Nitric Oxide in Membranes and Aqueous Media: Spin Trapping and Interaction with Spin Labels. An Electron Paramagnetic Resonance Spectroscopic Study

Ph. D. Thesis

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Contents

Abbreviations list:

ATP	adenosine triphosphate
BH_4	tetrahidrobiopterin
cGMP	cyclic guanosine monophosphate
cNOS	constitutive isoform of NOS
DETC	diethyldithiocarbamate
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DNIC	dinitrosyl dithiolato iron complex
DPPC	1,2-dipalmytoyl-*sn*-glycero-3-phosphocholine
DTC	dithiocarbamate
DTCS	N-(dithiocarboxy)sarcosine
eNOS	endothelial NOS
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
$Fe(DTC)_2$	complex of Fe with dithiocarbamate derivatives
FMN	flavin mononucleotide
FNOCT	fluorescent NOCT
Fremy's salt	potassium nitrosodisulfonate ($K_2[(SO_3)_2NO]$)
GC	guanylate cyclase
GSH	glutathione
GSNO	nitrosylglutathione
GTP	guanosine triphosphate
GTN	glyceryl trinitrate
Hb	hemoglobin
iNOS	inducible isoform of NOS
LDL	low-density lipoproteins
L-NOHA	N^{ω}-hydroxy-L-arginine
LPS	lipopolysaccharide
MGD	N-methyl-D-glucaminedithiocarbamate
NADH	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate

nNOS	neuronal NOS
NO	nitric oxide
N_2O	nitrous oxide
NO_2	nitrogen dioxide
NO_2^-	nitrite anion
NO_3^-	nitrate anion
NOCT	NO cheletropic trap
NO-Fe(II)$(DTC)_2$	nitrosyl iron-dithiocarbamate complex
NOHA	N-hydroxy-L-arginine
NOS	nitric oxide synthase
n-PCSL	1-acyl-2-[*n*-(4,4-dimethyloxazolidine-N-oxyl) stearoyl]-*sn*-glycero-3-phosphocholine
O_2^-	superoxide
$ONOO^-$	peroxynitrite
PBN	phenyl-N-*t*-butyl nitrone
PDTC	pyrrolidine dithiocarbamate
PMTMA	1-(pyrenyl)methyl-trimethylammonium
ProDTC	L-proline dithiocarbamate
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-yloxyl-3-oxide
PUTMA	11-(1-pyrenyl)undecyltrimethylammonium
RNOS	reactive nitrogen oxide species
ROS	reactive oxygen species
SIN-1	3-morpholinosydnonimine
SL	spin label
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase
TEMPO	2,2,6,6-tetramethylpiperidine-*N*-oxyl
XO	xanthine oxidase

Chapter I
Introduction

"From diabetes to hypertension, cancer to drug addiction, stroke to intestinal motility, memory and learning disorders to septic shock, sunburn to anorexia, male impotence to tuberculosis, there is probably no pathological condition where nitric oxide does not play an important role", this is how Dr. Jack R. Lancaster, Jr. advertises the home page (www.apnet.com/no) dedicated to this Janus-faced molecule: NITRIC OXIDE.

This very small and simple diatomic molecule, nitric oxide, has been the subject of thousands of articles, projects, meetings and congresses in the last decades. In 1987 it was first claimed that endothelium-derived relaxing factor could be nitric oxide and, in the meantime, it was discovered as a product of enzymatic biosynthesis of L-arginine in animals [Palmer et al, 1987]. These facts have yielded more than 90,000 scientific papers (just within the last 15 years) dealing with this remarkable molecule. Declared "the molecule of the year 1992" by Science magazine [Koshland, 1992], nitric oxide gained even more attention when, in 1998 the Nobel Prize in Physiology or Medicine was awarded to L. J. Ignarro, R. F. Furchgott, and F. Murad for their discovery concerning "nitric oxide as a signalling molecule in the cardiovascular system."

I.1. Physical and chemical properties of NO

The first mention of NO as a chemical compound led to very early writings of the Belgian scientist, J.B. van Helmont from 1620 or 1648 [Encyc.], and later, R. Boyle (1671) and G.E. Stahl (1703), who independently generated NO by heating KNO_3 ('nitre') with glowing charcoal in the absence of air [Partington, 1961]. The chemical properties of this gas were first characterized in 1772 by J. Priestley who prepared the gas by the action of nitric acid ('spirit of nitre') on metals (e.g. copper). Collecting it over water, Priestley reported its ready reaction with "common air" to produce a soluble brown gas (now known to be nitrogen dioxide, NO_2) [Bonner et al., 1996]. The naming of 'nitrous air' as nitric oxide is attributed to J.A. Murray in 1805 [Partington, 1962].

The nitric oxide, NO (IUPAC: nitrogen monoxide) molecule, contains nitrogen in the +2 oxidation state and an arrangement of 11 valence shell electrons. In the ground state of NO, its 11 electrons occupy these orbitals giving the molecule eight bonding and three antibonding electrons for the equivalent of 2½ bonds, consistent with the fact that the N-O bond length is 1,1508 Å, which is intermediate between that of typical double (1,18Å) and triple bond (1,06Å) distances. The unpaired electron is localized on the N-O bond [Bonner et al., 1996].

Removal of the single unpaired π^* electron from NO results in the oxidation product, NO^+ (nitrosonium ion). When NO is reduced by the addition of a twelfth electron, the product is NO^- (nitroxyl ion). The reactivity of NO is related to the redox couples it forms with these ions [Bonner at al, 1996]. NO can act as an oxidant (rarely): E'_7 =+1.18 V for $NO \rightarrow N_2O$ (nitrous oxide) or a reducer: E'_7 =+0.35 V for $NO_2^- \rightarrow NO$, depending on the redox environment [Henry at al., 1991].

NO is a rather hydrophobic molecule, poorly soluble in water. Its aqueous solubility depends on temperature from 3.3 mM at 0 ^{0}C to 0.1 mM at 60 ^{0}C (according to Merck Index) and 1.9-2 mM at 1 atm and 20 ^{0}C under anaerobic conditions [Archer, 1993]. NO is more soluble in organic solvents such as methanol (14 mM), toluene (11 mM) and *n*-hexane (18 mM) [Nagano et al., 2002]. NO is thermodynamically unstable, as indicated by its large positive Gibbs energy of formation ($\Delta_f G^o_{298}$=86.32 kJ/mol) [Bonner at al., 1996].

Unlike the other radicals, such as NO_2, superoxide (O_2^-) or ascorbyl (As^-), NO is unusually stable for a paramagnetic molecule and has little tendency, at standard temperature and pressure, to dimerize or disproportionate, except when it is coordinated to transition metals [Henry and Singel, 1996]. NO gas must be handled with extreme caution, being a violent poison due to its high reactivity toward molecular oxygen (O_2) yielding strongly oxidizing species such as:

- NO_2 radical, which dimerizes into N_2O_4, a reddish brown gas described as "one of the most insidious gases" [Budvari et al., 1989];
- derivatives of NO_2: N_2O_3 and N_2O_5
- anions: nitrite (NO_2^-) and nitrate (NO_3^-) and their corresponding acids: HNO_2 and HNO_3.

NO forms complexes with various transition metals: Mo and Co (in various oxidation states), Mn (II), Ni (II), Cu (I and II), Fe (II and III), which are crucial to the function of many cytochromes and oxidases [Archer, 1993; Henry and Singel, 1996].

NO forms adducts with nucleophiles (amines, sulfite, thiols, heme group etc.) [Henry et al., 1993].

I.2. Synthesis of nitric oxide

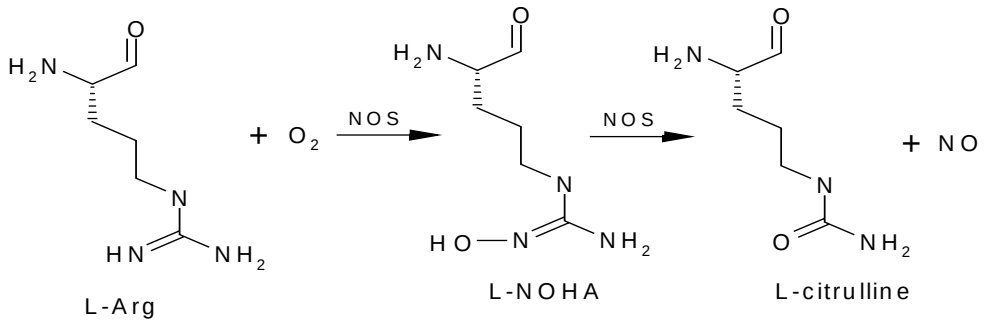
Methods for the synthesis of NO have been used since the 1840s, when Crum devised a method for preparing pure NO by shaking together nitric acid, concentrated sulfuric acid and mercury [Partington, 1964a]. Then, in 1908, Haber described the synthesis of NO in electric arc [Partington, 1964b].

Nowadays, NO is usually prepared commercially by the oxidation of ammonia (NH_3) at 500 ^{0}C over platinum gauze, used as a catalyst, although it can still be produced by passing an electric arc through the air [Braker et al., 1975]. On a laboratory scale one of the convenient methods to prepare NO is by reducing acidified nitrite with a suitable reducing agent [Bonner et al., 1996], such as a mixture of Cu and nitric acid [Zhao et al., 1996]. To produce a saturated aqueous NO solution (1.5-1.9 mM), a standard procedure can be used. Through deoxygenated double distilled cold water (or desired buffer), pure NO gas (>99.0% NO) is bubbled in a glass sampling bulb [Archer, 1993].

I.3. Biosynthesis of NO

NO is synthesised inside organisms through different enzymatic or nonenzymatic pathways or can be introduced by exogenous sources.

Mainly, NO is biosynthesed by the oxidation of L-arginine to L-citrulline, reaction catalysed by particular class of enzymes called nitric oxide synthases (NOSs), with the intermediate formation of N^{ω} -hydroxy-L-arginine (L-NOHA) [Nathan, 1992; Stuehr et al., 1992].



Scheme 1. The chemical reaction of biosynthesis of NO from L-arg in biological systems.

This pathway for NO production has been found in a wide variety of organisms [Yoshimura et al., 1997] ranging from bacteria to invertebrates and also in plants and in mammals [Palmer et al., 1987].

NOSs (E.C. 1.14.23) are NADPH-dioxygen oxidoreductases binding tetrahidrobiopterin (BH_4), FAD, FMN, Ca^{2+} and calmodulin as cofactors and having a Fe(II)-heme active center [Stuehr et al., 1992, Henry et al., 1993]. There are three isoforms of NOSs [Greenwood et al., 1997]: a neuronal isoform (nNOS or NOS I) (first identified in neuronal cells), an endothelial isoform (eNOS or NOS III), both being constitutive isoforms (cNOS), and an inducible isoform (iNOS or NOS II) (expressed in various cells in response to inflammatory cytokines and endotoxins and first identified in macrophages). All three NOS isoforms are dimeric and contain a reductase and an oxidase domain. Several cell types may contain both cNOS and iNOS. nNOS and iNOS are found mainly in the soluble portion of the cell or tissue homogenates and eNOS is largely associated with endothelial cell membranes containing an N-terminal myristoylation site and also being palmitoylated [Griffith et al., 1995].

NO is continuously produced in stimulated endothelial and neuronal cells at low concentrations (10-100 nM), relative to those produced in macrophages (10-100 μM), for short periods of time [Katayama et al., 2001]. It was found that the total daily production of NO by eNOS could be 1.7 mmol/person [Kelm and Yoshida, 1996].

It has been shown that NOS can synthesize both NO and O_2^- (superoxide anion) because of uncoupling of the electron transfer system when the amount of either L-Arg or BH_4 is limiting [Gross et al., 1995].

Other enzymatic pathways for the biosynthesis of NO were also identified. It was demonstrated that two enzymes from the urea cycle, arginosuccinate synthetase and arginosuccinate lyase, can produce NO, from citrulline, in the absence of any exogenous L-Arg [Hattori et al., 1994]. Another pathway for NO formation is the reduction of nitrite (NO_2^-) to NO either enzymatically, catalysed by xanthine oxidase (XO) under oxygenated or hypoxic conditions, or nonenzymatically under acidic medium which requires NADH as an electron donor [Zhang et al., 1998]. Exogenous sources of NO, dietary nitrate/nitrite undergo enterosalivary circulation [McKnight et al., 1999]. In humans, nitrate is concentrated in saliva and reduced to nitrite by facultative Gram-positive bacteria (*Staphylococcus sp.*) on the surface of the tongue. Salivary nitrite is swallowed into the acidic stomach where it is reduced into large quantities of NO and other nitrogen oxides that protect against gastrointestinal

pathogens [McKnight et al., 1999]. Exogenous NO can be inhaled from ambient air [Gaston et al., 1994].

NO can be produced biologically in plants and in microbes by the reduction of nitrite during denitrification [Anderson et al., 1986], reactions catalyzed by metalloenzymes, mainly nitrite reductase (NIR) [Payne et al., 1997] or by the oxidation of hydroxylamine during nitrification [Zumft et al., 1992].

I.4. Donors of nitric oxide

In order to study NO metabolism, NO donor compounds, rather than authentic NO, are used for *in vitro* and even *in vivo* experiments, due to the fact that NO gas is expensive and difficult to handle [Archer, 1993]. Any compound producing NO-related activity when applied in biological systems, and thus mimicking endogenous NO responses, is called a NO donor. Most of the NO donors behave as relaxing agents (called nitrovasodilators) [Dasgupta et al., 1998]. From the most widely clinically used glyceryl trinitrate (GTN), better known as the “explosive“ TNT, various compounds that release NO were synthesised and classified according to their chemical structures [Feelish et al., 1996].

The pathways leading to NO formation differ significantly among individual compound class reactions, such as:

- enzymatic or nonenzymatic catalysis,
- interaction with thiols or reduction/oxidation reaction,
- susceptibility to changes in pH, oxygen, light or temperature
- possibility of formation of different by-products during decomposition.

For the choice of a NO donor several factors should be considered:

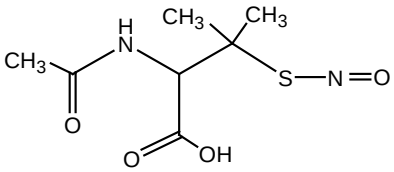
- the diversity of the pharmacological tools used as NO donors,
- the highly variable rate of NO release by these compounds,
- the possibility that they release other radicals, in addition to NO [Schmidt et al., 1997].

NO concentrations released from donor compounds are difficult to predict as they are determined by formation and inactivation reactions, which usually take place with very fast rates. It is important to know how the NO release from NO donors depends not only on the chemical and physical conditions of the surrounding environment, but also where this process takes place, in the aqueous (cytosol) or hydrophobic cellular compartment (membrane).

In this work, in order to mimic a wider range of NO behaviour *in vivo*, we have used different NO donors (SNP, SNAP and $NaNO_2$) with various properties (amount and half-life of NO released, sensitivity to various conditions).

SNP (sodium nitroprusside, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$) is one of the most widely studied of the iron nitrosyls and it is used clinically to reduce blood pressure in hypertensive emergencies [Feelish et al., 1996]. SNP is an inorganic complex where Fe(II) is bound to NO^+. One-electron reduction of the Fe centre leads to the formation of $[Fe(CN)_5NO]^{3-}$ which can be reoxidized, by O_2 for example, or can release its trans-cyanide ligand to form $[Fe(CN)_4NO]^{2-}$ with the release of NO and cyanide anions. SNP requires either irradiation with light or one-electron reduction to release NO. It was shown that SNP is a NO^+ donor in the dark and can simultaneously release NO, NO^+ and cyanide anions in the light [Cooper et al., 1995]. The predominant reactions occurring during photolysis of SNP are the photooxidation of the metal centre (i.e. Fe(II) $-e^- \rightarrow$ Fe(III)) followed by the reduction of the nitrosyl cation (i.e. $NO^+ -e^- \rightarrow {}^{\cdot}NO$) [Singh et al., 1995].

In biological systems, both nonenzymatic and enzymatic NO release from SNP may occur, most probably involving membrane-bound thiols. The presence of nucleophiles, redox-active agents (such as thiols, amines, ascorbate and superoxide) and ambient light has a great impact on the extent and rate of NO release from SNP. Since nitroprusside reduction is accompanied by cyanides release and SNP is usually administrated by continuous infusion, cyanide toxicity may constitute a complication with long-term use.



SNAP

S-nitroso-N-acetylpenicillamine (SNAP) is a nitrosothiol which can release NO spontaneously. It has been reported that the half-life ($t_{1/2}$) of SNAP, in buffer at pH 7 and 37 ^{0}C, is 5 h and the factors that influence its stability are temperature, buffer preparation, aerobic condition, presence of metal impurities and light [Ramirez et al., 1996]. In 50 mM phosphate buffer at room temperature, SNAP solubility is about 2 mg/ml [Ramirez et al., 1996] and 25 mg/ml in dimethylsulfoxide or ethanol. It has been intensively used as a NO donor in *in vitro* and *in vivo* experiments on animals. It has been shown that infusion of SNAP plays a cardioprotective role *in vitro* reducing cardiomyocyte death [Zhang et al., 2002] and reactive oxygen intermediates in isolated rat hearts [Suessenbacher et al., 2002]. SNAP can also decrease the mean arterial blood pressure and induce a

significant increase in heart rate [McGrowder et al., 2001]. In atherogenesis, SNAP was demonstrated to reduce the uptake of acethylated LDL (low density lipoprotein), attenuating the formation of atherosclerotic plaques [Berkels et al., 2000].

