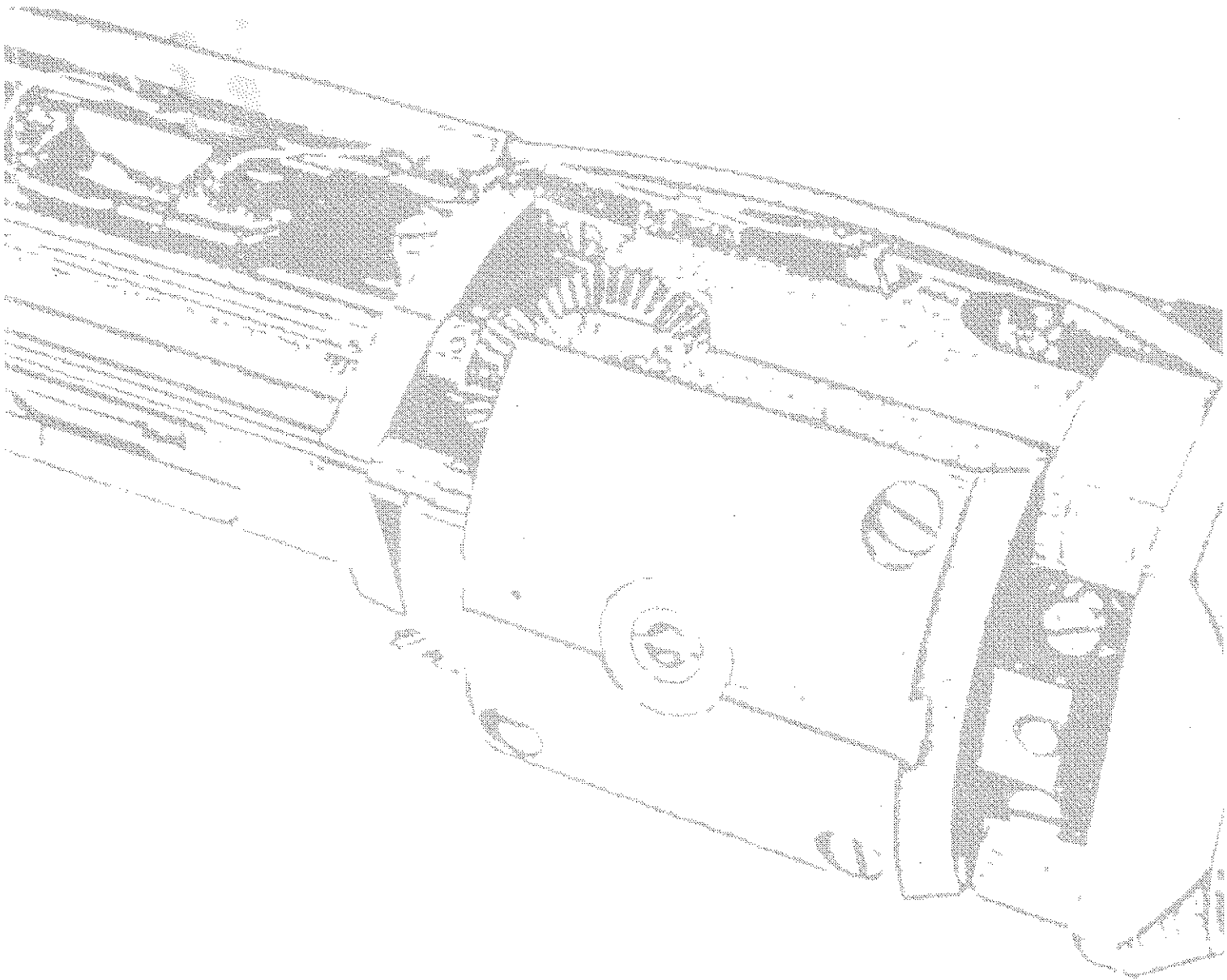
Thirteenth Meeting of the Benelux EPR Group



13th Meeting of the Benelux EPR Group 2005
Leiden – April 29

Room 427
Huygens Laboratory
University of Leiden
Niels Bohrweg 2, 2333 CA Leiden

10:00 Registration and coffee/tea on ground floor

10:30 Welcome by Edgar Groenen

10:35 Development and characterization of microdispersed forms for the measurement of oxygen and nitric oxide using *in vivo* EPR
Nicolas Charlier and Bernard Gallez (Brussels, B)

