**Summary of the Ph.D. Thesis**

# NITRIC OXIDE IN MEMBRANES AND AQUEOUS MEDIA: SPIN TRAPPING AND INTERACTION WITH SPIN LABELS. AN ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPIC STUDY

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# INTRODUCTION

One of the smallest molecules, nitric oxide (NO), studied from the XVII century and known in the past mostly as a toxic compound (a toxic gas, a pollutant from cigarette smoke and smog, a precursor of acid rain and one of the compounds responsible for destruction of ozone layer). It was discovered in 1980's also inside the mammalian body, where it plays an important role in various metabolic processes. It can function as: an endogenous vasodilator, a neurotransmitter, an inhibitor of platelet aggregation, a modulator of inflammation and immunity, being implicated in numerous diseases and metabolic processes such as, the inhibition of key enzymes in DNA synthesis, mitochondrial respiration, iron and calcium metabolism. Different pathways, enzymatic and non-enzymatic, are known for its synthesis not only in animals, but also in microbes and plants.

The metabolism of NO at the molecular level and its movement from the site of formation to its targets in different tissues still pose many open questions. NO has many important targets inside biological systems at various locations. It has a high affinity for transitions metals, especially for Fe and also for heme and nonheme-proteins, for molecules containing thiol, amino, or sulfite groups and other free radical-containing compounds, such as tyrosyl radical containing proteins. NO reacts with $O_2$, forming $NO_2$ and $N_2O_3$, with superoxide anion ($O_2^-$), forming peroxynitrite ($ONOO^-$), all these products are usually more reactive than NO itself and can induce various metabolically dysfunctions. Main questions remain as to whether all these effects are the results of NO itself or its related compounds, called generically reactive nitrogen oxide species (RNOS) making it difficult to determine which is the pertinent source *in vivo*. NO has a high diffusibility in biological systems and can permeate cell membranes. Biological membranes and hydrophobic compartments are thus, important locations for the nitrosative chemistry.

Since NO is such an important mediator of organisms metabolism, it is very important and necessary to have very sensitive and specific methods for its detection and quantitation. There are several analytical assays for the direct detection NO and related nitric oxides, as well as indirect methods. NO is a free radical, but not directly detectable by conventional electron paramagnetic resonance (EPR) method since its lifetime is too short (0.01-6 s) in biological systems. To solve this problem a spin

trapping EPR assay was applied, as the nitrosyl-adduct formed by the interaction of NO with a spin trap complex, can give a characteristic and stable spectrum. To extend our study of NO effects on its possible targets (proteins and lipids) a spin labelling EPR method has been used. Important biological components, such as proteins and lipids, are diamagnetic (or EPR-silent), but by attaching a stable free radical-compound (called spin label) at a specific site, these compounds became paramagnetic and detectable by EPR technique. The most used spin labels are nitroxide-containing molecules. Nitroxide spin labels should also to be useful tools in understanding the mechanisms induced by either $O_2$ and/or NO or their metabolites (oxidation, nitrosylation, nitration) and the reductive or protective effect induced by a reducing agent. None of these effects have been studied so far.

The EPR technique seems to be the best method for providing high sensitivity and specificity to follow the NO formation and disappearance, in both aqueous and lipid environments. It is biologically and physiologically important to understand the differences in the generation and trapping of NO by different compounds.

## OBJECTIVES

The aim of the present study was to provide a better understanding of the generation of NO from its donors, the interaction of NO with different compounds and its behaviour both in aqueous and membranous environments, because such knowledge is biologically and physiologically important.

In order to achieve this objective, various experiments were designed using the most sensitive technique for detection of NO, spin trapping EPR, in combination with spin labelling EPR, which has the unique advantage of the water-soluble and lipid-analogue spin labels being extremely sensitive to their environment, as well as susceptible for spin-spin interaction with NO.

Specific studies were designed with the following main objectives:

1. To investigate the formation and stability of the nitrosyl-adducts formed by binding of NO to iron-dithiocarbamate ($Fe(DTC)_2$) spin trapping complexes in aqueous solutions and in membranes.

2. To characterize the influence of various physical and chemical factors, the presence of reductive agents on the formation of NO-$Fe(DTC)_2$ adducts, in particular the stoichiometry of compounds involved in the reactions.
3. To determine the trapping efficiency and the limit of detection of EPR method for NO using these spin traps.
4. To assess the effect of NO, $O_2$ and ascorbic acid and their combination on stable water-soluble spin labels.
5. To investigate the diffusion and penetration of NO and its pharmacologically important donor, SNAP, in phospholipid membranes and their interaction with spin-labelled lipid analogues in comparison with that of $O_2$.