In biological systems, nitrite (NO_2^-) was found to produce NO enzymatically (by xanthine oxidase, XO) or nonenzymatically, under acidic conditions, such as decreased pH during ischemia (from 7.1 to 5.5) [Zweier et al., 1995a], in phagocytes [Harvey, 2000] and probably under normal conditions in the stomach (pH=3) and in the mouth [Zweier et al., 1999], since nitrite is one of exogenous sources of NO (nutritional sources of NO, NO_2^-/NO_3^- are provided by food, especially meat, green vegetables, and drinking water). Some authors suggested that the reduction of NO_2^- to NO could take place even at physiological pH induced by exogenous Fe-citrate, when the compound was injected [Vanin et al., 2001].

I.5. Role of nitric oxide in biological systems and membranes

The NO production inside mammalian bodies at different locations under normal and different pathophysiological conditions has been highly investigated, even in real time [Ye et al., 2003, Berliner el al., 2001, Yoshimura et al., 1996], but 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. The metabolism of inhaled, injected or endogenously formed NO in the body depends on diffusion, convection, distribution and chemical reactions within the different compartments. Its life-time depends on the cell type and the composition of the environment in which NO exists.

NO is a molecular “double-edged sword”; although excess of NO can cause many organ dysfunctions and diseases, it remains an important modulator of a wide variety of functions in the cardiovascular, central nervous and immune systems. Excessive and unregulated NO synthesis has been implicated as causal or contributing to pathophysiological conditions including many lethal and debilitating diseases: vascular or septic shock [Lai et al., 1994], stroke, diabetes, neurodegeneration, arthritis, reperfusion injury, chronic inflammation [Gross et al., 1995] multiple organ failure and eventually death. 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* [Wink et al., 2000].

A new concept appeared, the “chemical biology of NO”, which separates the chemical reactions of NO with various compounds into two basic categories: direct and indirect effects [Wink et al., 1998]. Direct chemical reactions are those in which NO interacts with biological targets, most commonly heme-containing proteins, metal-oxygen complexes and radical species. These reactions are generally rapid, require low concentrations of NO and most likely account for the majority of the physiological effects [Wink et al., 2000]. Indirect effects involve RNOS, derived from reactions between NO and either O_2 or O_2^-, rather than NO itself, reacting and modulating critical intracellular molecules. In these reactions higher, sustained NO concentrations are implicated, leading to either nitrosative (induced by N_2O_3) or oxidative stress (mediated by $ONOO^-$ and NO^-) [Wink et al., 1997]. Both direct and indirect reactions occur close to cells, such as macrophages, that produce high levels of NO, while cells farther away primarily experience direct effects.

Of the reactions of NO with a metal centre, the most relevant is that with Fe and implicit with heme-proteins, such as cytochrome P450 [Adams et al., 1992], NOS [Griscavage et al., 1995], catalase [Wink et al., 2000], prostaglandin H synthase and cyclooxygenase (COX) [Tamir et al., 1996]. However, the most notable is the soluble guanylate cyclase (GC) (found in almost all mammalian tissues) that leads to the conversion of GTP to cyclic GMP (cGMP) and to a cascade of molecular events within target cells, which depends on the cell types, such as the relaxation of smooth muscle, platelet function and neurotransmission [Gross et al., 1995, Ignarro, 1990, 1991]. NO is also known to disrupt, depending on signalling pathways, Fe-S cluster-containing enzymes, such as mitochondrial aconitase, complex I and II of the mitochondrial respiratory chain, transcription factors and proteins implicated in iron metabolism: ferritin and transferrin [Tamir et al., 1996]. Dinitrosyl dithiolato iron complexes, together with other nitrosyl-thiol compounds (glutathione and cysteine) are suggested to be NO carriers inside the body and to transfer the NO group to other specific targets (i.e. metalloproteins) by transnitrosylation [Ueno et al., 2002].

NO reacts also with other radical-containing molecules, such as a variety of lipid-oxy and lipid-peroxy adducts formed during lipid peroxidation, that resulte in damage of the cell-membranes. NO can act as an inhibitor of peroxidation by reacting with peroxide-mediated lipid radicals [Puppo et al., 1988; Padmaja et al., 1993], but might also be a promotor via its reaction with redox-active metal ions and the production O_2^- [Stuehr et al., 1992]. Lipoxygenase, which mediates a variety of lipid oxidation steps, and XO are also inhibited by NO [Wink et al., 2000]. NO reacts

with tyrosyl radical ($Tyr^{\cdot}$)-containing proteins, such as ribonucleotide reductase (RNR) [Lepoivre et al., 1992], the inhibition of which only seems to occur in the membrane [Wink et al., 2000], or prostaglandin H synthase, with which NO forms a tyrosine iminoxyl radical [Henry et al., 2000].

The reaction with superoxide radical (O_2^{-}) dominates the fate of NO because the kinetics is diffusion-limited and the product of the reaction, peroxynitrite ($ONOO^{-}$), decomposes rapidly into NO_2· and HO·. These oxidants are all substantially more toxic than NO itself [Henry et al., 1993]. $ONOO^{-}$ can react with DNA, proteins and cell membrane lipids [Tamir et al., 1996]. This instability of NO *in vivo* is partially ameliorated by presence of superoxide dismutase (SOD) that decreases the amount of O_2^{-}· [Archer et al., 1991].

One of the mechanisms responsible for the disappearance of NO is its reaction with O_2. The autooxidation of NO yields to NO_2^{-}, NO_3^{-}, under physiological conditions of temperature, O_2 tension and pH. In water, nitrous and nitric acids are the final products, via N_2O_3 formation [Caccia et al., 1999]. The half-life of NO depends on the concentration of both NO and O_2 and was reported in biological systems to be 0.01-5 s [Katayama et al., 2001, Henry et al., 1993], depending also on the cellular source [Lancaster, 1994], although in physiological salt solutions, its half-life is about 6 to 60 s [Gross et al., 1995]. This it happens because in an aqueous solution NO reacts much more slowly with O_2 than in a lipid medium [Stuehr et al., 1992].

NO is a hydrophobic molecule with a high diffusibility in biological systems and can permeate cell membranes. The reaction between NO and O_2 is much accelerated within the membrane because of the increased reactant concentrations (10-50 times) [Liu et al., 1998, Denicola et al., 1996] and higher solubility of both NO and O_2 in membranes, even if the intrinsic rate constant of the reaction within the membrane hydrophobic phase is the same as in the aqueous cytosol [Liu et al., 1998; Wink et al., 1998]. In the presence of micelles, the rate of autoxidation is increased by more than three orders of magnitude, relative to the rate in aqueous solution. Relatively low amounts (3 mg/ml) of hydrophobic phase (soy phospholipid vesicles, a nonionic detergent or isolated membranes from rat hepatocytes) dramatically increase the total rate of NO disappearance [Liu et al., 1998]. For NO producing tissues, such as brain and liver with a phospholipid content of approximatively 30 mg/ml, that means that the rate of NO reaction with O_2 is 8 times

more rapid than if the phospholipids were not present and that 90% of the reaction takes place within the membrane, as opposed to the cytosol [Liu et al., 1998]. Using an EPR method, it has been found that for a pure lipid bilayer (unsaturated phosphatidylcholine), the NO diffusion-concentration product in the hydrocarbon region of the membrane is higher than in water, while in the polar headgroup region, it is about the same [Subczynski et al., 1996]. Addition of cholesterol (30%) decreases this product in and near the polar headgroup region, while significantly increasing it in the centre of the lipid bilayer, as well as increasing the membrane permeability coefficient for NO. A similar profile was found for O_2 [Subczynski et al., 1996]. It seems that the presence of cholesterol and membrane proteins facilitates the diffusion of NO, as well as O_2, toward the membrane centre. Using an average value of the NO diffusion coefficient in the membrane, an initial NO concentration of 1 μM at the site of production and 10 nM at the site of action, the biological NO diffusion path could be estimated to be 10-70 times the mean diameter of a cell (10 μM) [Denicola et al., 1996, Lide, 1990], suggesting that endogenously produced NO may be able to exert its biological effects far from its source of production. Steady-state gradients of NO within tissues and cells are controlled by rates of NO synthesis, diffusion, and decomposition [Gardner et al., 2001].

It is clear, also from the above overview, that biological membranes and hydrophobic phases are important locations for the nitrosative chemistry, which occurs upon NO autooxidation, formation of nitrosothiol, nitrosamine and lipid peroxide, DNA damage and genotoxicity. The membrane-bound proteins that are functionally and structurally dependent on certain thiols or amines would be the most affected by nitrosative stress (induced by N_2O_3) [Wink et al., 2000]. It has been suggested [Liu et al., 1998] that the reaction of NO with HO_2, the conjugate acid of O_2^- (pK=4.8) may occur within the lipid bilayers, resulting in the formation of ONOOH, which could react further with CO_2, also within the hydrophobic core of the membrane. For example, two cysteine-containing proteins from the sarcoplasmic reticulum (SR) implicated in Ca^{2+} homeostasis, a calcium-released channel (ryanodine receptor) and Ca-ATPase, were found to be activated or inhibited, by NO and $ONOO^-$, depending on concentration [Viner et al., 2000]. NO might also interfere in the interaction between Ca^{2+} and membrane-associated proteins such as spectrin [Sheetz, 1983]. There are still many other cytosolic or membrane-bound proteins, others than Fe-containing proteins, which were more intensively studied. Different components of specific internal and external membranes are to be investigated as

possible targets of NO or its related compounds (RNOS) under various physiological and pathological conditions. It follows then that it is absolutely essential to understand the generation, distribution and trapping of NO in membranes. Its distribution in the membrane is particularly important in comparison with that of oxygen, whereas its generation and trapping is important in comparison with those in aqueous environments.

I.6. Methods for detection and quantitation of NO

Since NO is such an important mediator of organisms metabolism, very sensitive and specific methods are needed for its detection and quantitation. Measurement of NO in biological specimens is difficult, because of the small amount present (1-50 nM up to 10μM), its lability in the presence of O_2 [Archer, 1993] and its reactivity with various compounds.

There are several analytical assays for the direct detection NO and related nitric oxides, as well as indirect methods, which measure different products of its formation or those formed during its interaction with various biological compounds. Techniques for direct identification of NO are:

1) chemiluminiscence reaction with ozone;
2) electrochemical measurements;
3) mass spectrometry (MS), or combined with gas-chromatography (GC-MS)
4) fluorimetric assays;
5) electron paramagnetic resonance (EPR).

1) *Chemiluminescence assay*

NO reacts with ozone producing NO_2 and light [Zafiriou et al., 1980]. This assay was originally developed to measure gaseous NO as an atmospheric pollutant [Katayama et al., 2001]. It is also an assay for nitrite [Aoki, 1990]. This method is sensitive to $(1\text{-}5)x10^{-12}$ M NO, with a detection threshold of roughly 20 pmol of NO [Zafiriou et al., 1980]. This technique requires relatively large sample volumes (> 1 ml) and is incapable of monitoring intracellular NO. In place of ozone luminol can be used, but this technique has other physiological limitations for the functional analysis of NO, such as the use of cytotoxic H_2O_2 [Katayama et al., 2001].

2) *Electrochemical assay using microelectrodes specific for NO*

There are currently two amperometric methods available for measuring NO from intact tissues and single cells, using either a classical Clarck electrode (with a detection limit of 1-3 μM NO) or a porphirinic microsensor (with a detection threshold of 10^{-20} M and a response time of 10 ms) [Archer, 1993]. Even if this technique offers great promise for the measurement of NO production in single cells, both in culture and *in situ*, [Malinski et al., 1992] and in real time the only spatial information is directly at the electrode tip and it is therefore influenced by the placement of the electrode [Katayama et al., 2001].

3) *Mass spectrometry*

This method is detecting NO in gaseous form using isotope labelling (^{15}N) allows for continuous measurements with short time responses that are especially useful for the calculation of kinetics [Payne et al., 1996]. MS can be used to investigate NO metabolism in complex biological systems as well as in reaction mixtures containing purified enzymes [Payne et al., 1996].

4) *Fluorometric assay*

It is a sensitive technique for the detection NO and oxidative products of NO based on their reaction with a (non)fluorescent compound forming a fluorescent nitrosyl adduct. 2,7-dichlorofluorescin, the hydrophilic DAN (an aromatic diamino compound) or its hydrophobic analog DAN-1 EE, are just few examples of nonfluorescent compounds oxidized by NO to fluorescent ones (dichlorofluorescein, NAT or DAN-1 T, respectively) having different degrees of specificities and various applications [Meineke et al., 2000; Nagano et al., 2002]. Other fluorescent probes for NO are pyrene derivates, PMTMA (1-(pyrenyl)methyl-trimethylammonium), a hydrophilic and PUTMA (11-(1-pyrenyl)undecyl-trimethylammonium), a hydrophobic compound, used to study the diffusion coefficient of NO inside the membrane [Denicola et al., 1996].

5) *EPR spectroscopic assay*

NO, though being paramagnetic, is not usually detectable by itself in liquid or frozen solutions by conventional electron paramagnetic resonance (EPR) under standard conditions as the relaxation time of the stimulated electron-spin to ground state is too rapid to be detected [Maples et al., 1991]. To solve this problem, one uses spin-trapping methods that allow the detection of free NO in different biological systems,

both *in vitro* and *in vivo*, in liquid phase at room temperature or in frozen samples. (Since spin trapping was our method of choice, section I.7.2 is devoted entirely to this assay.)

6) There are several assays that detect different products of NO metabolism, indirectly reflecting the presence of NO, but not always specific for NO, since these compounds could be products of other pathways:

- measurement of cGMP asseses the effect of NO on GC [Archer, 1993];
- measurement of nitrite/nitrate (NO_2^-/NO_3^-) accumulation using UV-visible spectroscopy (known as the Griess reaction) is indicative of NO oxidation [Ignaro et al., 1987, Shikano et al., 1988].
- measurement of citrulline, a coproduct of the conversion of L-arginine by NOS [Hecker et al., 1990].
- infrared spectroscopy (IR) for the identification of NO-bound compounds such as: S-nitrosothiols (RS-NOs) and metal nitrosyls (M-NO) [Nagano et al., 2002];
- resonance Raman (RR) spectroscopy for M-NO [Nagano et al., 2002];
- capillary electrophoresis which is the newest method for detecting NO_2^-/NO_3^- and RS-NOs [Nagano et al., 2002].

These assays evaluate the NO pathway at varying levels, but none is sufficiently specific to be considered a "stand alone" assay for NO.

I.7. EPR method for detection and quantitation of NO

I.7.1. General remarks

Electron paramagnetic resonance (EPR) is a spectroscopic technique that deals with the transitions induced between the Zeeman levels of a paramagnetic system situated in a static magnetic field (B). Transitions can occur between the Zeeman levels if the sample is irradiated with an electromagnetic field of proper frequency (ν) such that the photon energy ($h\nu$) matches the energy level separations (ΔE). This field is polarized in a plane perpendicular to the static field direction [Berliner, 1976]. The resonance condition is leading to energy absorption by the sample from the oscillation magnetic field is met when:

$$\Delta E = h\nu = g\beta B \qquad (1)$$

in which β is the Bohr magneton, a physical constant of the electron related to its charge (e) and mass (m_e) and the Planck constant (*h*):

$$\beta = eh/4\pi m_e \text{ , } \beta_e = 9.274 \times 10^{-24} \text{ J T}^{-1} \quad (2)$$

and *g* is the *g*-factor, the Zeeman splitting factor, a dimensionless constant. For the free electron, *g* = 2.0023. The *g*-value is used to characterize EPR spectral features. A specific property of EPR spectra derives from the coupling of the electron spin with the nuclear magnetic moments, so called hyperfine coupling (*A*). The hyperfine pattern depends on the nuclei with which the unpaired electron interacts, hence on the chemical structure of the paramagnetic molecule.

The linewidth of an EPR line is governed by contributions from two kinds of relaxation processes, i.e. relaxation times. The unpaired electron of a paramagnetic species does not only interact with B and the oscillating magnetic field leading to resonance, but also with their surroundings, the “lattice”. Thus, the first contribution to the linewidth is the spin-lattice or longitudinal relaxation time, T_1, which is a measure of the recovery rate of the spin populations after a perturbation. Since T_1 is governed by the electronic properties of the paramagnetic center and its surroundings, its determination can help to identify, and further characterize, the paramagnetic center [Campbell and Dwek, 1984]. The second contribution to the linewidth is given by the spin-spin or transverse relaxation time, T_2, which is related to the peak-to-peak separation of a resonance and gauges the thermal contact among the spins [Berliner, 1976]. Both relaxation times, T_1 and T_2, contribute to the line broadening of an EPR spectrum, but since for nitroxide spin labels T_2 is typically about two orders of magnitude smaller that T_1, it is T_2 that primarily determines the linewidth.

A simple method for characterizing the relaxation times is the “continuous microwave power-saturation” (CW-saturation) technique. If the microwave power, *P*, applied to the sample is sufficiently small that the transitions between the spin levels are driven at a rate significantly less than $1/T_1$, then the population adheres to its thermal equilibrium value, and the signal scales linearly with $\sqrt{P}$. If the microwave power is much larger, then the system will be driven into ‘saturation’ and the increase in signal with increasing microwave power is depressed. The shape of saturation curve is related to the product of relaxation times, T_1T_2.

NO is a free radical and its unpaired electron is in the antiboding π orbital. The EPR technique is considered to be the most appropriate tool for its detection. However, direct EPR measurements of NO are only possible under two special conditions: NO in a pressure-controlled gas phase or adsorbed in inorganic matrixes. Beringer and Castle were first to observe a X-band spectrum of gaseous NO consisting of 27 lines [Beringer et al., 1950]. Lunsford reported the EPR spectra of NO adsorbed on MgO, ZnO, ZnS and zeolite and recently Q- (35 GHz) and W-band (95 GHz) EPR spectra and electron nuclear double resonance (ENDOR) spectroscopies have identified new adsorbed stated of NO in zeolites [Katayama et al., 2001].