11:00 Understanding the vacuolar proton pump – spin labelling of peptides from V-ATPase
Louic Vermeer, Werner Vos, Igor Borovykh, Ruud Spruijt, Cor Wolfs, Marcus Hemminga (Wageningen, NL)

11:25 EPR spin trapping of nitric oxide and superoxide anions in cultured endothelial cells. eNOS regulation by Angiotensin II.
Irina I. Lobysheva, Chantal Dessy, Mamadou Ndiaye, Jean-Claude Stoclet, Valerie B. Schini-Kerth, Olivier Feron, Bernard Gallez, and Jean-Luc Balligand. (Brussels, B)

11:50 Effect of various salts on the structure and function of bacteriorhodopsin: site-directed spin labelling and EPR spectroscopy study
Igor V. Borovykh, Vitali Zielke, Aliaksei Krasnaberski, Matthias Pfeiffer, Dieter Oesterhelt and Henz-Juergen Steinhoff (Wageningen, NL)

12:15 LUNCH - and group photograph

14:15 Tracing the structure and possible function of human neuroglobin and its E7 mutants
Florin Trandafir, M. Fittipaldi, S. Dewilde, L. Moens, S. Van Doorslaer (Antwerp, B)

14:40 Microsecond freeze hyperquenching (MHQ): A new method to study enzyme catalysis at the microsecond timescale.
Simon de Vries (Delft, NL)

15:05 Photoinduced and pulsed EPR spectroscopy in polymer/fullerene derivative blends
Aranzazu Aguirre, G. Janssen, E. Goovaerts, S. Van Doorslaer, L.Lutsen, Vanderzande (Antwerp, B)

15:30 Pulsed and High-Field EPR for distance information: ATPsynthase as an example
Martina Huber, Stefan Steigmiller, Michael Börsch, Peter Gräber (Leiden, NL)

15:55 General discussion

16:00-18.00 Drinks on 8th floor, with visit to 275 GHz EPR

ABSTRACTS

Development and characterization of microdispersed forms for the measurement of oxygen and nitric oxide using *in vivo* EPR

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Measurements of Oxygen and Nitric Oxide (NO) within biological systems are essential in regard to their very important roles in mammals in physiological states as well as in pathological states. Our laboratory is continuously developing new EPR methods to quantify non invasively these parameters in vivo.

In vivo EPR oximetry is a powerful minimally invasive method that allows the measurement of oxygen in tissues through the use of a paramagnetic probe. In the present study, we investigated new strategies for preparing biocompatible inks containing carbon black particles (Printex U), which could be used as oxygen sensors. The carbon black particles were dispersed in solutions of biocompatible polymers of CarboxyMethyl Cellulose (CMC), HydroxyPropylMethyl Cellulose (HPMC) or PolyVinylPyrrolidone (PVP). A total of 12 polymers with different molecular weights were tested. A physico-chemical characterization of the inks was carried out to assess the sedimentation of the particles, the rheological behavior of these inks, and the relative diffusion of the inks. The preparations with CMC and PVP had the highest viscosity and stability. The presence of the polymers did not modify the calibration curves (EPR linewidth as a function of the pO_2) of the carbon black. In vivo, the oxygen sensors were stable for at least one month in muscles as the EPR linewidth remained fully sensitive to induced ischemia or carbogen challenge. The calibration curve was not modified after this period of implantation. A first study of biocompatibility was carried out in vitro (hemolysis and cytotoxicity assay) and in vivo (histological examination). No sign of toxicity was observed using these inks. These preparations are good candidates for future in vivo studies including clinical trials.