## MATERIALS AND METHODS

*Spin trapping EPR method for detection of NO in aqueous media and in membranes.*

*Spin trapping in aqueous media:* For the spin trapping EPR technique, two iron-dithiocarbamate ($Fe(DTC)_2$) spin traps specific for NO were used, a water-soluble ($Fe(MGD)_2$) and a lipid-soluble ($Fe(DETC)_2$) compound, respectively. Different sources of NO were applied, either NO gas or one of three different NO donors: $NaNO_2$, SNP or SNAP. To assess the trapping of NO in aqueous media different buffers (TRIS or PBS, 50 mM) or double distilled water (DDW) were used. To prevent the precipitation of the lipid-soluble $Fe(DETC)_2$ complex in solution, it was either included in 10% BSA or was extracted with an organic solvent, ethyl acetate. To avoid oxidation of the spin trapping complexes, a reducing agent was used, either sodium dithionite ($Na_2S_2O_4$) or ascorbic acid (AsA). The nitrosyl-adduct formed (NO-$Fe(DTC)_2$) presented a characteristic triplet EPR spectrum that was recorded at different temperatures (298 K or 165 K) and the integrated intensity of each EPR spectrum was analysed in order to measure EPR spectral parameters, such as g-values, linewidths, hyperfine splittings.

*Spin trapping in membranes:* In order to increase the stability of the nitrosyl-adducts lifetime, they were encapsulated in lipid vesicles (liposomes), formed from

multilayers of saturated phospholipids, either DMPC or DPPC. The spectra of these samples containing the NO-Fe(DTC)$_2$ complex inside liposomes, were recorded at different temperatures for various periods of time.

*Spin-labelling EPR method using water-soluble and lipid-soluble spin labels:*

*Water-soluble spin labels:* Three different nitroxides: TEMPO, 3-maleimido-PROXYL or Fremy's salt were used as water-soluble spin labels (SL). The effect of NO under aerobic and anaerobic conditions were determinated analysing the changes on their EPR spectra. In these experiments, SNAP was used as a NO donor. The formation of nitrite ($NO_2^-$), as a product of NO oxidation, was measured by the Griess reaction. Effect of AsA, as a reductive agent, was also analysed. CW power-saturation EPR spectroscopy was used to examine the effects of NO, either released by a donor, SNAP or from NO gas, molecular $O_2$ and their nitrogen-related metabolites on the saturation behaviour of a nitroxide SL at RT.

*Lipid-soluble spin labels:* As lipid spin labels, nitroxide spin-labels attached on a chain of phosphatidylcholine, (*n*-PCSL) at various positions of the *sn*-2 acyl chain (*n*=4-14) were used and incorporated in bilayer membranes of DMPC. A continuous-wave power saturation (CW-sat) EPR approach has been applied to determine the spin-label relaxation times ($T_1T_2$), also the diffusion and penetration of NO into the membrane, under anaerobic or aerobic conditions.

## RESULTS AND DISCUSSIONS

I. SPIN TRAPPING EPR ASSAY

*1. Nitric oxide in aqueous media*

The nitrosyl-adduct formed by interaction of NO with a Fe(DTC)$_2$ spin trap complex, either Fe(MGD)$_2$ or Fe(DETC)$_2$, showed a typical triplet EPR spectrum. The spectrum characteristics were not affected by the type of buffer used for sample preparations in our experiments: either TRIS or phosphate, or simply double distilled water (DDW). However, the formation of the complex is very sensitive to the presence of $O_2$, reducing agents, Fe(II):DTC ratio, temperature and concentration of the participants in the reaction. Each of these factors and parameters were studied in detail. The detection limit of this spin trapping EPR method, using SNP as NO donor,

was determined to be as low as $4x10^{-10}$ M NO, under anaerobic conditions. The concentration of the Fe(II)$(DTC)_2$ complex should be always in excess of the [NO] to ensure that all NO is trapped.

*2. NO-Fe$(DETC)_2$ complex extracted in organic solvent*

Results showed that a 60 μl volume of ethyl acetate is enough to extract all the Fe(II)$(DETC)_2$ complex formed in 0.3 – 1 ml. It was also found that a ratio of [Fe]:[DETC] as low as 1:1000 can form enough spin trapping complex to bind the NO released by 1 mM SNAP. These data suggest that very low concentrations of Fe (1 μM or less) are enough to form a Fe$(DETC)_2$ adduct capable of trapping physiological amounts of NO (0.01-10 μM) inside the body. The extraction of Fe$(DETC)_2$ or NO-Fe$(DETC)_2$ with ethyl acetate also presents the advantage of forming a very highly stable nitrosyl-adduct over several days.