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I.7.2. Spin trapping technique

Direct EPR detection of endogenous free radicals in biological samples requires that at steady state the free radical of interest is present at a concentration more than the practical EPR detection limit (0.1 – 0.01 μM) and that it has a relatively long lifetime [Katayama et al, 2001]. Although such direct detection may be possible at cryogenic temperature, the *in vivo* detection of NO at ambient temperature seems almost impossible because bioradicals do not have sufficiently long lifetimes to survive the measuring period. NO, having a relaxation time of the stimulated electron to ground state too rapid, is itself undetectable by conventional EPR [Maples et al., 1991]. For its detection in solutions or biological samples, one can use spin-trapping methods conceptually similar to those used for detection of $O_2^{-}\cdot$ and HO· radicals [Henry et al., 1993]. Spin traps are compounds that interact with the unstable radicals, producing a more stable adduct which can then be detected by EPR. The spin trapping agents can detect NO directly and they can be applied to real-time monitoring and spatial imaging of biological NO [Katayama et al., 2001].

In EPR, the commonly used frequency range is 10^9 – 10^{11} s^{-1} (1-100 GHz). EPR signals from large biological samples cannot be detected with a conventional X-band (9.5 GHz) spectrometer because of the high dielectric loss of water at these frequencies and small size of the EPR cavity resonator. Thus, EPR spectrometers operating at lower frequencies (L-band (1.2 GHz), S-band (3.5 GHz)) are now applied to examine larger, inhomogeneous objects, such as *in vivo* measurements of whole body of small animals. Short half-lives and low concentrations of endogenously synthesized and metabolically produced free radicals in physiological and pathophysiological processes make *in vivo* EPR detection difficult and this is

exacerbated by poor sensitivity of the spectrometer. Despite the facility of *in vitro* spin trapping of oxygen-centred radicals, its use for *in vivo* detection has been accomplished with difficulties because of the low rate of radical adduct formation and the low stability of the adduct in biological situations [Katayama et al., 2001].

I.7.2.1. Nitroxide spin traps

For the detection of NO and oxygen-centred radicals, nitroxide spin traps can be used [Arroyo et al., 1991, Greenberg et al., 1990]. There are two types of nitroxide spin traps: nitrones and nitroso compounds. The nitroso spin traps (MNP = 2-methyl-2-nitrosopropane and DBNBS = 3,5-dibromo-4-nitrosobenzene) are better suited for the identification of NO-related signals than the nitrones (DMPO = 5,5-dimethyl-pyrroline-N-oxide and its related spin trap, DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide), PBN (α-phenyl-N-tert-butyl-nitrone) and POBN (α(4-pyridyl-1-oxide)-N-tert-butyl-nitrone)) [Arroyo et al., 1991]. DMPO, MNP, DBNBS spin traps were applied to the measurement of NO in cell cultures [Katayama et al., 2001]. Nitrones are unstable at acid pH and tend to yield NO-type spectra even in the absence of NO.

A new class of compounds, called NO cheletropic traps (NOCTs) (double carbon-centered biradical equivalents), were designed to trap NO with high affinity and were used in cell culture experiments [Korth et al., 1992; Katayama et al., 2001]. These compounds can also trap NO_2 and the EPR spectrum of NO_2 adducts is distinguishable from that of the NO adducts. Unfortunately, these NOCTs are not available commercially. Another class of spin traps for NO detection are nitronyl nitroxides, stable radicals originally developed as spin-labelling reagents, which react with NO to yield imino nitroxides radicals. Their disadvantage is that both nitronyl and imino nitroxides are nonspecifically reduced by various biological reductants such as ascorbate, thiol-containing compounds and superoxide anions or NO_2 and the rate of reaction with NO is low (10^4 M^{-1} s^{-1}) [Katayama et al., 2001].

I.7.2.2. Heme proteins as spin traps

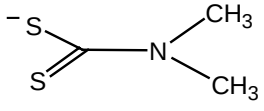
As shown previously, NO also reacts with the iron center of heme-proteins, forming nitrosyl derivates, which are EPR active and exhibit a characteristic EPR spectrum. [Katayama et al., 2001]. In the blood, NO interacts, with high affinity with hemoglobin (Hb) from erythrocytes [Eriksson, 1994], the product of the reaction being either NO-Hb (nitrosyl-haemoglobin) or methemoglobin (Hb-Fe^{3+}), depending on the oxidative state of Hb [Henry et al, 1991]. NO-Hb is a paramagnetic (S=1/2) complex and can

be detected by EPR spectroscopy offering a convenient method of spin-trapping for NO. OxyHb can be oxidized by NO to paramagnetic (S=5/2) EPR detectable methaemoglobin Hb(Fe^{3+}) and also yielding nitrate and nitrite, by a autocatalytic reaction. NO also binds slowly and reversibly to Hb-Fe(III) to form diamagnetic nitrosyl-metHb (Hb-Fe(III)NO) which eventually autoreduces to a paramagnetic species Hb-Fe(II)NO [Bonner et al., 1996]. Reducing agents (dithiothreitol, ascorbate, cysteine, gluthatione) maintain heme-iron in the ferrous form (Fe^{2+}) and favour the formation of paramagnetic nitrosyl-heme complexes [Craven et al., 1978]. The observed complex (Hb-NO or Mb-NO) spectra are sensitive to many factors, such as quaternary and tertiary structures of the proteins, concentrations of NO and O_2, pH, degree of hydration, temperature, isolated species etc. [Hall et al., 1996, Sanches, 1988, Martin et al., 1988].

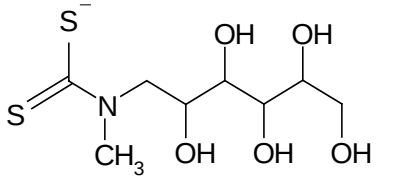
I.7.2.3. Iron-Dithiocarmabate complexes as spin traps

A relatively recent class of compounds used as high affinity NO spin-traps are the iron dithiocarbamate type of complexes. Dithiocarbamates (DTCs) are strong chelators of heavy metals and have been used as fungicides, herbicides and insecticides, but they and their disulfides also have antioxidant and prooxidant properties in biological systems [Katayama et al., 2001]. Their biological activities are attributed to their metal-chelating and/or thiol-delivery properties. DTCs have been clinically used, safely and efficiently, in the treatment of HIV-infected patients [Hosni et al., 1992]. Some biological activities of DTCs may affect the physiological roles of NO or other biological compounds.

The dithiocarbamate derivatives are classified after the solubility of the complex formed with iron. Thus, there are water-insoluble Fe-DTCs (e.g. DETC (N,N-diethyldithiocarmabate) and PDTC (pyrrolidine dithiocarbamate)) and water-soluble Fe-DTCs (e.g. MGD (N-methyl-D-glucamine dithiocarmabate), DTCS (N-(dithiocarboxy)sarcosine), ProDTC (L-proline dithiocarbamate), and HED (bis(hydroxyethyl)) complexes.



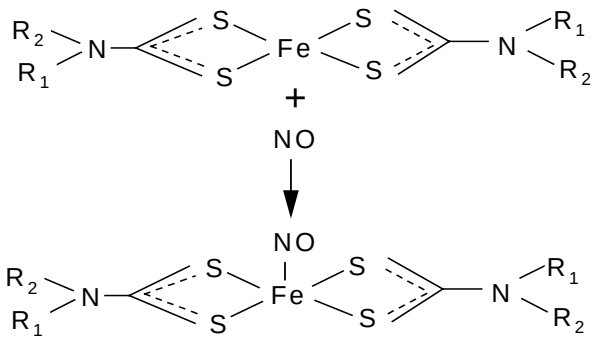
N,N-diethyldithiocarbamate
(DETC)



N-methyl-D-glucamine dithiocarbamate
(MGD)

Scheme 2. Chemical structures of the derivatives of dithiocarbamates used experimentally.

NO interacts with high affinity with Fe(II)-DTC complexes, forming stable nitrosyl iron-dithiocarbamate complexes with a three line EPR spectrum (g_{av}~2.04, A_N~1.27 mT) at room temperature and a spectrum with axial symmetry ($g_{\perp}$~2.037, g_{II}~2.015) in frozen state. The triplet (three-line) splitting originates from the hyperfine interaction of an unpaired electron with the ^{14}N nucleus (I=1) in NO. If nitrogen of the NO source is ^{15}N (I=1/2), a doublet (two-line) signal (g_{av}~2.04, A_N~1.8 mT) is observed [Nagano et al., 2002]. The structure of the Fe(II)-DTC complex and the model of its reaction with NO is presented in Scheme 3:



Scheme 3. Model of reaction between NO and iron-dithiocarbamate complex.

$Fe(II)(DTC)_2$ is rapidly oxidized by traces of O_2 to yield $Fe(III)(DTC)_3$ complex. It was observed that NO reacts not only with $Fe(II)(DTC)_2$ complex but also with $Fe(III)(DTC)_3$ to form the stable NO-$Fe(II)(DTC)_2$ adduct in aqueous solution, suggesting that the reaction occurs via reductive nitrosylation [Tsuchiya et al., 1999; Vanin et al., 2000]. Three mechanisms for the reductive nitrosylation of $Fe(III)(DTC)_3$ complexes were proposed by three independent groups [Yordanov et al., 1982; Vanin et al., 2000; Fujii et al., 2000a].

The addition or administration of exogenous NO traps can cause disturbance to biological specimens to some extent. Thus, exogenous Fe, supplied in NO trapping through $Fe(DTC)_2$ complexes, may affect NO production in cells and tissues, as well as increasing the amount of $Fe(II)(DETC)_2$ available to trap it [Komarov et al, 1997; Kubrina et al., 1992]. An interesting observation is that free Fe^{2+} destroys NO in solution [Moncada et al., 1990]. DETC was found to be an exogenous antioxidant that attenuates NO production in mouse liver *in vivo* [Mikoyan et al., 1995]. It could also react with oxyHb, producing several products, such as ROS, metHb, sulfHb,

oxidized DETC and lipid peroxidation products besides inhibiting Cu/Zn-SOD [Katayama et al., 2001].

The LD_{50} for acute toxicity of most used DTCs in mice was estimated as 1.9 g/kg (orally) [Merck Index, 1996] or 1.3 g/kg (intravenously, i.v.) [Nagano, et al., 2002] for DETC·Na·$3H_2O$ and more than 2.5 g/kg (intraperitoneally, i.p.) [Pieper et al., 1996] or over 7 g/kg (i.v.) for MGD·Na [Nagano, et al., 2002]. The toxicity of $Fe(DTC)_2$ complexes has not been determined [Nagano, et al., 2002].

$Fe(DTC)_2$ complexes seem to be the best spin traps for detection of NO *in vivo* and in real-time measurements, since their rate constant of reaction (10^8 M^{-1} s^{-1}) [Nagano et al., 2002] is much larger than other used spin traps, such as PTIO (~10^4 M^{-1} s^{-1}) [Akaike et al., 1993], oxy-Hb ($3.7x10^7$ M^{-1} s^{-1}) [Doyle et al., 1981] and deoxyHb ($1.5x10^7$ M^{-1} s^{-1}) [Gibson et al., 1957].

In solution, NO trapped by $Fe(MGD)_2$ forms a stable and water soluble NO-$Fe(MGD)_2$ complex with an EPR spectrum characterized by a^N=12.5 G and g_{iso}=2.04 at 22 ºC [Komarov et al., 1993]. $Fe(DETC)_2$ complex forms with NO a stable hydrophobic paramagnetic complex, NO-$Fe(DETC)_2$, characterized by an EPR signal at $g_\perp$ = 2.035 and $g_{||}$ = 2.022 with triplet hyperfine structure (HFS) at $g_\perp$ [Xia et al., 1997]. $Fe(MGD)_2$ complex has been used to detect NO in different organs of LPS-treated animals [Reinke et al., 1996; Lecour et al., 1999], *in vivo* in real time using L-band EPR [Fujii et al., 1997], in urine [Komarov et al., 1995] and in septic shock [Lai et al., 1994], in LPS-stimulated macrophages [Kotake et al., 1995, Kotake et al., 1996] and neural cell cultures [Kotake et al., 1996]. The spin trapping method was found to be very sensitive, with a lowest limit of the detection of 3 pmol/min for *in vivo* measurements [Kotake et al., 1996]. NO production was detected in real time in the tail blood circulation of the conscious mouse (using a S-band EPR spectrometer) [Komarov et al., 1993], in mice bodies after exposure to high-dose of X-ray irradiation [Nakagawa et al., 2001] and in the bile flow of anesthetized rats [Kotake et al., 1999].

Vanin and his colleagues [Vanin et al., 2000] were the first to use $Fe(DETC)_2$ complex as a NO trap complex. It is known that DETC is soluble in aqueous media, but the ferrous and the mononitrosyl ferrous complexes of DETC precipitate at neutral pH. Another disadvantage appeared when DETC was administred alone in biological systems, since it penetrates the cell wall and binds, not only the free intracellular Fe^{2+}, but also to the Cu^{2+}, forming a Cu(II)$(DETC)_2$ complex characterised by four-line EPR spectrum (with a^{Cu}~4.9 mT [Henry et al., 2000]) that

overlaps with the NO-iron adduct signal at low temperature [Suzuki et al., 1997]. To avoid this problem DETC can be incorporated into yeast membranes [Mikoyan et al., 1994] which then serve as carriers for the [NO-Fe$(DETC)_2$] complex [Vanin et al., 2000] or Fe$(DETC)_2$ can be used in the colloidal form which seems to be more sensitive for quantification of NO *in vitro* [Kleschyov et al. 2000].

When injected in the animal body, the Fe$(DETC)_2$ complex seems to be well distributed throughout the body, as observed by the detection of the NO-Fe$(DETC)_2$ complexes in different organs *in vitro* and *in vivo* in animal models under normal conditions [Wallis et al., 1996] or various stress and shock pathologies [Fujii et al., 1999a], cancer processes, heart and brain ischemia-reperfusion [Kuppusamy et al., 1995a; Sato et al., 1993; Sato et al., 1994], autoimmune diseases, viral [Zweier et al., 1995b; Bune et al., 1995; Quaresima et al., 1996] and bacterial infections [Fujii et al., 1999b], in solid tumors and ascites [Henry et al., 2000] and gamma-irradiation [Voevodskaya et al., 1992].

I.7.3. Spin labelling EPR technique

As mentioned previously, the EPR method is characteristic for paramagnetic molecules such as free radicals, compounds containing unpaired electrons. But most compounds of biological interest, such as proteins and lipids, are not detectable by EPR technique (being diamagnetic) unless stable free radicals (called spin labels) are attached to them at a specific site in their structure; the technique is known as spin labelling EPR.

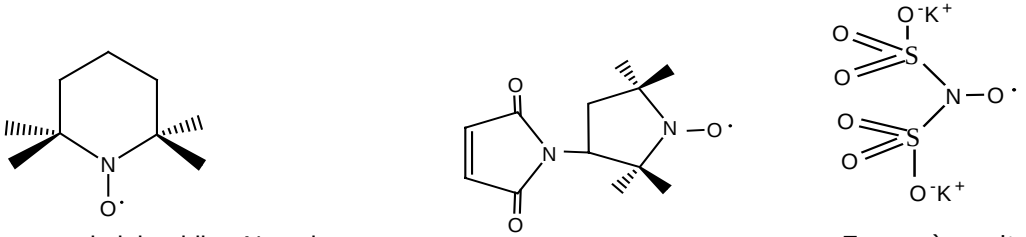
The term spin label (SL) was introduced by McConnell to describe a stable free radical. The most commonly used SLs contain a nitroxide moiety [Campbell and Dwek, 1984]. Nitroxide spin labels are unusually stable free radicals, due to the fact that their radical centre is sterically protected. They are, however, very sensitive to the physico-chemical environment which surrounds them and thus, used to study molecular mobility of proteins and lipids in membranes, enzyme active sites, DNA synthesis and measure electrical potential, pH and temperature, as well as distribution of paramagnetic molecules (metal ions or triplet state molecules like oxygen) in cells, membranes or tissues, and more recently, as contrast agents in magnetic resonance imaging (MRI) and EPR imaging (EPRI) in studies of biochemical interest [Iannone et al., 1993].

As presented previously, NO, being a hydrophobic molecule, can freely diffuse through the biological membranes unless it meets some of its specific targets associated with them. Numerous studies concerning NO diffusion through the cellular membrane and its role on different molecules inside or associated with the membrane using different approaches have been done, but it's not very clear whether NO or its metabolites (RNOS) are responsible for the effects attributed to NO. In order to study and obtain a better characterization of NO effects on a cytosolic level or its penetration into lipid membrane, we have used various nitroxide spin label compounds. In addition, being free radicals, spin labels are also directly exposed both to NO-related chemistry and spin-spin interactions with NO.

Attempts have been made to study the effect of NO [Nakajima et al., 2002], NO_2 radical [Goldstein et al., 2003] and $ONOO^-$ [Wróbel, 2001] on different nitroxides in various biological systems (microsomes, erythrocytes) but the kinetics and mechanisms of the reaction of nitroxides with nitrogen-derived reactive species are far from being elucidated.

I.7.3.1. Soluble nitroxide spin labels in aqueous media

We have used three different water-soluble nitroxide spin labels, with different chemical structures (shown in Scheme 4.) and properties: two cyclic compounds: 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and 3-maleimido-PROXYL and a linear compound: Fremy's salt. All these compounds are relatively stable paramagnetic molecules and in solution, they exhibit the typical nitroxide EPR triplet spectrum.



Scheme 4. Chemical structures of soluble nitroxide-spin labels.