For NO measurements in biological systems, Iron-chelates based on dithiocarbamate structure (i.e. the hydrophilic compound ferro-di(N-methyl-D-glucamine-dithiocarbamate, Fe(II)-MGD, and the lipophilic compound ferro-di(diethyldithiocarbamate), Fe(II)-DETC) have been widely used as spin traps for EPR measurments. However, the efficiency of the detection is hampered by a complex redox chemistry for Fe(II)-MGD, and by the insolubility of Fe(II)-DETC in water and the impossibility to administer this pre-formed spin trap agent in vivo. To circumvent these problems, 2 new formulations based on DETC-Fe(II) have been developed: nano-emulsions stabilized by lecithin and inclusion complexes in hydroxypropyl-ß-cyclodextrin. When incubated in the presence of NO donor, the efficiency of both systems was about 4 times higher compared to MGD-Fe(II). Both systems were administrable by IV without any acute toxicity. After induction of a septic shock in mice, the EPR signal intensity recorded in blood samples was higher for both systems compared to MGD-Fe(II). The trapping efficiency in the liver and in the kidney was dependent on the form injected: the nano-emulsions of DETC-Fe(II) were the most effective for trapping NO in the liver while the inclusion complexes in cyclodextrins were the most effective in the kidneys.

Understanding the vacuolar proton pump – spin labelling of peptides from V-ATPase

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The vacuolar H+-ATPase or V-ATPase is a proton pumping membrane protein that has been linked to many diseases, including osteoporosis. A single amino acid substitution in the proton-translocating channel of this protein completely inhibits proton transport.

The membrane spanning peptide containing this crucial R-735 residue was chemically synthesized because the entire protein is too complex to purify in the amount necessary for ESR measurements. The peptide was doubly spin labelled with MTSL on two naturally occurring cysteines and reconstituted in micellar systems.

The labelling efficiency was determined by MALDI-ToF MS analysis and was optimized until efficiencies of 50% and higher were achieved. The conformation of the peptide was investigated by both CD and ESR spectroscopy. The results indicate that the structure of the peptide is a 3_0-helix. The relevance of this finding is discussed in the light of the proton transport mechanism of V-ATPase.

EPR spin trapping of nitric oxide and superoxide anions in cultured endothelial cells. eNOS regulation by Angiotensin II.

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Nitric oxide (NO) is produced by the endothelium and regulates vascular homeostasis. Bioavailability of NO decreases under pathologic conditions associated with endothelial dysfunction such as hypertension, diabetes, and atherosclerosis. Interaction of NO with superoxide anions ($O_2^{\cdot-}$) was demonstrated as one of the factors disturbing NO formation in cells.

We studied formation of NO and $O_2^{\cdot-}$ by EPR spin trapping technique using colloid solution of [Fe(II)-(Diethyldithiocarbamate)$_2$] complex and 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), respectively, in bovine aortic endothelial cells (BAEC) after angiotensin II (AngII) stimulation.

We observed that NO production was tightly correlated with that of $O_2^{\cdot-}$, i.e.:

i) Ang II increased NO and $O_2^{\cdot-}$ to 158 +/- 12% and up to 219 +/- 5% relative to control production in unstimulated cells; the former was inhibited by L-NAME (a NOS inhibitor) to 89 +/- 10% relative to control and the latter by apocynin, a NADPH oxidase inhibitor to 68 +/- 23% of control. The NO signal was also abrogated by apocynin to 67 +/- 10% of control, suggesting the implication of ROS production by NADPH oxidase in the activation of eNOS. Conversely, the $O_2^{\cdot-}$ signal was inhibited by L-NAME to 47 +/- 10% of Ang II stimulated activity, suggesting superoxide production by uncoupled eNOS in response to Ang II.

ii) Both signals were also blunted by the PI3K inhibitor, LY 294002, to 74+/- 14% relative to control, suggesting the implication of this kinase in the coupling of Ang II to eNOS and $O_2^{\cdot-}$ production.

On the other hand, several antioxidants, which may preserve NO or NO adducts from interaction with ROS, affected NO detection in cells in several ways:

i) The relatively hydrophobic and membrane-restricted antioxidants, PEG-SOD, MnTBAP or alpha-tocopherol increased the NO signal with AngII up to 156 +/- 14 % relative to the signal in cells stimulated by Ang II without antioxidants, whereas antioxidants increased the signal by only 36 +/- 10% in cells stimulated by Bradykinin.

ii) Conversely, the hydrophilic and cell diffusible N-acetyl-cysteine (NAC) or Trolox abrogated the NO signal with AngII to 21 +/- 7% and 88 +/- 5% of control, pointing out to the inhibition, by these antioxidants, of an intracellular pool of $O_2^{\cdot-}$, which is critical for eNOS activation by AngII. NAC and Trolox had no effect on the NO signal with the receptor-independent calcium ionophore, A23187 (no significant signal change).