*3. Spin trapping of NO in lipid multilayer vesicles*

To further increase the life-time of these iron-dithiocarbamate nitrosyl (NO-Fe$(DTC)_2$) complexes, we have embedded them into lipid multilayer vesicles (liposomes), thus improving their air-stability which also facilitates handling. The NO-Fe$(MGD)_2$ adduct encapsulated into DMPC-liposomes presented a very weak triplet signal, which disappeared after few hours, suggesting that only a low concentration of NO-Fe$(MGD)_2$ complex remained inside lipid vesicles. In the case of the NO-Fe$(DETC)_2$ complex embedded into multilayer lipid vesicles, the intensity of the triplet EPR spectrum was very strong and decreased with less than 20% after first 5-8 days after that it reached almost a constant level for more than 30 days.

II. SPIN LABELLING EPR ASSAY

*1. Soluble nitroxide spin labels*

The EPR spectra of water-soluble paramagnetic nitroxide spin labels (SLs): TEMPO, 3-maleimido-PROXYL and Fremy's salt were recorded under different chemical conditions at room temperature ($25^0$C). Typical EPR triplet spectra observed are characteristic for nitroxide SLs. The linewidths and hyperfine coupling constant ($A_0$) of each soluble SL used in these experiments are slightly different since

their chemical structure and properties are also different. Two soluble SLs are cyclic aromatic nitroxides (TEMPO and 3-maleimido-PROXYL) and the third one is a linear nitroxide compound, Fremy's salt. SNAP, as a NO donor, or NO gas was used as the NO source in these experiments.

*1.1. Effect of NO and $O_2$ on soluble SLs*

Unlike the cyclic nitroxide SLs (TEMPO and 3-maleimido-PROXYL), the nitroxide group of linear Fremy's salt seemed to be less influenced by either NO or $NO_2^-$. The results showed that the cumulative effect of SNAP and $O_2$, or $NO_2^-$, as its presence has been confirmed by positive Griess reactions, seemed to induce an enhanced decay of the EPR triplet signal intensity of nitroxides SLs, especially the cyclic nitroxides (TEMPO and 3-maleimido-PROXYL), and this decay was not dependent on the concentration, but on time. Using CW power saturation measurements it was found that the spin relaxation enhancement induced by $NO_2^-$ on nitroxide SLs, was a result of different types of spin-spin interactions as shown by the decrease of $T_1T_2$ product values. NO, $O_2$, $NO_2^-$ and $NO_2$ radical also induced a broadening effect on the nitroxide EPR signals that occurred due to Heisenberg spin exchange.

*1.2. Effect of reducing agent (AsA) on soluble SLs*

As the most potent reductant of nitroxide SLs in biological systems, we used AsA in our experiments to induce the reductive effect and to analyse its effect, if any, on the model-system used: soluble nitroxide SLs under the influence of a NO donor (SNAP) under aerobic and aerobic conditions. AsA reduced the nitroxide group as observed by the decrease of its EPR triplet signal intensity at RT. AsA affected the three nitroxide SLs, used in our experiments, in a different manner, as they were affected by $O_2$ and/or SNAP. In the case of AsA, the Fremy's salt was the most sensitive, since its EPR signal could not be detected after the addition of AsA. In the case of cyclic nitroxide SLs, the addition of AsA induced a decrease in the EPR signal intensity by 38% and 70% for 3-maleimido-PROXYL and TEMPO, respectively. It has been found that AsA was more effective in reducing the nitroxide group of TEMPO and addition of both AsA and SNAP led to the disappearance of the EPR signal of this SL. Thus, AsA at an AsA:SL molar ratio of 10:1, seems to be very effective in reducing the nitroxide group of SL, most probably by forming a

hydroxylamine. Both in the case of 3-maleimido-PROXYL and TEMPO, intensive oxidation of samples treated with SNAP decays close to zero the EPR triplet signal of nitroxide group contained by SLs. Addition of AsA after SNAP and $O_2$, but not before, seems to prevent this effect bringing up the intensity of EPR signal. The addition of AsA to any combination of SNAP and $O_2$ led to the loss of the EPR signal of the Fremy's salt.