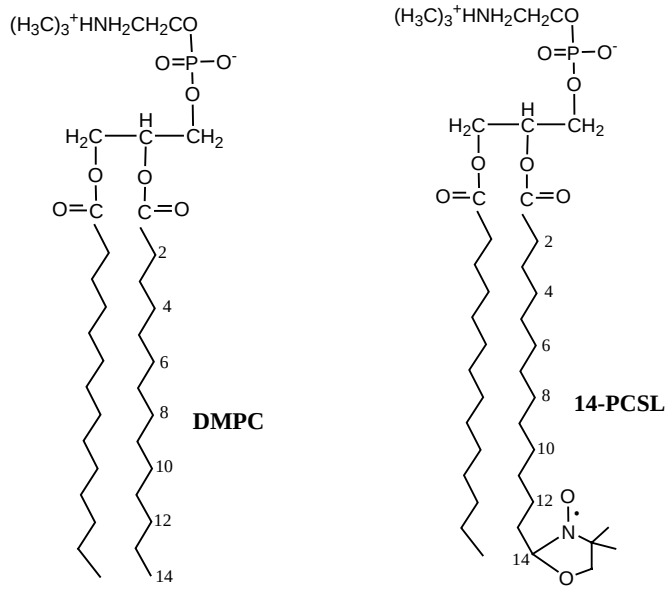
It has been observed that cyclic nitroxides can protect biological systems from radical-induced damage, playing an antioxidant role. Besides this role, 3-maleimido-PROXYL can be attached to thiol groups of proteins thus, being useful in the determination, by EPR methods, of dynamics and mobility properties of different

soluble or membrane associated proteins and polipeptides. Fremy's salt is used as an EPR standard and it was proposed as a model of the peroxyl radical because both are isoelectric in structure and react in a similar way with ascorbic acid [Colacicchi et al., 2000]. Nitroxide radicals are transformed to EPR-silent hydroxylamines in biological systems by various reductants. The most potent reductive agent in biological systems seems to be ascorbic acid (AsA).

Thus, nitroxide spin labels seem 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 ascorbic acid. None of these effects have been studied so far.

I.7.3.2. Lipid spin labels in lipid vesicles

Phosphatidylcholine (PC) or lecitin, a saturated lipid, is one of the most abundant phospholipids in various biological membranes and is mostly used in mimicking membrane behaviour. By attaching a spin label to a lipid molecule, various spin labelled lipids analogues are obtained having a doxyl group at position ranging from membrane-water interface to the centre of the hydrophobic core of the bilayer. Using phosphatidylcholine nitroxide spin-labelled at various positions of the *sn*-2 acyl chain (*n*-PCSL, with *n*=4-14) incorporated in bilayer membranes of DMPC, the translational and rotational mobility of lipids can be studied, e.g. through membrane phase transtitions [Páli et al, 1996] or when exposed to interaction with membrane proteins [Marsh and Horváth, 1998]. A continuous-wave power saturation (CW-sat) EPR approach has been applied to determine the spin-label relaxation times (T_1T_2) [Marsh et al., 1997]. This allows detection of the effect of water-soluble and membrane-permeable paramagnetic compounds on the spin labels in membranes providing information on the spin label location and the penetration of such compounds into the membrane [Livshits et al., 2001; Páli et al., 1992].



Scheme 5. Chemical structures of a lipid (DMPC) and a spin label analogue (14-PCSL) having a nitroxide group (SL) attached at position 14 on its carbon chain.

The physical state of the membrane (e.g. fluidity, rotational and translational diffusion) can be tested at different depths in the phospholipid bilayer using spin labelled lipids. These labelled lipids are also sensitive to redox reactions, which mainly occur in the membrane, an important aspect in NO biochemistry. In addition, the spin relaxation times (T_1T_2) of spin-labelled lipids are sensitive to spin-spin interactions between the spin label molecules or with “external” fast-relaxing paramagnetic molecules like NO or O_2 [Dzikovski et al., 2003; Páli and Marsh, 2002; Subczynski et al, 1996].

We aimed to analyse the diffusion and penetration NO and O_2 into lipid vesicles at different depths of the bilayer and also for the rather hydrophobic SNAP, a NO donor. The characterization of the membrane penetration of this donor is interesting because it is expected to release NO in the membrane during *in vivo* applications. Since there are numerous hydrophobic targets for NO, this investigation will be relevant not only to the use of SNAP-based S-nitrosothiols as NO donors, but also to the reactivity of NO in membranes and the consequent effects on the cellular function.

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 it is biologically and physiologically important to understand the differences in the generation and trapping of NO by different compounds.

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, into phospholipid membranes and their interaction with spin-labelled lipid analogues in comparison with that of O_2.

Chapter II

Materials and methods

II.1. Chemicals

The water-soluble spin labels: 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl (3-maleimido-PROXYL) and potassium nitrosodisulfonate ($K_2[(SO_3)_2NO]$) (Fremy's salt) were purchased from Aldrich Chemical. Co., Inc. (Milwaukee, WI). Acid ascorbic (AsA), potassium carbonate (K_2CO_3), Tris (hydroxymethyl)-aminomethane (TRIS) and organic solvents: chloroform ($CHCl_3$) and methanol (CH_3OH) were all purchased from Merck (Darmstadt, Germany). Ethyl acetate was purchased from J.T. Baker B.V. (Deventer, Holland). Diethyldithiocarbamate (DETC), iron sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), sodium dithionate (hydrosulfite, $Na_2S_2O_4$), sodium nitrite ($NaNO_2$), bovine serum albumin (BSA), naphthylethylenediamine, sulphanilamide were obtained from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). The saturated lipids 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC) and 1,2-Dipalmytoyl- *sn*-Glycero-3-Phosphocholine (DPPC) were purchased from Avanti Polar Lipids, Inc. Sodium salt monohydrate of [*N*-(dithiocarbamoyl)-*N*-methyl-*D*-glucamine] ($MGD{\cdot}Na{\cdot}H_2O$) and S-nitroso-N-acetylpenicillamine (SNAP) were purchased from Alexis Biochemicals (Lausen, Switzerland). Sodium nitroprusside (SNP) has been kindly donated by Dr. Peter Ferdinandy from the University of Szeged, Hungary. Spin-labelled phosphatidylcholine (*n*-PCSL, with *n* = 4-14) (1-acyl-2-[*n*-4,4-dimethyloxazolidine-*N*-oxyl)steroyl]-*sn*-glycero-3-phosphocholine) was synthesized according to Marsh and Watts, (1982). Oxygen and argon gas (both of 99.9% purity) tanks were purchased from Messer Griesheim GmbH (Frankfurt, Germany). NO gas was purchased from Praxair (Westerlo, Belgium).

II.2. Spin trapping of NO in aqueous media

All solutions were freshly prepared in an Ar-saturated solution, before use. For the preparation of the solution of water-soluble spin trapping complex, the $Fe(II)(MGD)_2$, $FeSO_4$ and MGD were dissolved separately in a small aliquots of degassed double distilled water (DDW) or buffer (pH 7.4), either TRIS 50 mM or PBS (phosphate buffer

saline, KH_2PO_4 and Na_2HPO_4, 100 mM) at desired concentrations. The solution of the hydrophobic spin trapping complex, i.e. $Fe(II)(DETC)_2$, was prepared by mixing a solution of DETC in 10% BSA with a solution of $FeSO_4$ at desired concentrations. To avoid oxidation, $Na_2S_2O_4$ or ascorbic acid (AsA) were used as reducing agents. A NO donor ($NaNO_2$, SNP or SNAP) was added to the spin trap solutions under anaerobic conditions. After preparation a 5 µl aliquot of the nitrosyl spin trap complex solution was loaded into a 1 mm (i.d., inner diameter) capillary tube and the EPR spectrum was recorded immediately at room temperature (298 K) or frozen in liquid nitrogen and recorded at lower temperatures (165 K or 190 K).

In the low temperature measurements (165 K), a small volume (not more than 30 µl) of solution containing either $NO\text{-}Fe(DETC)_2$ or $NO\text{-}Fe(MDG)_2$ was introduced into a 4-mm diameter EPR quartz tube and the mixture was frozen in liquid nitrogen. Spectra of empty quartz tubes were also recorded under the same conditions for baseline correction. All spectra were recorded at microwave powers in the range of 0.51-128 mW for continuous wave (CW) saturation measurements.

The settings of the EPR spectrometer were: microwave frequency 9.3 GHz, centre field 3350 G over a scan range of 340 G, modulation amplitude 2.85 G, modulation frequency 100 kHz, conversion time 40.96 ms, time constant 20.48 ms, magnetic field resolution 1024 points. The final spectra were the result of the accumulation of either 4 (for spectra recorded at 298 K) or 9 scans (for spectra recorded at 165 K) with a sweep time of 41.943 s per scan.

II.3. Spin trapping of NO in lipid media

Multilayer vesicles (MLVs) or liposomes of either DMPC or DPPC (1mg/ml) were prepared following a standard method [Páli et al., 1996]. Stock solution of $FeSO_4$, MGD and DETC in 50 mM $Na_2S_2O_4$, $NaNO_2$, SNP and SNAP were freshly prepared before use, in either Tris buffer 50 mM (pH 7.4) or phosphate buffer 50 mM (pH 7.4), and used on the same day. Depending on the type of the compound to be encapsulated inside liposomes, two categories of MLVs were made. (i) For $Fe(II)(DTC)_2$ in MLVs, the spin trapping complex (either $Fe(II)(MGD)_2$ or $Fe(II)(DETC)_2$) was incorporated into the lipid vesicles during hydration. Unencapsulated spin trapping complex was removed by rewashing with a solution containing 1 mM NO donor compound ($NaNO_2$ or SNP or SNAP). (ii) For NO donor in MLVs, 1 mM NO donor compound ($NaNO_2$ or SNP or SNAP) was incorporated into

the lipid vesicles and the resulting pellets were rewashed with a solution containing a spin trapping complex (either Fe(II)$(MGD)_2$ or Fe(II)$(DETC)_2$) (also in order to remove the unincorporated NO donor). The final multilayer vesicles (MLVs) were pelleted in EPR capillaries in a bench centrifuge at 10000 rpm for 10 min. The supernatant was removed and small aliquots (about 10 μl) were loaded into an EPR capillary. These capillaries were inserted into the EPR cavity and the EPR signal of these samples was recorded at different temperatures.

In the case of DPPC-MLVs, a supplementary freeze-thaw procedure was applied and repeated several times for a better encapsulation of the desired compound into the lipid vesicles. Each set of samples was incubated at the specific temperature at which they were recorded.

The settings of the EPR spectrometer were: microwave frequency 9.3 GHz, centre field 3350 G over a scan range of 340 G, modulation amplitude 2.85 G, modulation frequency 100 kHz, conversion time 40.96 ms, time constant 20.48 ms, magnetic field resolution 1024 points and 4 scans recorded for each spectrum with a sweep time of 41.943 s per scan.

II.4. Preparation of samples with water-soluble spin labels

The water-soluble spin label (TEMPO, 3-maleimido-PROXYL or Fremy's salt) was dissolved in DDW at either 3 or 30 mM concentration yielding stock solutions that were used immediately. Care was taken to protect Fremy's salt (dissolved in 50 mM K_2CO_3 solution, forming a characteristic violet colour) from light, in order to avoid photodecomposition. The concentration of Fremy's salt was determined spectrophotometrically at 248 nm (molar extinction coefficient, ε=1670 M^{-1} cm^{-1}) and 540 nm (ε=20.8 M^{-1} cm^{-1}) at room temperature in a 1 cm light-path quartz micro-cuvette using a UV/vis Shimadzu UV-160 spectrometer.

For the oxygen effect, the DDW was flushed with O_2 gas for more than 30 min before and, when desired, after the spin label was dissolved. For the NO effect, a small aliquot of a NO donor (SNAP), at a concentration of 2 mM or 30 mM, was added to the spin label solution. According to the desired interaction, in different experiments, either Ar gas or O_2 gas was flushed over the spin label solutions for more than 30 min. For some experiments, a small volume of ascorbic acid (AsA), at a concentration of 3 mM, was added to the spin label solutions. A 5 μl volume of the

final solution was loaded into a 1 mm (i.d.) capillary that was sealed and the EPR signal was recorded immediately at room temperature (298 K).

The settings of the EPR spectrometer were: microwave frequency 9.3 GHz, centre field 3350 G over a scan range of 120 G, modulation amplitude 2.85 G, modulation frequency 100 kHz, conversion time 40.96 ms, time constant 20.48 ms, magnetic field resolution 1024 points and 4 scans recorded for each spectrum with a sweep time of 41.943 s per scan.

II.4.1. Determination of nitrite by Griess reaction

The Griess reaction reagents, sulphanilamide 2% and naphthylethylenediamine 0.2%, were dissolved in DDW as stock solutions. These solutions can be stored at 2-4^{0}C for several weeks. Aliquots of 450 μl of each Griess reagent were mixed with 100 μl of a probe and the final solution of was incubated at 56 ^{0}C for 30 min. A typical red-colour of this solution is characteristic for a positive Griess reaction. The optical density of each sample was measured spectrophotometrically at 540 nm, at room temperature (25 ^{0}C), in a 1 cm light-path quartz micro-cuvette, using a UV/vis Shimadzu UV-160 spectrometer.

II.5. Lipid spin labels (*n*-PCSL) in DMPC membranes

DMPC (10 mg/ml) and lipid spin labels (*n*-PCSL) (1 mg/ml) were prepared as a stock solution in a organic solvent mixture of $CHCl_3$:CH_3OH=2:1, (v/v) and kept in the freezer before use. DMPC vesicles were labelled with *n*-PCSL at a molar ratio of either 1:800 or 4:100, as follows. A defined amount of *n*-PCSL (*n*=4-14) was added to 100 μl DMPC stock solution and after mixing, the solution was dried under N_2 gas and then, under a vacuum overnight. The dry lipid-film obtained was hydrated with 50 or 100 μl of water (DDW), in the low- and high-label concentration experiments, respectively, and loaded into a standard 1 mm (i.d.) EPR glass capillary. According to the particular experiment, Ar or O_2 gas was flushed into DDW before and after hydration of the lipid-films. In some experiments, the dry lipid-film was hydrated with either Ar or O_2 gas flushed DDW containing either 2 mM SNAP (for *n*-PCSL:DMPC ratio of 1:800) or 30 mM SNAP (for n-PCSL:DMPC ratio of 4:100). DMPC vesicles were pelleted in the EPR capillaries in a benchtop centrifuge at 10000 rpm for 10 min at room temperature. The supernatant and some lipid material (if necessary) was

removed to leave a uniform pellet of 5 mm in length and the capillary was filled with the requisite gas before being sealed. Sealed capillaries, containing the spin-lipid probes, were kept at 2-4 ^{0}C before the EPR spectrum was recorded. EPR continuous wave (CW)-saturation spectra of each probe were recorded at a temperature of 10 ^{0}C, 20 ^{0}C or 30 ^{0}C, over a microwave power range from 0.020 to 200 mW.

The settings of EPR spectrometer were: centre field 3307 G over a width of 120 G, modulation amplitude 1 G, modulation frequency 100 kHz, conversion time 41.96 ms, time constant 20.48 ms, magnetic field resolution 1024 points and 4 scans recorded for each spectrum with a sweep time of 42 ms per scan.

II.6. EPR spectroscopy

The EPR spectra were recorded using a Bruker (Rheinstetten, Germany) ECS106 (in Szeged) and EMX (in Göttingen) X-band (9.3 GHz) spectrometer, equipped with a computer controlled thermostat and TE_{102} cavity, using 100 kHz field modulation and an ER041 X-band microwave bridge. Samples were positioned along the symmetry axis of the 4-mm standard quartz sample tube, which contained light silicone oil for thermal stability. The sample temperature was computer-controlled by a nitrogen gas-flow system that regulated the temperature of the silicone oil. Samples were centered in the TE_{102} microwave cavity.

For CW-power saturation experiments, a calibration was made between the microwave power output and the microwave field (H_1) incident on the sample, and corrections were also made for differences in the cavity Q between different samples as described in Fajer et al., (1982).

Simulation of frozen and solution EPR spectra of nitrosyl spin trap complexes, including the determination of peak-to-peak distances, as well as fitting, processing and presentation of the data and CW saturation curves, was performed using Igor (WaveMetrics, Lake Oswego, OR) with software written by T. Páli. Integration of the spectra was done either with Igor or with WinEPR (Bruker, Karlsruhe).

Results and Discussions

Chapter III
Spin Trapping

III.1 Nitric oxide in aqueous media

As it has been previously shown (section I.7.2), in order to be detectable by EPR techniques, NO has to be stabilized as a polyatomic adduct by using spin trap compounds or complexes. We limited our studies to the spin traps that are most widely used and suitable for *in vivo* and clinical applications, namely the iron-dithiocarbamate ($Fe(II)(DTC)_2$) complexes. The two dithiocarbamate compounds, we have used were the diethyl- (DETC) derivate and the N-methyl-D-glucamine- (MGD) derivate (for chemical structures see Scheme 2), both of them being soluble in aqueous media, but via their reactions with Fe(II), they form complexes with different solubility. $Fe(II)(DETC)_2$ is liposoluble and $Fe(II)(MGD)_2$ is hydrosoluble. NO reacts with these $Fe(II)(DTC)_2$ complexes forming a stable paramagnetic adduct characterized by a typical EPR triplet spectrum (see Fig. 3.1.1). Without NO as a ligand these ferrous dithiocarbamate complexes ($Fe(II)(DTC)_2$) are diamagnetic and thus, EPR inactive.