We conclude that superoxide anions (derived from NAD(P)H oxidase) mediate the stimulatory effect of Ang II on the endothelial NO formation in a PI3-kinase-dependent manner. The differential effect of membrane-restricted versus intracellular antioxidants on the NO signal suggests that two different fluxes of ROS are stimulated by AngII in endothelial cells and play a different role in NO formation.

Effect of various salts on the structure and function of bacteriorhodopsin: site-directed spin labelling and EPR spectroscopy study

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Site directed spin labelling EPR (SDSL-EPR) spectroscopy has been used to study the influence of the salts on the structure of the membrane protein bacteriorhodopsin which has been selected as a model system. This light driven proton pump is one of the best-studied membrane proteins at the moment and the wealth amount of information about structure and function of bacteriorhodopsin obtained with different techniques are available. In our study we used three cysteine mutants which were modified with a methanethiosulfate spin label at position 101 in the beginning of cytoplasmic C-D loop and at position 158 and 167 in the first α-helical turns of helix E and F, respectively. A decrease of the mobility of spin label side chains at positions 101 and 158 and no changes in the mobility of spin label at position 167 have been observed when increasing NaCl (Hofmeister neutral salt) concentration. This observation indicates that structural changes take place in the vicinity of these labels: movement of the nearby helices or changes of the loop structure are possible explanation. When chaotropic salts NaI and KSCN are used, decreasing of the mobility has been observed for spin label side chains at position 158 (similar to NaCl). On the contrary, spin labels at positions 101 and 167 show a strong increase of the mobility. The use of the kosmotropic salt $NaCH_3COO$ results in decreasing of the mobility of spin labels for all three mutants used in our study. All together the results indicate that various salts induce similar changes of the protein structure in the vicinity of one site (position 158) but induce different changes of the protein structure in the vicinity of the two other sites. We demonstrated that SDSL-EPR technique is capable to probe the changes of protein structure and to identify the sites in the protein where those changes take place.

→ Is there a possibility to detect the HF interaction with I, Il, Br, Cl ?

Tracing the structure and possible function of human neuroglobin and its E7 mutants

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Neuroglobin (Ngb) is a newly discovered member of the globin superfamily and its function is yet unknown. Several possibilities have been suggested, amongst which an involvement in the NO metabolism or a possible oxygen-carrier function under hypoxia condition. EPR studies and optical measurements show that the heme iron in Ngb is hexacoordinated in both the ferrous and ferric form of protein.

Escherichia coli cell cultures with low O_2 concentration overexpressing proteins generate nitric oxide (NO). When wild-type (wt) human Ngb is overexpressed in *E. coli* cell cultures grown under anaerobic conditions, the protein is predominantly in the F8His-Fe^{2+}-E7His form, whereby a small percentage of the protein is in the nitrosyl ferrous form (F8His-Fe^{2+}-NO). In contrast, under the same conditions, the E7-Leu and E7-Gln mutants of the protein are mostly in the nitrosyl ferrous form. This indicates an important role of the E7 residue in stabilizing the exogeneous NO ligand. The differences in the temperature dependence of the CW-EPR spectra of wild-type, E7-Leu and E7-Gln hNgbNO will be related to the heme pocket structure.

After purification, the proteins are found to be in different states: wt hNgb shows two ferric low-spin (LS) forms whereas the E7-Gln mutant is predominantly in a ferric high-spin (HS) state. In order to derive more structural information HYSCORE measurements were done on both hNgb variants. The superposition of the two different LS forms in wt hNgb renders the interpretation of the spectra more difficult. For the mutant, the interpretation is even harder due to the almost inexistence of other studies using pulsed EPR on high-spin Fe(III) systems. In order to facilitate the analysis of the latter spectra, HYSCORE experiments and simulations were done on aquomet myoglobin for which single crystal ENDOR data are available. These experiments will be discussed in detail and related to the hNgb case.

Microsecond freeze hyperquenching (MHQ): A new method to study enzyme catalysis at the microsecond timescale.