*2. Spin labelled-PC (n-PCSL) in DMPC multilamellar vesicles. Effect of $O_2$ and NO*

In order to better understand the effect played by NO on lipids of cellular membrane, at different depths of the lipid bilayer, the influence on rotation and diffusion motions and relaxing times, spin labelled lipids-analogues, such as phosphatidylcholine (PC) labelled with a nitroxyl group at different positions of the lipid chain were used. EPR spectra of spin-labelled PC (*n*-PCSL) incorporated in DMPC multilamellar vesicles at a label:lipid ratio of 4:100, in the absence and presence of SNAP, as NO donor, were recorded at 10 $^0$C and 30 $^0$C. The control spectra with deoxygenated samples, show relatively broad lines in the gel phase (at 10 $^0$C), which is partly due to spin-spin interaction between the spin labels and partial spin-label segregation for positions *n*=8-12 of the *n*-PCSL. The presence of SNAP, at a saturating aqueous concentration (9 mM), reduces this spin-spin interaction significantly. In fluid-phase DMPC (at 30 $^0$C), where spin-label segregation is absent, SNAP exerts a strong perturbing effect on the spectra that increases progressively towards positions C10-C11 of the PC chain. The effect of SNAP on lipid chain dynamics was quantified by measuring the outer hyperfine splitting of the *n*-PCSL, in the presence and absence of the NO donor. It was observed that in the gel phase, SNAP has largest effect in the middle of the bilayer (*n*=10-12) and this effect shows a monotonic dependence on *n*. In the fluid phase, the positional dependence of the chain perturbation is qualitatively similar (but has the opposite sign) and the largest effect appears at positions *n*=10-14. The penetration profiles of NO and $O_2$ into DMPC were monitored by the relaxation enhancement of the *n*-PCSL that is induced by these paramagnetic relaxants. The spin-label:lipid molar ratio was 1:800 and the aqueous concentration of SNAP was 2 mM. Curves that saturate less readily (e.g. 14-PCSL at 10 $^0$C or 4-PCSL at 30 $^0$C) indicate faster relaxation rate products, which mean larger $1/T_1T_2$. The value of $T_1T_2$ is determined primary by the dynamics of the spin-label group, i.e. by the rate, amplitude and symmetry of its rotational diffusion.

These dynamic features increase in intensity towards the centre of the bilayer and thus the $T_1T_2$ product decreases monotonically with increasing temperature. These intrinsic values serve as references for SNAP- and $O_2$-treated samples. SNAP perturbs the mobility of the lipid chains, suggesting its incorporation into the hydrophobic interior of the membrane. Most of chemical reactions of NO are diffusion controlled and depend on the rate of collision of NO with the target molecules or functional groups. The collision rate between NO and its target is proportional to the concentration-diffusion product of NO which is determined at high vertical resolution for a phospholipid bilayer in the present study. This allowed us to observe differences between NO and $O_2$ and also to locate SNAP in the membrane. In addition, this is the first systematic study to look at the effect of this important NO donor (SNAP) on a phospholipid membrane.

## CONCLUSIONS

Our results demonstrate that EPR spectroscopy is one of the most, if not the best, method for detection of NO production and its interaction with various compounds. $Fe(DTC)_2$ complexes are very sensitive and specific spin traps for NO and together with the spin trapping EPR method, we successfully applied them to better characterise different parameters implicated in the formation of nitrosyl-adducts NO-$Fe(DTC)_2$. The trapping efficiency and the limit of detection for NO using these $Fe(DTC)_2$ spin trapping complexes were determined.

The stability of the hydrophobic NO-$Fe(DETC)_2$ complex was further improved either by extraction with an organic solvent (ethyl acetate) or by inclusion in lipid multilayer vesicles (liposomes). It is generally accepted that the effects of many drugs encapsulated in liposomes, as well as the delivery to their targets, are enhanced when applied *in vivo*. Thus, the encapsulation of these compounds (either $Fe(DETC)_2$ or a NO donor) in liposomes make them potentially applicable for clinical purposes.

Using the spin labelling EPR technique, the effect induced by NO on many of its possible targets can be studied. We have used two categories of nitroxide spin

labels; one type, which are water-soluble and can be attached to specific sites of proteins and another type, which are lipid-analogues. Nitroxide spin labels (SLs) are reduced in a different manner, in solution and in biological systems, depending on their structure, concentration and reductive/oxidative effectors found in their environment. The effects induced by NO and its metabolites (RNOS) under oxidative conditions appear to act by a complex redox mechanism on the nitroxide group of soluble SLs. Lipophilic nitroxides are especially useful for the analysis of metabolic processes because they localize in membranes, the regions of high redox activity and, with the use of structural analogues, can probe various sites in membrane where the metabolism occurs.