The EPR signal of the nitrosyl complex ($NO\text{-}Fe(II)(DTC)_2$) presents a typical g_{iso} = 2.04 at room temperature (RT) and an axial symmetry at low temperature with $g_{\perp}$ = 2.037 and g_{II} = 2.015 [Fujii et al., 2000b]. The spectrum characteristics are 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 [Nedeianu and Páli, 2002]. Each of these factors and parameters will be discussed in detail in the following sections. We have used two model systems: an aqueous media, using either a buffer (pH=7.4) or DDW; and a hydrophobic media, via the use of multilayer phospholipid vesicles (liposomes), in order to mimic the behaviour of these complexes inside cells, as close as possible.

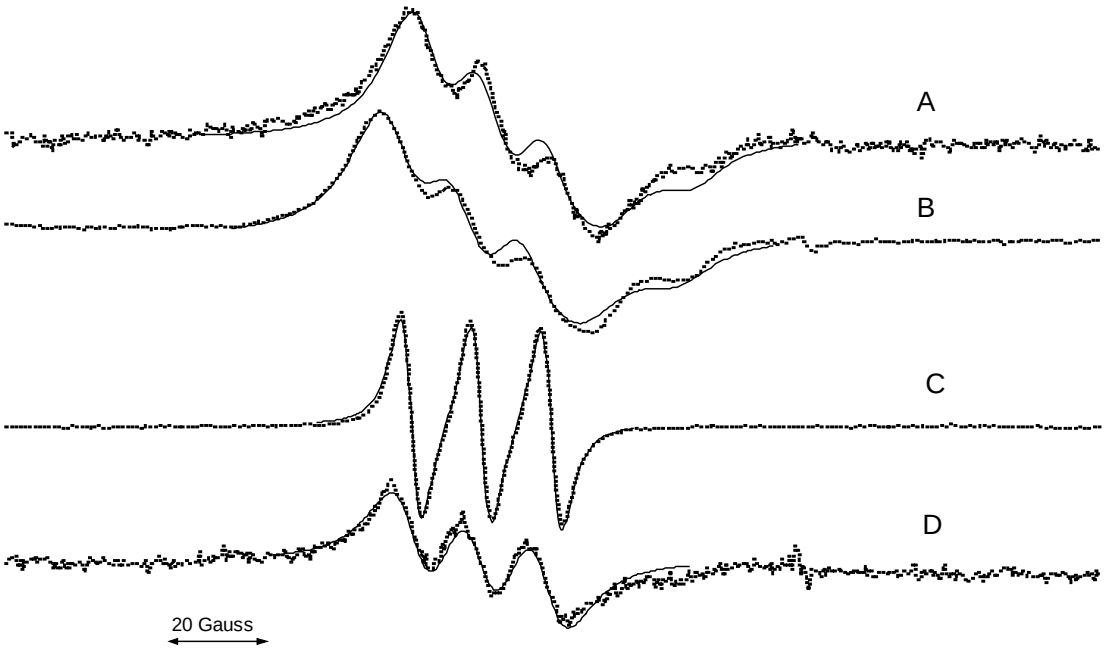


Fig. 3.1.1. Experimental EPR spectra of the NO-Fe(DTC)$_2$ complex in solution (A, B) (dashed line) with their best Lorentzian fit (solid line). Low temperature spectra (C, D) were fitted with the powder pattern (solid line) [Nordio, 1976]. Spectra were recorded for the NO-Fe(II)-(MGD)$_2$ complex at 165 K (A) and 298 K (C) and for the NO-Fe(II)-(DETC)$_2$ complex at 165 K (B) and 298 K (D).

III.1.1 NO source

The shape of the EPR spectrum of the NO-Fe-(DTC)$_2$ complex was not affected by the NO source, which was either a NO saturated solution (below 2 mM) or a NO donor. We used three different compounds as NO donors: sodium nitrite ($NaNO_2$) and two compounds used as nitrovasodilators, namely sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP).

As shown previously, NO_2^- is a source of NO *in vivo* in biological systems (chapter I.1.4). Based on some controversial literature data, we raised the question whether NO_2^- would lead to formation of NO, or if not, whether Fe(II)(DTC)$_2$ complex would form a typical nitrosyl-adduct directly with NO_2^-, probably because its redox potential value. To investigate this problem Fe(DTC)$_2$ spin trapping complexes were mixed with a solution containing $NaNO_2$.

We have found that $NaNO_2$, in solution, at pH 7, forms a paramagnetic EPR active NO-Fe(DTC)$_2$ complex which can be detected immediately after formation at RT or in frozen samples. Moreover, the presence of reducing agents ($Na_2S_2O_4$) or

acidic conditions, led to the increase of the intensity of the signal with 2-3 times orders of magnitude relative to the physiological pH conditions.

In the case of SNP, we could detect the nitrosyl triplet EPR signal for concentration as low as 10^{-7} M, fig. 3.1.2. Considering results presented in the literature [Ioannidis et al, 1996], that 10^{-3} M SNP releases approx. $4x10^{-6}$ M NO under anaerobic conditions, our results provide a limit of detection of $4x10^{-10}$ M NO with spin trapping EPR method.

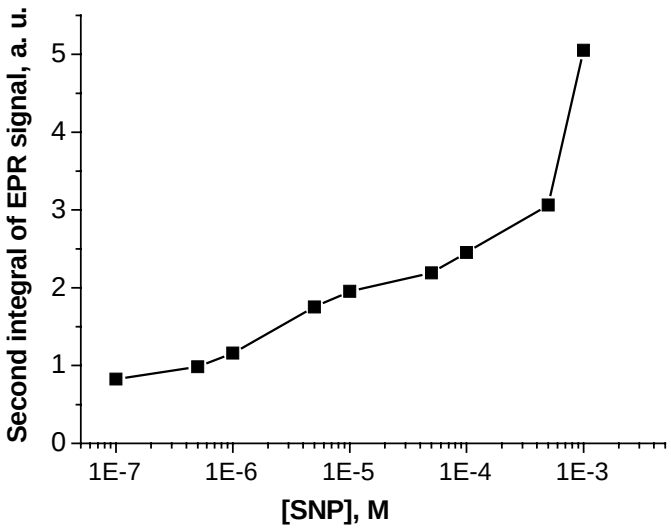


Fig. 3.1.2. Intensity of NO-Fe(DTC)$_2$ EPR signal as a function of SNP concentration. Spectra were recorded at RT under anaerobic conditions.

SNAP seems to be a more suitable NO donor for *in vivo* studies, being also a hydrophobic compound. Data related to its stability and efficiency in releasing NO in both aqueous and lipid media will be presented later.

III.1.2 O_2 effect

The preparation of spin trapping complexes must be made under strictly anaerobic conditions since the Fe(II) solution and the Fe(II)(DTC)$_2$ solution are very sensitive to presence of oxygen. The Fe(II)(DTC)$_2$ complexes are rapidly oxidized under aerobic conditions, as it is visible when the colour of the solution turns from colourless to dark orange or black, due to formation of Fe^{3+} complexes. The diamagnetic NO-Fe(III)(DTC)$_3$ complex can gradually self-transform by reductive nitrosylation into a paramagnetic NO-Fe^{2+}(DTC)$_2$ complex and NO_2^-. More efficient transformation of NO-Fe^{3+}(DTC)$_2$ into NO-Fe^{2+}(DTC)$_2$ was observed after the addition of reducing equivalents, such as ascorbate (AsA) or dithionite ($Na_2S_2O_4$).

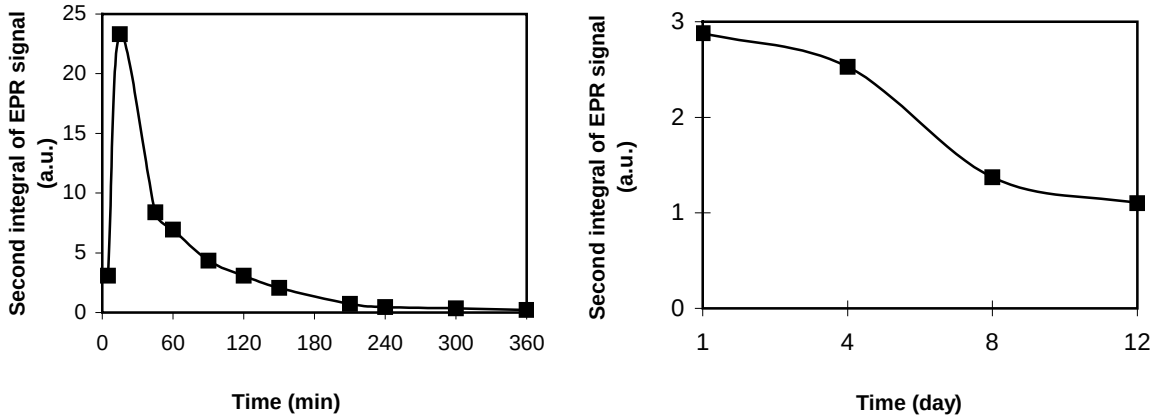


Figure 3.1.2. Decay in time of the EPR signal of the NO-Fe(II)-$(MGD)_2$ complex under aerobic conditions for SNP (left) and anaerobic conditions for SNAP (right). Left: [SNP] = 1 mM, [$FeMGD_2$] = 5 mM and right: [SNAP] = 2 mM, [$FeMGD_2$] = 2 mM with [Fe]:[MGD]=1:5 in both cases. Spectra were recorded at RT.

We have observed that, once formed, the NO-Fe(II)$(DTCs)_2$ complexes are less sensitive to O_2 and their life-time in ambient air is much longer (a few hours) than that of the spin adducts of most of the organic spin traps (minutes). As it can be observed from Fig. 3.1.2 (left panel), the intensity of the EPR signal of the NO-Fe(II)$(MGD)_2$ complex, formed under aerobic conditions using SNP as a NO donor, increases during the first 15 min and decreases to almost the initial value during the first hour, but the triplet signal can be still detected after 5 hours. Under anaerobic conditions, the stability of the nitrosyl-adduct in solution increases, from a few hours to days, depending on the NO donor. When SNAP is used as a NO releasing agent, the intensity of EPR signal of NO-Fe(II)$(MGD)_2$ shows only a 12% decrease during the first 4 days and could be detected even after almost two weeks (fig. 3.1.2 (right panel)). This effect is in agreement with data presented in the literature, since it was observed in biological systems that both SNP and SNAP, release higher concentrations of NO under hypoxic than normoxic conditions [Ioannidis et al., 1996].

Inside an animal body the presence of O_2, (detected at low concentrations (20-30 μM) [Vanin et al., 2002]), cannot be avoided, but there are also other reducing agents besides ascorbate (AsA), such as hydroquinone, cysteine or glutathione (GSH), to protect against oxidation.

III.1.3 Reducing agents

Since the EPR signal of the NO-Fe(II)$(DTC)_2$ complex is sensitive to the redox state of Fe and the presence of O_2, it is necessary to prevent oxidation by using a reducing agent. Ascorbic acid (AsA) or dithionite ($Na_2S_2O_4$) were used in our experiments for

this purpose. It was shown in the literature that, in solution, dithionite ($Na_2S_2O_4$) reacts with both free NO and O_2 and can remove all the O_2 from the quencher solution. A minimum concentration of 100 μM $Na_2S_2O_4$ was found to be enough to completely reduce O_2 and maintain the iron in Fe(II) state. This concentration was also well tolerated by the cell culture preparation (e.g. macrophages, data not shown) and it is much lower than that previously predicted in the literature: 550 μM [Caccia et al., 1999] or 2 mM [Tsuchiya et al., 1996].

In biological systems, the most abundant and efficient reductant is AsA. It has been shown that concentrations in the range of mM levels are required in order to efficiently protect against free radicals formed in the cellular media, so we have used 10 mM AsA in order to prevent the formation of oxidative products.

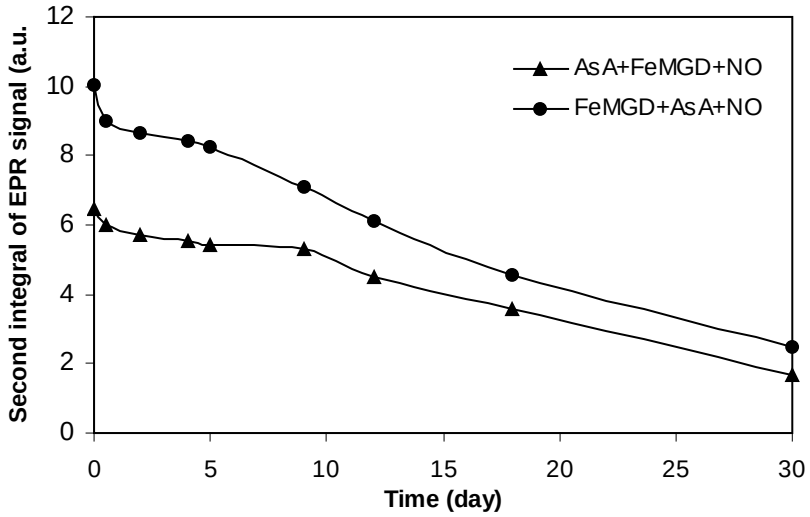


Fig. 3.1.3. Stability and the protective effect of AsA on the NO-Fe(II)$(MGD)_2$ complex under anaerobic conditions, at RT. [AsA]=10mM, [$FeMGD_2$]=2mM, Fe:MGD=1:5, using a saturated solution of NO (< 2 mM).

Results presented in fig. 3.1.3 show that AsA increased the stability of the EPR signal of the NO-Fe(II)$(MGD)_2$ complex when it was added to the spin trap complex, after its formation and not before. This effect could be explained by the total reduction of possible Fe^{3+}, formed during reactions, to Fe^{2+} and also by decreasing the oxidative process that could not be totally avoided over such a long period (around a month). It is notable that the $Fe(MGD)_2$ spin trap complex was not stable in solution under acidic conditions, even for short period of time (data not shown).

III.1.4 Trapping efficiency

Vanin et al. claim that the stability of NO-Fe(II)$(MGD)_2$ complexes increases with increasing the ratio of MGD to Fe [Vanin et al., 2000]. We have found that at ratios of [Fe]:[MGD] ranging from 1:5 to 1:3, the trapping efficiency of the spin trapping complex for NO is maximum for a given concentration of NO (approx. 100 μM). When iron was in excess, yielding to higher ratios between [Fe] and [MGD], i.e. [Fe]:[MGD]>1:3, the spin trapping complex formed did not shown a further improvement in the NO trapping affinity in solution (see fig. 3.1.4.1). For ratios [Fe]:[MGD]<1:5 the ability of the spin trapping complex to bind NO decreases showing that a certain amount of Fe is necessary for the formation of the $Fe(DTC)_2$ complex to effectively detect NO. A 100-fold decrease in the Fe concentration (0.1 mM) used for $Fe(MGD)_2$ complex formation, induced a 95% decrease in the intensity of the EPR signal of NO-$Fe(MGD)_2$ adduct formed.

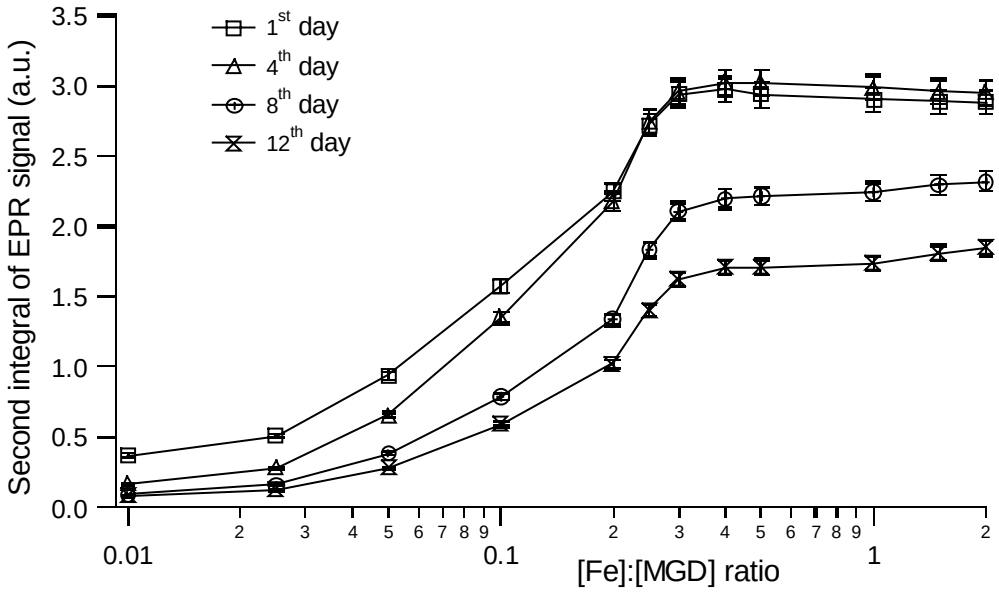


Fig. 3.1.4.1. Fe:MGD ratio- and time-dependency of the intensity of EPR signal for NO-$Fe(MGD)_2$ adduct at RT. Samples were prepared under anaerobic conditions in Tris buffer (pH= 7.4) containing 10 mM AsA. [SNAP]=2 mM, [Fe]=2 mM.

We have investigated the optimum ratio between the concentration of $Fe(DTC)_2$ and NO that should be used for a more efficient trapping. As it is shown in figure 3.1.4.2, a high NO donor concentration, (2 mM SNAP, which release approx. 100 μM NO) and at least 2 mM $Fe(DTC)_2$ complex, at the near-optimum ratio of 1:5 between [Fe] and [MGD], has to be used to trap all NO from solution.