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We have recently developed a novel ultrafast freeze-quench technique, named microsecond freeze-hyperquenching (MHQ). The current setup is 100 times faster than the classical rapid freeze quench, and samples with ageing times as short as **60 µs** (and up to 500 ms) can be prepared routinely and analyzed by low-temperature UV-Vis, resonance Raman, Mössbauer and multifrequency EPR spectroscopy (1,2). Since the great majority of enzyme conformational changes and transient intermediates occur at the µs time scale, MHQ is the first mixing/sampling technique making this time range accessible for the study of (bio)catalysis. With our generally applicable MHQ technology we can –potentially answer both new and longstanding questions in enzymology, many of which remained or have remained owing to the time limitation (~ 1 ms) of e.g. stopped-flow instrumentation.
Oxygen plays a very important role in the metabolism of numerous organisms in all three Domains of Life. O_2 acts as final electron acceptor in aerobic respiration (CcO), serves as a biosynthetic agent in primary and in secondary metabolism and in the detoxification of xenobiotics (P450); O_2 is also a signaling molecule involved in gene expression. Both CcO's and P450 have highly complex reaction mechanisms involving O=O bond activation and cleavage. Intermediates like Fe(IV)=O are known c.q. speculated to be formed on the µs time scale.
With MHQ, new intermediates in the catalytic cycle of CcO and P450 have been detected. Their roles in the catalytic mechanism will be discussed.

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2) F. G.M. Wiertz, O.-M. H. Richter, A. V. Cherepanov, F. MacMillan, B. Ludwig and S. de Vries (2004) An Oxo-ferryl Tryptophan Radical Catalytic Intermediate in Cytochrome *c* and Quinol Oxidases trapped by Microsecond freeze-HyperQuenching (MHQ) FEBSLett. 575, 127-130

Photoinduced and pulsed EPR spectroscopy in polymer/fullerene derivative blends

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In the last years a large technological effort is made to develop solar cells based on organic materials for low-cost and energy production. The active layer is made of blends of conjugated polymers with a soluble fullerene derivative (PCBM). During illumination, charge transfer processes give rise to positive radicals (or polarons) on the polymer chains and negative radicals on the fullerene molecules. The production and electronic structure of these radicals can be investigated by various EPR techniques. Both the polarons and fullerene radicals have previously been investigated by EPR in blends of a soluble PPV-polymer (MDMO-PPV) with PCBM [1,2].

We have performed 9.5~GHz (X-band) and 95~GHz (W-band) EPR measurements on composites of different conjugated polymers with PCBM to determine the effect of varying polymer/fullerene ratios, of annealing treatments and of illumination. In addition, the positive polarons in several polymers, which can be produced by doping with an electron acceptor like iodine, have been studied by W-band EPR. This yields an accurate determination of the symmetry and principal values of the g matrix of the polymer radical. A nearly axial symmetry has been observed for PPV type polymers whereas for poly-thiophene type polymers the g tensor shows orthorhombic symmetry.

Pulse EPR experiments were performed to get a more detailed insight in the electronic states of the polymer radicals, in particular in the interactions of the unpaired electrons with surrounding nuclear spins.

Proton hyperfine interactions unresolved in continuous-wave EPR, could be revealed by ESEEM and HYSCORE measurements in the doped MDMO-PPV polymer. These measurements show a high delocalization of the hole in the polaron radical.

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Pulsed and High-Field EPR for distance information: ATPsynthase as an example

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The arrangement of the b-subunits in the holo-enzyme F_0F_1-ATP synthase from *E. coli* is investigated by site-directed mutagenesis spin-label EPR. F_0F_1-ATP synthases couple proton translocation with the synthesis of ATP from ADP and phosphate. The hydrophilic F_1-part and the hydrophobic membrane-integrated F_0-part are connected by a central and a peripheral stalk. The peripheral stalk consists of two b-subunits. Cysteine mutations are introduced in the tether domain of the b-subunit at b-40, b-51, b-53, b-62 or b-64 and labeled with a nitroxide spin-label. Conventional (9 GHz), high-field (95 GHz) and pulsed EPR spectroscopy reveal: All residues are in a relatively polar environment, with mobilities consistent with helix sites. The distance between the spin-labels at each b-subunit is 2.9 nm in each mutant, revealing a parallel arrangement of the two helices. They can be in-register but separated by a large distance (1.9 nm), or at close contact and displaced along the helix axes by maximally 2.7 nm, which excludes an in-register coiled-coil model suggested previously for the b-subunit. Binding of the non-hydrolysable nucleotide AMPPNP to the spin-labeled enzyme had no significant influence on the distances compared to that in the absence of nucleotides.

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