem. Biophys.* **303**, 185-194 (1993).
S. Pou et al., *Analyt. Biochem.* **177**, 1-6 (1989).