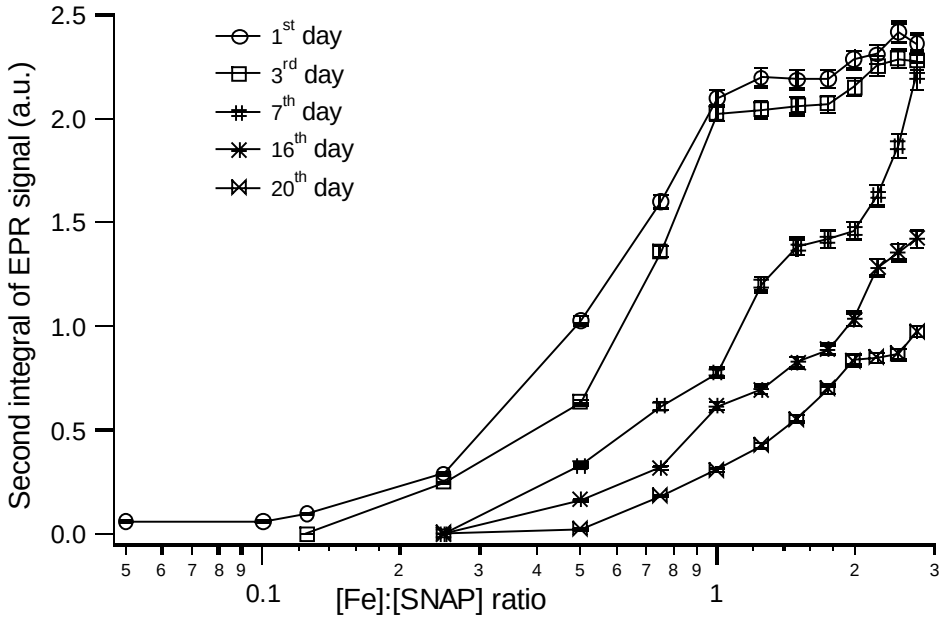


Fig. 3.1.4.2. [Fe]:[SNAP] ratio- and time-dependence of the intensity of EPR signal for NO-$Fe(MGD)_2$ adduct at RT. Samples were prepared under anaerobic conditions in Tris buffer (pH= 7.4) containing 10 mM AsA. [SNAP]=2 mM, [Fe]:[MGD]=1:5 mM.

The concentration of the $Fe(II)(DTC)_2$ complex should be always in excess of the [NO] to ensure that all NO is trapped. If the trapping efficiency is known, the amount of the NO production can be estimated, since the double integrated areas or the peak height of EPR spectra varies linearly with [NO].

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

Zhang and co-workers proposed that the optimal organic solvent used to extract the $Fe(DETC)_2$ complex from aqueous solution should have three properties: (i) a high partition coefficient, to extract most of the NO-$Fe(DETC)_2$ complex into organic phase, (ii) the NO-$Fe(DETC)_2$ complex should be easily separable from the protein and then easily separable from the water phase after extraction and (iii) the trapping complex should have a strong signal [Zhang et al., 2001]. It was found that ethyl acetate was the best organic solvent to fulfil these conditions. The NO-$Fe(DETC)_2$ complex extracted with ethyl acetate is very sensitive to sunlight (after 10 min, intensity of the EPR signal decreased about 50% and after 3 h it disappeared completely), to temperature (decomposed at RT) and very stable if kept in the dark at 0-4 ^{0}C [Zhang et al., 2001].

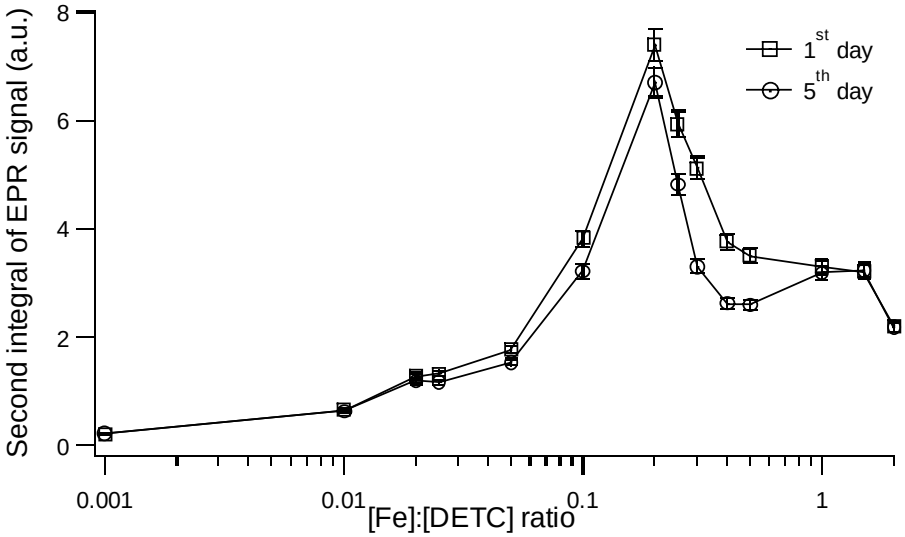


Fig. 3.2. [Fe]:[DETC] ratio- and time-dependence of the intensity of the EPR signal for the NO-Fe(DETC)$_2$ adduct extracted with ethyl acetate. Samples were prepared under anaerobic conditions in Tris buffer 50 mM (pH= 7.4) containing 10 mM AsA. [SNAP]=1 mM, [Fe]=2 mM, 60 μl ethyl acetate. EPR spectra were recorded at RT.

Using the same system, we carried out a more detailed characterization of the efficiency and sensitivity of the method. For this purpose, different ratios of [Fe] and [DETC] were mixed together and the resulting complex was extracted with different volumes of ethyl acetate. The EPR spectra presented the same characteristic triplet signal as the NO-Fe(MGD)$_2$ complex in aqueous media. 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. Data presented in fig. 3.2 show that the optimal ratio between [Fe] and [DETC] is 1:5 as given by the maximum value for the second integral of the triplet EPR signal. It was also found that a ratio of [Fe]:[DETC] as low as 1:1000 can form the spin trapping complex able to bind the NO released by 1 mM SNAP, but the intensity of the EPR signal of NO-Fe(DETC)$_2$ adduct would be decreased with 97%. These data suggest that very low concentrations of Fe (2 μM or less) would still form with 1-5 mM DETC a Fe(DETC)$_2$ adduct capable of trapping physiological amounts of NO inside the body. It should be also mentioned that excess of Fe (i.e. [Fe]:[DETC] > 1:5) decreased the trapping efficiency as observed by the decrease of the intensity of EPR signal of nitrosyl-adduct formed. The extraction of Fe(DETC)$_2$ or NO-Fe(DETC)$_2$ with ethyl acetate also has the advantage of a nitrosyl-adduct, that is highly stable over several days. This could be useful for clinical applications when biological samples containing the nitrosyl-complex are drawn but cannot be recorded immediately. These samples can be preserved at 4 ^{0}C in the dark for few days until their EPR spectra are recorded.

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

Although the most used spin traps for NO (including Hb and Fe-complexes with other ligands) and their nitrosyl complexes rapidly degrade in the presence of O_2, NO-Fe(II)$(DTC)_2$ complexes, are less sensitive to O_2, and their life-time in the ambient atmosphere is much longer than that of the spin adducts of most organic spin traps. 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.

As liposomes, we have used multilayer lipid vesicles (MLVs) consisting of a saturated phospholipid, either DMPC or DPPC. Phosphatidylcholine (PC) is the predominant lipid found in internal membranes (40%) and in plasma membranes (17-24%) [Alberts et al, 2002]. The phase-transition temperature for DMPC is 23.5 ^{0}C and for DPPC it is 41 ^{0}C. The spectra of spin trap complexes embedded in MLVs were recorded at RT (25 ^{0}C) and at 37 ^{0}C, the physiological temperature of human body, in order to observe the formation of the nitrosyl-spin trap complex and its stability. Two types of samples were prepared in 50 mM phosphate buffer deoxygenated by flushing Ar gas, and the presence of atmospheric O_2 was avoided as much as possible:

(i) a type in which the Fe$(DTC)_2$ complex was encapsulated into MLVs and they were re-washed with a solution containing a NO donor,

(ii) and another type in which a NO donor (either SNP or $NaNO_2$) was trapped inside the MLVs and a solution containing Fe$(DTC)_2$ was used to re-washed them. For simplicity we will use conventional names for the two types of probes such as Fe$(DTC)_2$-MLVs and NO-MLVs for the first (i) and second type (ii), respectively.

EPR spectra of nitrosyl-iron-dithiocarbamate complexes in lipid media (MLVs) present typical triplet signals, characteristic to nitrosyl adducts, as shown in fig. 3.3.1. The shape of the triplet spectrum was not influenced by the type of dithiocarbamate (MGD or DETC), phospholipid (DMPC or DPPC) or NO donor (SNP or $NaNO_2$) used. The temperature, in the range of 298 K – 310 K, did not affect the spectrum. Although the intensity of the EPR spectrum was influenced by some factors, as shown in fig. 3.3.1C. In the case of NO donor (either SNP or $NaNO_2$) encapsulated in DMPC-MLVs, the Fe$(MGD)_2$ solution used for re-washing the liposomes, presented a very weak triplet signal, suggesting that only a low concentration of NO-Fe$(MGD)_2$ complex remained inside lipid vesicles.

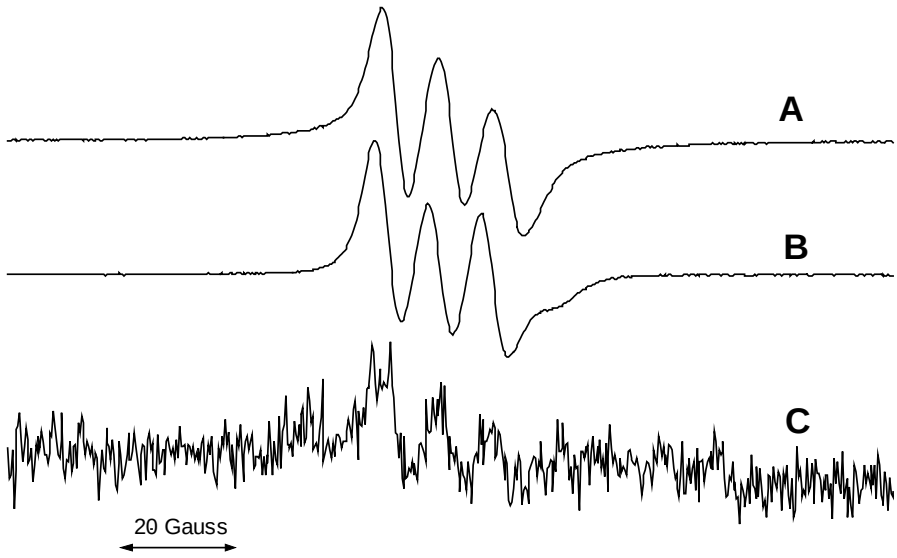


Fig. 3.3.1. EPR spectra of NO-Fe(DTC)$_2$ complex in DMPC-MLVs at RT. (A) Fe(MGD)$_2$ in DMPC-MLVs + NO donor, (B) NO donor in DMPC-MLVs + Fe(DETC)$_2$, (C) NO donor in DMPC-MLVs + Fe(MGD)$_2$. Samples were prepared in phosphate buffer 50 mM (pH=7.4). Spectra were recorded at 298 K.

Since the Fe(MGD)$_2$ complex is hydrophilic, it will remain in the aqueous phase of the MLVs during their formation. NO is lipophilic and when added to the previous preparation it will easily pass through the lipid layers until reaches the Fe(MGD)$_2$ with which it forms the nitrosyl-adduct and it will remain there. Most of Fe(MGD)$_2$ complex is washed out by addition of NO donor solution, a fact explained by the strong triplet signal observed in the supernatant of these type of samples (not shown). In comparison, the Fe(DETC)$_2$ complex is liposoluble and it will accumulate inside th lipid bilayers and only low amounts of it will be washed out when the solution containing a NO donor is added. This is observed by differences in the intensity of the EPR signals of either pellets or supernatants of MLVs samples.

The EPR triplet signal of the nitrosyl-adduct, coming from NO-MLVs rewashed with a solution containing Fe(II)(DETC)$_2$ spin traps, shows a characteristic line shape with hyperfine splitting at $g_{\perp}$ for powder samples or low temperature spectra, which is also typical for a more immobilized spectrum. This effect has been also observed in biological samples (not shown). Indeed, the EPR spectrum of NO-Fe(II)(DTC)$_2$ adduct in biological specimens at 298 K shows a more distorted line shape than that of the same complex in solutions or buffers because the free rotation of the complex

is restricted by the interaction of the NO complex with biological components such as membranes.

Strong EPR signals of nitrosyl adducts were recorded when $Fe(DETC)_2$-MLVs samples were rewashed with a solution containing a NO donor.

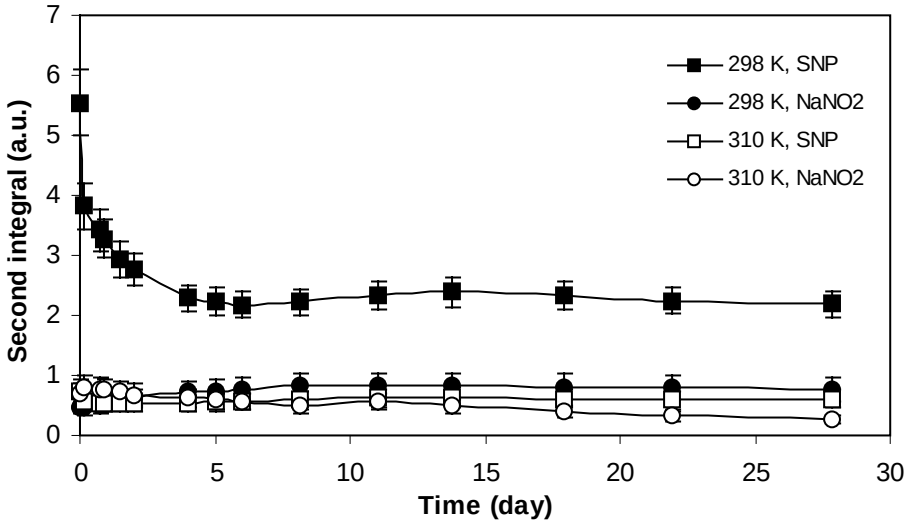


Fig. 3.3.2. Time-dependence of the Fe-$(DETC)_2$ complex in DMPC-MLVs rewashed with a NO donor. Samples were prepared in phosphate buffer and the NO donor used was either SNP (squares) or $NaNO_2$ (circles) at two different temperatures 298 K (solid symbols) and 310 K (open symbols), respectively, as indicated in the legend.

In this case, both NO and the $Fe(DETC)_2$ complex are hydrophobic and then the nitrosyl complex can enter the lipid layers of the MLVs, which constitute a proper medium for both of them and offer a higher stability and protection of the NO-$Fe(DETC)_2$. This is clear in fig. 3.3.2 which presents the time-dependence of this type of adduct in DMPC-MLVs at two different temperatures (25 ^{0}C and 37 ^{0}C, 298 K and 310 K, respectively). For spectra of $Fe(DETC)_2$ encapsulated in DMPC-MLVs and hydrated with a solution containing SNP, recorded at RT, the triplet signal intensity drops by almost 70% from the initial value during the first 5 days until it reaches a plateau, where it remains constant for more than one month. For equivalent samples with $NaNO_2$, the intensity of EPR signal is much lower (more than 4 times, compared with SNP) and after a small increase during first 5-7 days, it keeps the same level for the rest of time considered here, almost independent of temperature.

Temperature seems to be a determining factor in the case of NO-MLVs with $Fe(DETC)_2$ in the rewashing-solution, and SNP used as NO donor. As presented in fig. 3.3.3, the intensity of EPR triplet signal continued to decrease over the first 10 days and then remained almost constant at this level for another month. The profile is

similar to previous results (fig. 3.3.2) for $NaNO_2$-MLVs, showing a lower intensity but unchanged by temperature and time.

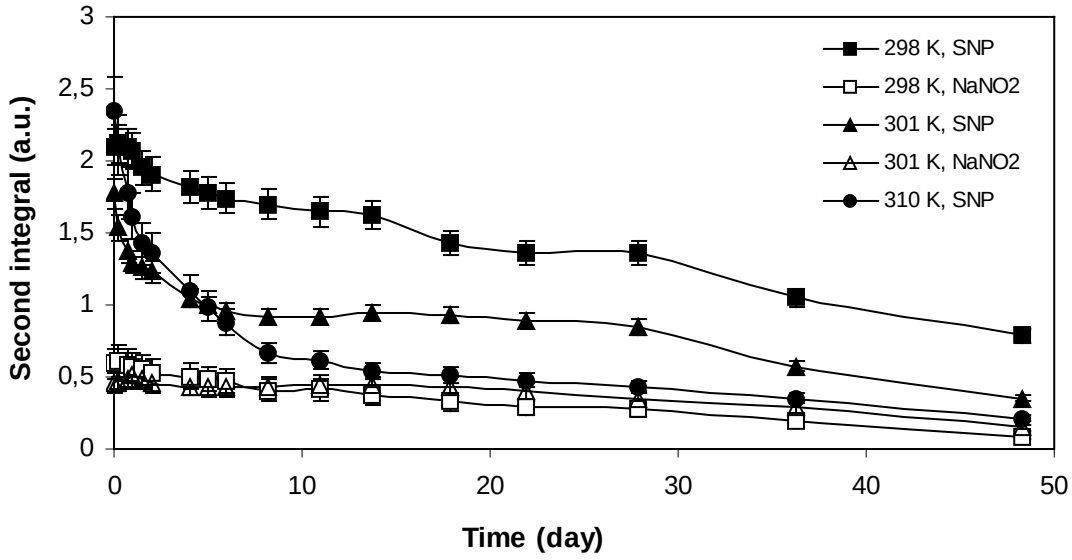


Fig. 3.3.3. Time-dependency of NO donor in DMPC-MLVs rewashed with a solution containing Fe-$(DETC)_2$ complex. Samples were prepared in phosphate buffer and the NO donor used was either SNP (solid symbols) or $NaNO_2$ (open symbols) at three different temperatures indicated in the legend.