Our results suggest that the hydrophobic core of the fluid membrane is one primary site of NO-dependent chemistry in biological systems that should have a strong dependence on the vertical position in the membrane. The large difference observed between the gel- and fluid-membrane phases suggests that NO and $O_2$ should display significant heterogeneity in their lateral membrane distribution, if the host biomembrane contains domains (patches, rafts etc.) of different composition or different lipid chain dynamics.

Overall, the above results are important for (i) improving spin trapping and generation of NO in aqueous and hydrophobic environments, (ii) for understanding interactions of NO with other free radicals and (iii) for understanding NO-related oxidative (bio)chemistry in biomembranes.

# List of publications

PUBLICATIONS ON WHICH THE PRESENT THESIS IS BASED:

1. **Nedeianu, S**. and Páli, T. (**2002**) “EPR spectroscopy of common nitric oxide - spin trap complexes”, Cellular and Molecular Biology Letters 7(1): 142-143.

2. **Nedeianu, S**., Páli, T., Marsh, D. (**2003**) „Membrane penetration of nitric oxide and its donor *S*-nitroso-*N*- acetylpenicillamine: a spin-label electron paramagnetic resonance spectroscopic study“, Biochim. Biophys. Acta (article in press).

3. **Nedeianu, S**., Ferdinandy, P., Marsh, D., Pali, T. (**2004**) “Characterization of common nitric oxide spin trap complexes: electron paramagnetic resonance features and relative trapping efficiency”, (in preparation).

4. **Nedeianu, S**., Pali, T., Marsh, D. (**2004**) „Interaction of nitric oxide and its donor, SNAP, with nitroxyl radicals commonly used in spin labelling: an EPR spectroscopic study“ (in preparation for publication in FEBS Lett.).

CONFERENCE ABSTRACTS:

1. **Saviana Nedeianu** and Tibor Pali,
*“Characterisation of paramagnetic nitric oxide complexes by spin trapping methods in polar and apolar environments”*.
First International Meeting of Romania Society of Biochemistry and Molecular Biology, Bucharest, Romania, September, 23-27, 1998. The abstract published in Rev. Roum. Biochim 35 (1-2) 145 poster position: S7-11, 1998.

2. **Saviana Nedeianu** and Tibor Pali,
*“Characterisation of paramagnetic nitric oxide complexes by spin trapping methods”*
FEBS Advanced Course co-sponsored by SFRR “Free Radicals, Nitric Oxide and Antioxidants in Health and Disease”, International Summer School for Postdoctoral Scientists and Advanced Students, Antalya, Turkey, September, 18-24th, 1999.

3. **Saviana Nedeianu** and Tibor Pali,
*"Characterisation of paramagnetic nitric oxide complexes by EPR spectroscopy and molecular modelling",* presentation at "Straub days", Szeged, Hungary, December, 7-9th, 1999.

4. **Saviana Nedeianu** and Tibor Pali,
*"Characterisation of paramagnetic nitric oxide complexes by spin trapping methods"* 6th INTERNATIONAL SYMPOSIUM ON SPIN TRAPPING "Spin Traps, Nitroxides and Nitric Oxide: Spectroscopy, Chemistry and Free Radical Biology", Marseille, France, August 27–31$^{st}$, 2000.

5. **Saviana Nedeianu** and Tibor Pali,
*"Characterisation of NO spin traps: EPR properties and trapping efficiency"* International School of Pharmacology, the 59th NATO/ASI summer course: "Nitric oxide from basic science to clinical applications", Erice, Italy, September, 7-17th, 2000. The abstract published in "Nitric Oxide – Basic Research and Clinical Applications" vol. 317, pp. 206, by Ed. R.J. Gryglewski and P. Minuz, IOS Press, Series A Life Sciences, 2001.

OTHER PUBLICATIONS:

1. Csont, T., Szilvássy, Z., Fülöp, F, **Nedeianu, S**., Páli, T., Tosaki, A., Dux, L. and Ferdinandy P. (**1999**) "Direct myocardial anti-ischaemic effect of GTN in both nitrate-tolerant and nontolerant rats: a cyclic GMP-independent activation of KATP", Brit. J. Pharmacol. 128 (7): 1427-1434.

2. Fejér, G., Lazar, G., Szalay, K., Gyory, I., Fejes, M., Kusz, E., **Nedeianu, S**., Schmidt, T., Kontoyiannis, D., Hobbs, M., Siklodi, B., Páli, T., Kollias, G., Horwitz, M., Duda, E. (**2004**) "Adenovirus infection dramatically augments bacterial endotoxin induced TNF-$\alpha$ production and sensitises to lethal shock." (to be submitted).