As it was previously claimed, O_2 is a very, if not the most, important factor in the formation and stability of NO adducts in aqueous media either with $Fe(DTC)_2$ spin traps or nitroxide SLs.

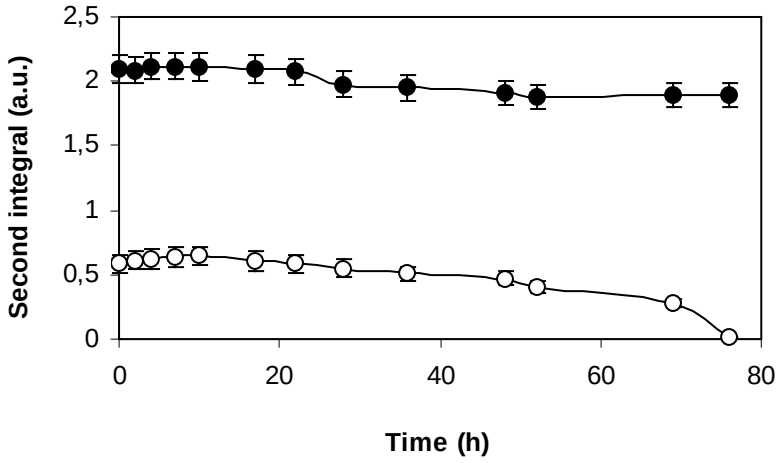


Fig. 3.3.4. O_2 effect and time-dependence of SNP in DMPC-MLVs rewashed with a solution containing Fe-$(DETC)_2$ complex. Samples were prepared in phosphate buffer and the samples were incubated and recorded under anaerobic and (solid circles) and aerobic (open circles) conditions.

Therefore, we cheeked the influence of atmospheric O_2 on sample preparation and incubation. Samples were prepared under either anaerobic or aerobic conditions and the capillaries containing the probes were sealed or left open, respectively.

The difference in signal intensity and the decay in time are shown in fig. 3.3.4 for NO-MLVs. Results were similar for both NO donors used. Interestingly, the signal intensity does decrease, reaching zero level after a few days in the case of NO-MLVs prepared and incubated under aerobic conditions (fig. 3.3.4).

Similar experiments, using the same procedure, were done on DPPC-MLVs.

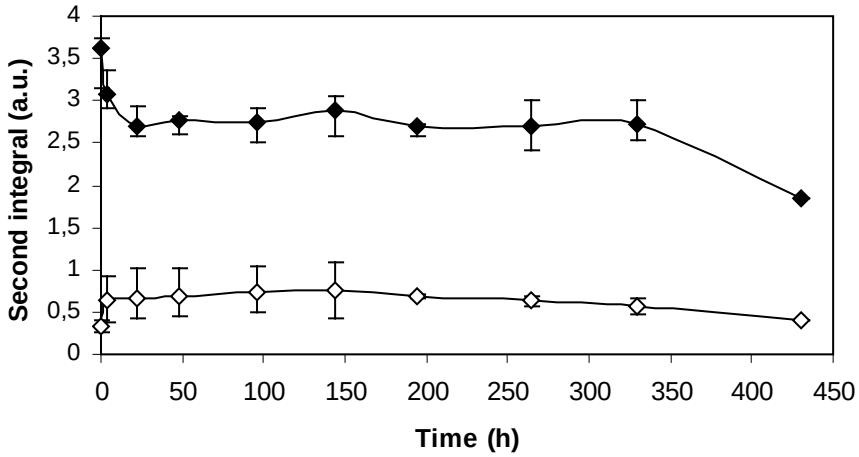


Fig. 3.3.5. Time-dependence of Fe-$(DETC)_2$ complex in DPPC-MLVs, re-washed with NO donor. Samples were prepared in phosphate buffer and the NO donor was SNP (solid circles) and $NaNO_2$ (open circles).

Time-dependence of $Fe(DETC)_2$-MLVs and NO-MLVs at 310 K with DPPC-MLVs are presented in fig. 3.3.5 and 3.3.6, respectively. The data for DPPC-MLVs showed the same profile as DMPC-MLVs, only the period of time seemed to be shorter than in the former case, although 310 K is a temperature only slightly below the phase-transition temperature of DPPC (314 K).

Overall, these results show that the stability of NO-$Fe(DETC)_2$ complexes incorporated in saturated phospholipid vesicles has been tremendously increased, from several hours or few days, as reported in the literature for the same complex in solution, to one month and even longer. The samples are still affected by aerobic conditions, but this is a slight effect compared to the long-term stability that was observed. The $Fe(DETC)_2$-MLVs probes might be used as tools for NO detection in *in vivo* experiments and, perhaps, in pathological situations where NO increases over the physiological levels and produces deleterious effects, these probes could trap the surplus of NO produced and contribute to maintaining the normal balance. For these

applications, further experiments are necessary to study the influence of various factors that could affect their stability inside biological systems.

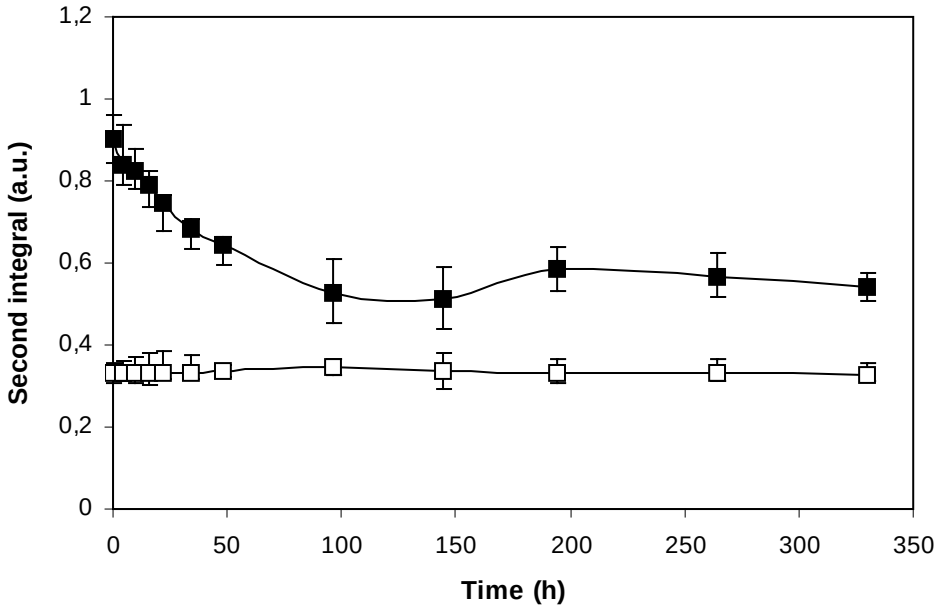


Fig. 3.3.6. Time-dependence of NO donor in DPPC-MLVs hydrated with a solution containing Fe-$(DETC)_2$ complex. Samples were prepared in phosphate buffer and the NO donors used were SNP (solid squares) and $NaNO_2$ (open squares). Spectra were recorded at 310 K.

A similar study was made with NO-$Fe(DTCS)_2$ complex (DTCS is a soluble dithiocabamate derivate in aqueous solution), embedded into liposomes prepared from L-α-phosphatidylcholine stearylamine and cholesterol [Yoshimura et al., 1997]. The signal intensity decreased during the first 30-40 min and then returned to the initial intensity after 120 min, the total time during which the spectra were recorded.

Although the lipophilic spin trap $Fe(DETC)_2$ has the tendency to be localized in the cell membranes, and thus, it can trap the highly diffusible NO crossing the membranes from inside to outside and vice versa [Sato et al., 1994], some NO may escape the trapping. In addition, in biological systems, there are other compounds that could affect its diffusion through the membrane. For example, NO can be oxidized by the simultaneously produced O_2^- inside the cells and the spin trap should compete with non-heme and heme nitroxyl complexes produced in the tissues of the LPS-treated animals [Moncada and Palmer, 1990] when high concentrations of NO are formed by the activation of iNOS by LPS. Fortunately, the EPR signals of these nitrosyl complexes arising from intrinsic protein targets of NO, can be simultaneously detected, together with nitrosyl-spin trap complexes, without quantitative interference [Mülsch et al., 1993; Lepoivre et al., 1992].

Results and discussions

Chapter IV

Nitric oxide and spin label EPR

IV. 1. Soluble nitroxide spin labels

Nitroxide spin labels (SLs) are useful compounds in measurements of chemical and biophysical properties and redox metabolism of components of the cellular membrane, as well as oxidative processes in living cells. The nitroxide spin labels are chemically stable with respect to other paramagnetic free radicals, because the radical centre is sterically protected, thus making them useful tools as spin labels for biochemical studies. It is known that nitroxides are remarkably persistent in cells, tissue homogenates and animals. Cyclic nitroxides seem to play an antioxidant role, their protective effects being attributed to their ability to detoxify the carbon-centred radicals [Brede et al., 1998], alkoxyl and peroxyl radicals [Offer et al., 2002] produced by various forms of organ damage in living organisms.

IV.1.1. Effect of NO and O_2 on soluble SLs

We have recorded the EPR spectra of water-soluble paramagnetic nitroxide spin labels (SLs): TEMPO, 3-maleimido-PROXYL and Fremy's salt under different chemical conditions at room temperature. Typical EPR triplet spectra were observed, which 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, as seen in Fig. 4.1.1.1. As it has been shown in Scheme 4 (in Chapter I) two soluble SLs are cyclic aromatic nitroxides (TEMPO and 3-maleimido-PROXYL) and the third one is a linear nitroxide compound (Fremy's salt). 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.

Samples were prepared in deoxygenated double distillated water (DDW), (except Fremy's salt, which was dissolved in a 50 mM K_2CO_3 solution), at two different final concentrations (0.3 and 8 mM) and then treated with either a NO donor

(SNAP) or flushed with NO gas in order to determine the effect of NO on the nitroxide group of soluble SLs.

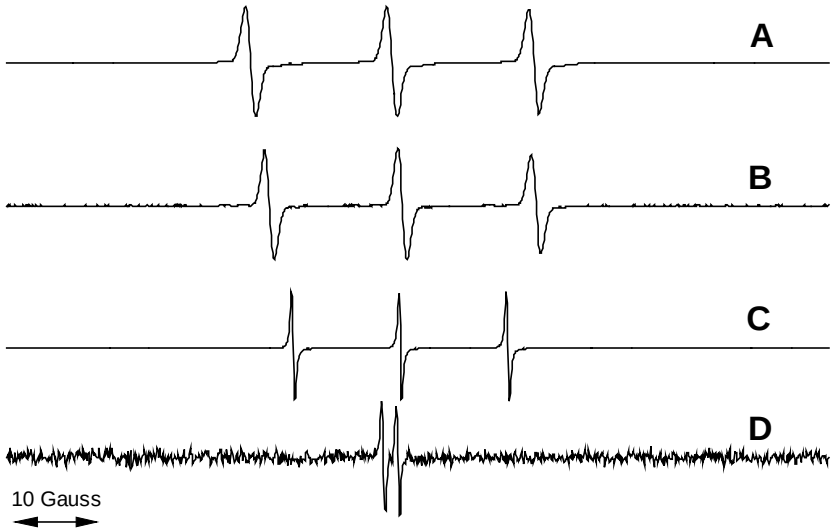


Fig. 4.1.1.1. EPR spectra of water-soluble spin labels at RT. (A) TEMPO, (B) 3-maleimido-PROXYL, (C) Fremy's salt in 50 mM K_2CO_3, (D) Fremy's salt (in 50 mM K_2CO_3) + O_2 + SNAP + AsA. Samples were prepared in Ar-DDW. [SL] = 0.3 mM, and for the D spectrum: [SNAP] =1 mM, [AsA] = 3 mM. Spectra were recorded at 298 K.

Experimental EPR spectra obtained show that NO gas treatment quenched the characteristic triplet signal to zero in the case of TEMPO and Fremy's salt SLs at concentrations of 0.3 mM, but for 3-maleimido-PROXYL the EPR signal could still be detected although its intensity was lower. The CW power saturation curves for 3-maleimido-PROXYL, in the absence (in deoxygenated DDW) and the presence of NO gas at different times, at room temperature, are presented in Fig. 4.1.1.2.

It can be observed that longer NO gas flushing over the 3-maleimido-PROXYL solution (0.3 mM) induces a lower degree of saturation of the EPR signal and thus, a faster relaxation rate. Also, the intensity of the triplet signal decreases with increasing time of flushing NO gas, and over time. This decrease in the peak signal of 3-maleimido-PROXYL was attributed to a redox process of the nitroxyl group induced by NO. We have also performed the same experiment using a NO donor (SNAP).

The CW power saturation curves for 3-maleimido-PROXYL in the absence (anaerobic conditions) and the presence of O_2 (aerobic conditions) and in the presence of the NO donor, SNAP, at a final concentration of 2 mM, under either aerobic or anaerobic conditions, at room temperature are presented in Fig. 4.1.1.3.

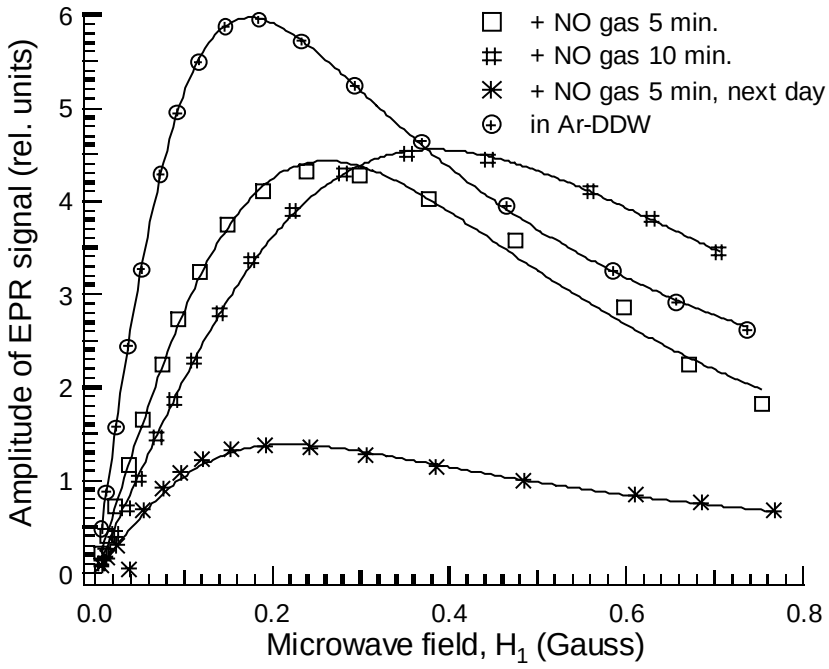


Fig. 4.1.1.2. CW power saturation curves for 3-maleimido-PROXYL (0.3mM) with NO gas for different periods of time. (⊕) control (in Ar-DDW), (□) + NO gas, 5 min, (#) + NO gas, 10 min, (*) the same as □, recorded the next day. EPR spectra were recorded at a range of 0.02-200 mW microwave power at RT.

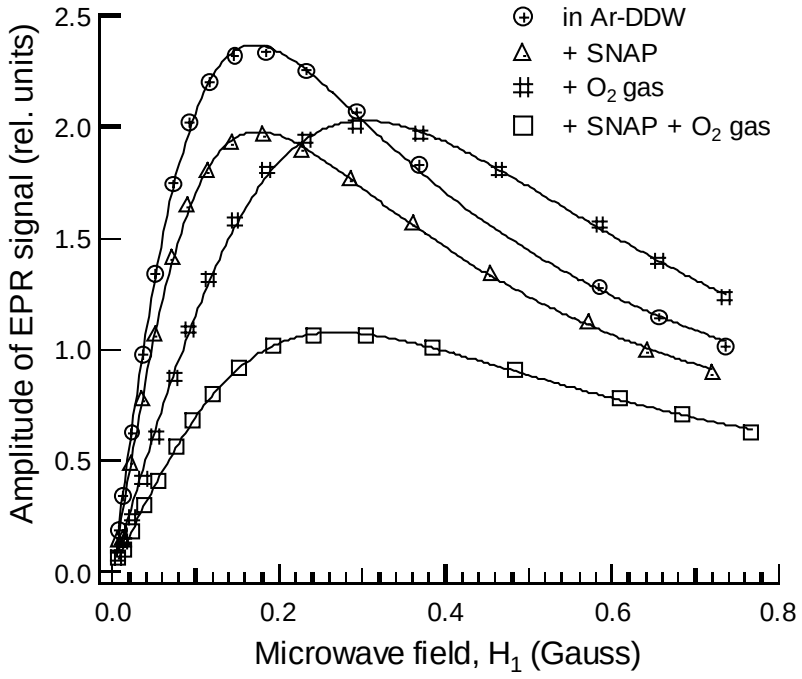


Fig. 4.1.1.3. CW power saturation curves for 3-mailemido-PROXYL (0.3 mM) under different treatments: (⊕) control (in Ar-DDW), (Δ) + SNAP 2 mM, (#) + O_2 gas, (□) + SNAP 2mM + O_2 gas. EPR spectra were recorded at a range of 0.02-200 mW microwave power at RT.

The CW-saturation curves show that SNAP (2 mM) seems to induce the same effect on the EPR signal of 3-mailemido-PROXYL (0.3 mM) as NO gas, but it is of lower intensity since the concentration of NO released from SNAP under these conditions is lower than that provided by 5 min of flushing with NO gas. The effect of SNAP under aerobic conditions, (SNAP + O_2 gas flushed for 30 min), which is suggested to form NO_2^-, induced a more pronounced effect similar to that induced by NO gas after one day, which suggest that some O_2 could enter the system.

Similar effects have been observed when experiments were performed with higher concentrations of both, nitroxide SL (8 mM) and SNAP (> 9 mM), under either aerobic or anaerobic conditions. The decay of the triplet EPR signal intensities of nitroxide SL radicals seems to be more significant for lower concentration of nitroxide SLs (0.3 mM) and SNAP (2 mM) and especially under aerobic conditions, as is shown in fig. 4.1.1.4. Spectra of each nitroxide SLs under anaerobic conditions were measured as controls in a set of experiments (+/- SNAP under either anaerobic or aerobic conditions) and their second integral considered as 100%. It can be observed that EPR signal intensity of 3-maleimido-PROXYL and Fremy's salt, but not of TEMPO, is lower under anaerobic conditions than under aerobic (when O_2 gas was flushed for 30 min) for both concentrations used.

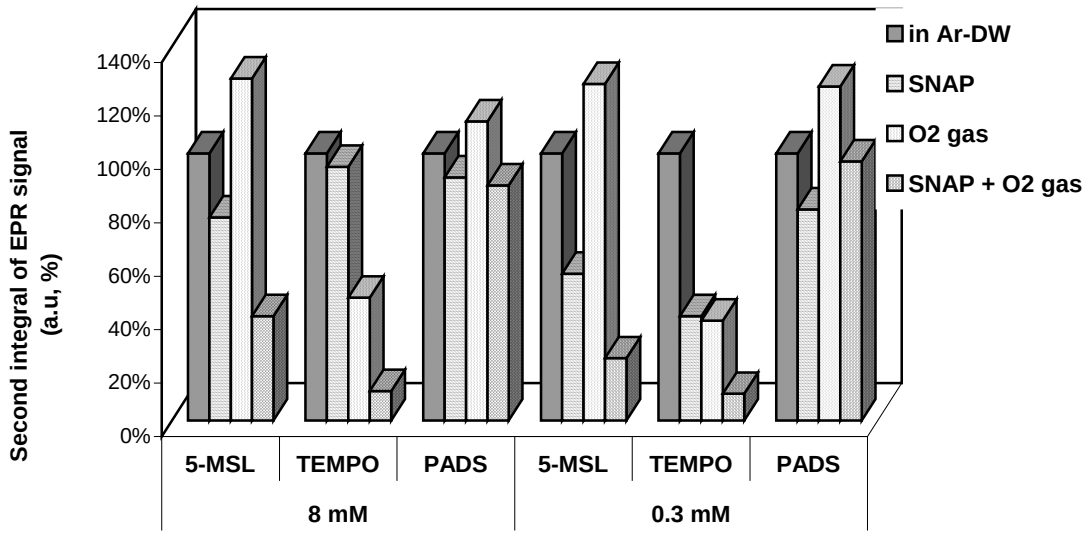


Fig. 4.1.1.4. Soluble nitroxide SLs under different treatments at RT. [SNAP] was 2 mM and 30 mM for [SL] of 0.3 mM and 8 mM, respectively. O_2 gas has been flushed for 30 min, in order to create the aerobic conditions. Spectra were recorded at RT.

This is consistent with the observation found in the case of TEMPOL, the 4-hydroxy-derived of TEMPO, under hypoxic and normoxic conditions in microsomes [Nakajima et al, 2002]. The effect of NO, released from SNAP, reduces the EPR triplet signal by 24% and 45% in the case of 3-maleimido-PROXYL, by 5% and 61% in the case of TEMPO, and by 9% and 21 % in the case of Fremy's salt, for 8 mM and 0.3 mM, respectively. The effect of NO_2^- (SNAP under aerobic condition) accentuates the decay of the EPR signal intensities, as follows: by 61% and 76% in the case of 3-maleimido-PROXYL, by 89% and 90% in the case of TEMPO, and by 12% and 3 % in the case of Fremy's salt, for 8 mM and 0,3 mM, respectively. These data suggest that TEMPO, a piperidine nitroxide, is the most sensitive, among the nitroxide SLs used in these experiments, to redox effects induced by NO and NO_2^-, except in the case of SNAP treatment under anaerobic conditions at the high concentration of TEMPO, 8 mM, when the EPR signal intensity is hardly affected. This is supported by data presented in the literature that piperidine nitroxides are not affected by the addition of NO donors [Nakajima et al., 2002]. These effects induced by O_2, NO and NO_2^-, seem to be time-dependent and to induce a further decay of the EPR signal intensities of nitroxide SLs.

The effect of SNAP on the 3-maleimido-PROXYL spin label, under both anaerobic and aerobic conditions, was also recorded over a longer period of time. The second integrals of the triplet signal of the nitroxide recorded at RT, are presented in Fig. 4.1.1.5.

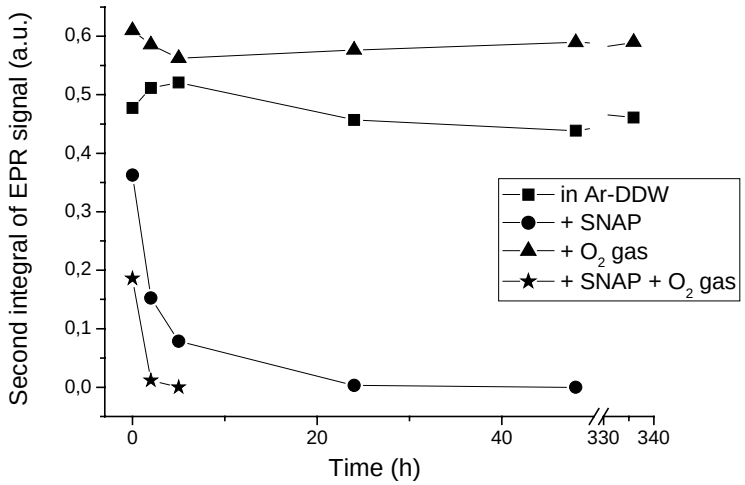


Fig. 4.1.1.5. Stability in time of EPR signal of 3-maleimido-PROXYL under different treatments. Concentrations of the components were: 8 mM for 3-maleimido-PROXYL, 30 mM for SNAP. Samples prepared in deoxygenated DDW were flushed with O_2 gas for 30 min (when it was required, as indicated). Spectra were recorded at RT.

The results show that the effect on the EPR signal induced by SNAP was enhanced in time, but the characteristic triplet spectrum of nitroxide was detectable for 24 h. In the case of NO_2^-, the reduction effect is very accentuated and the triplet signal for nitroxide disappeared after about 3 h.

Similar effects of SNAP under aerobic (i.e. NO_2^- production) and anaerobic conditions (i.e. NO production) on the other soluble nitroxide SLs, TEMPO and Fremy's salt, have been observed at RT and are presented in fig. 4.1.1.6.

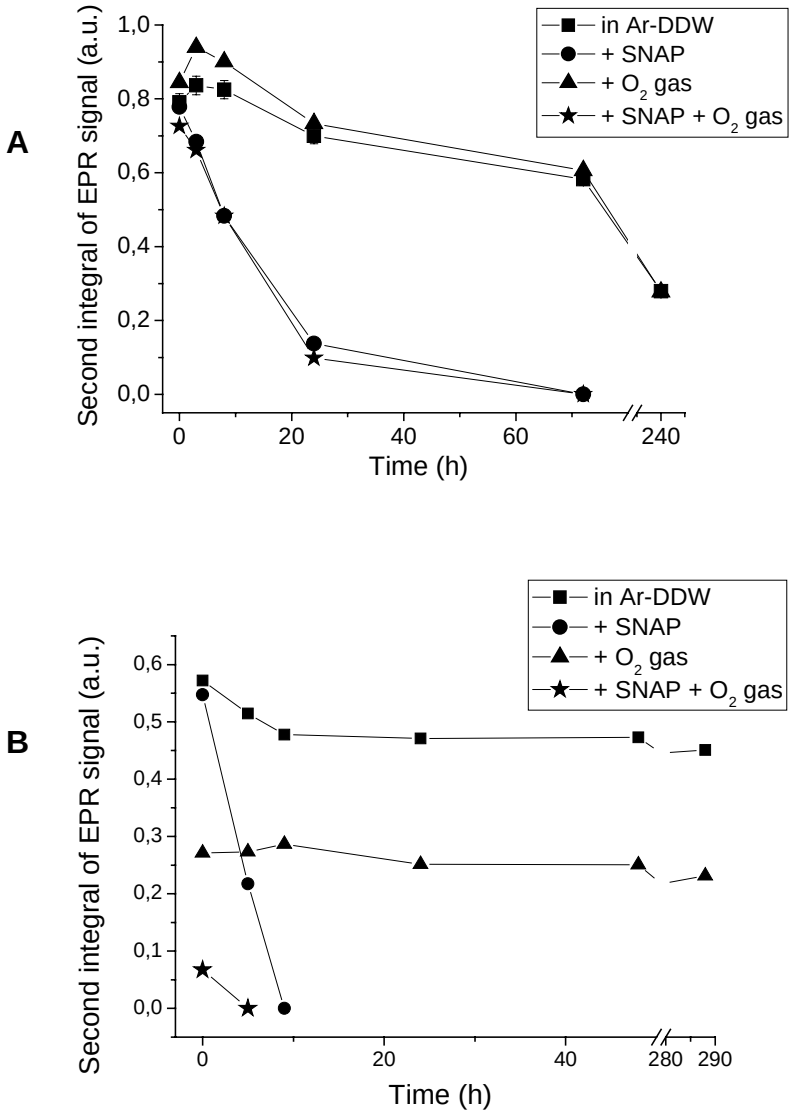


Fig. 4.1.1.6. Stability in time of EPR signals of Fremy's salt (A panel) and TEMPO (B pannel) under different treatments. Concentrations of the components were: 8 mM for TEMPO and Fremy's salt, respectively 30 mM for SNAP. Samples prepared in deoxygenated DDW were flushed with O_2 gas for 30 min (when it was required, as indicated). Spectra were recorded at RT.

Unlike the cyclic nitroxide SLs (TEMPO and 3-maleimido-PROXYL), the nitroxide group of linear Fremy's salt seemed to be less affected by either NO or NO_2^- (see fig. 4.1.1.6. A panel). SNAP, under either aerobic or anaerobic conditions, had the same effect and the decay in EPR signal intensity of Fremy's salt, which was around 20% after 5 h. The spectrum was still detectable for 3 days. The decay was 100% in the case of the other nitroxide SLs.

Spin relaxation time product (T_1T_2), from CW progressive saturation experiments at RT, for all three nitroxide SLs at low concentration (0.3 mM) under different treatments, are presented in Fig. 4.1.1.7. It can be seen that O_2 and NO, as well as their metabolite, NO_2^-, induced a relaxation enhancement as 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, i.e. the effect on T_2, on the nitroxide EPR signals that occurred due to Heisenberg spin exchange, as observed in the case of O_2 in ischemic hearts [Kuppusamy et al, 1995b]. At high concentration (8 mM), the T_1T_2 relaxation time product was not affected by the presence of O_2 for any of nitroxide SLs used in these experiments, as shown in fig. 4.1.1.8.

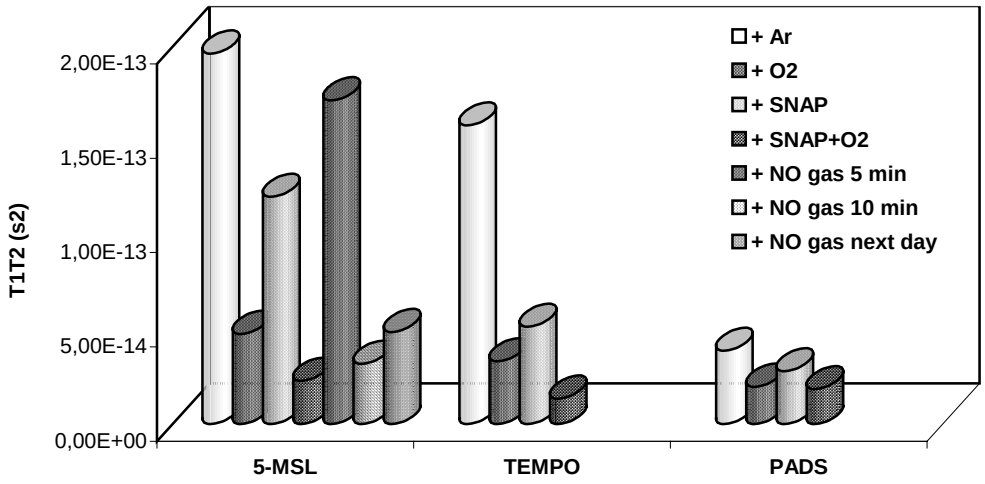


Fig. 4.1.1.7. Spin relaxation time product (T_1T_2) for all three nitroxide SLs under different treatments, as indicated in legend, at RT. [SL] = 0.3 mM, [SNAP] = 2 mM, O_2 gas has been flushed for 30 min, when indicated. NO gas has been flushed at indicated times under anaerobic conditions. EPR spectra were recorded at RT and at a range of 0.02-200 mW microwave power.

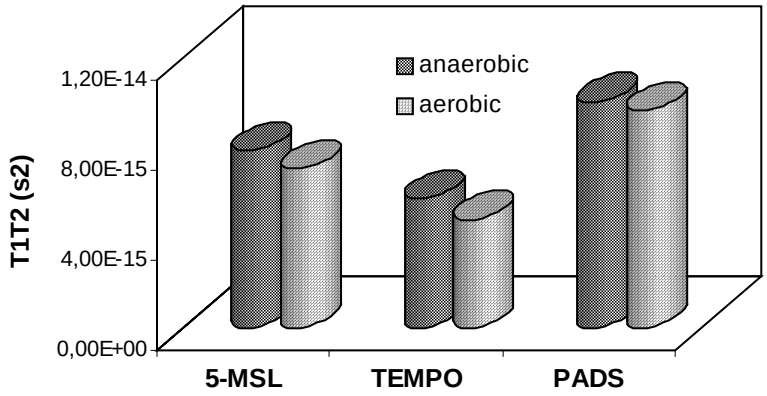


Fig. 4.1.1.8. Spin relaxation time product (T_1T_2) for all three nitroxide SLs under either anaerobic or aerobic conditions, at RT. [SL] = 8 mM, O_2 gas was flushed for 30 min, when indicated as aerobic conditions. EPR spectra were recorded at RT and at a range of 0.02-200 mW microwave power.

These data suggest that the cumulative effect of SNAP and O_2, or NO_2^-, as its presence has been confirmed by positive Griess reactions, induced an enhanced decay of the EPR triplet signal intensity of nitroxides SLs, especially the cyclic nitroxides (TEMPO and 3-maleimido-PROXYL). This decay was not dependent on the concentration, but on time. Since the profile of CW power saturation curves of the 3-maleimido-PROXYL signal with addition of SNAP under oxidative conditions are similar to those induced by NO gas treatment after a period of time, we suppose that some molecular O_2 entered the sample and NO_2 radical might be formed. It is known that the NO_2 radical is a much stronger oxidant than the NO radical, thus the nitroxide SLs should have been more affected by NO_2. This could explain the disappearance of the EPR signal in the case of TEMPO and Fremy's salt. The rate constant for the reaction of NO_2 radical and derivates of cyclic nitroxides was determined to be 7-8 x 10^8 M^{-1} s^{-1} [Goldstein et al., 2003].

Thus, our results, using CW power saturation EPR spectroscopy and the time-dependence of second integrals of EPR spectra of nitroxide soluble SLs, indicate a redox process induced by nitrogen-derived radicals (NO and NO_2) and metabolites. These nitrogen derived compounds are found in living organisms under various conditions, especially under different pathological conditions (cancer, hypoxia, ischemia-reperfusion, aging etc.), and nitroxide compounds were shown to have protective effects against them and other oxidative products and radicals. We also wanted to analyse the effect of 'natural' reductive agents found in biological systems that might affect the redox process in which they participate.

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

In biological systems, nitroxide compounds are in contact with various reductants that can transform them into EPR-silent molecules. The reaction is a one-electron reduction involving the unpaired electron of the nitroxide and produces a diamagnetic hydroxylamine.

Biological systems have various reductive compounds (glutathione and glutathione-peroxidase, catalase, SOD, cyt P450, transition metal ions, vitamin E and even Hb) [Zhang et al., 1994] and systems (e.g. enzymes from mithocondrial respiratory chain) but among all of them ascorbic acid (Asc. Ac. Or AsA) seems to be the most effective. Ascorbic acid appears to be the principal reductant in most types of cells (it has been found at levels of 100 μM in erythrocytes and at high concentration in the brain, in the order of mM). Relatively minor reduction pathways of nitroxides include the mitochondrial respiratory chain and thioredoxin reductase in skin [Mehlhorn, 1991]. AsA is also a potent free radical chain-terminating antioxidant, as judged by its effect on the spin trapping of oxygen-radicals. The reduction of free radicals by AsA yields the ascorbyl radical ($As^{-\cdot}$) which dismutates to yield AsAs and DHAsA (dehydroascorbic acid) [Mehlhorn, 1991].

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.

In this series of experiments we used only a low concentration of each soluble nitroxide SL, namely, 0.3 mM. The concentration of SNAP, the NO donor, was 1 mM and AsA concentration was 3 mM, in excess over the other components of the reaction. As previously, all samples were prepared in DDW under anaerobic conditions at RT. To create aerobic conditions, O_2 gas was flushed for 30 min over the desired samples.

IV.1.2.1. Effect of AsA on SNAP-treated nitroxide SLs probes under anaerobic conditions.

Each solution of a soluble SL, 3-maleimido-PROXYL, TEMPO and Fremy's salt, at a final concentration of 0.3 mM in deoxygenated DDW (Ar-DDW), (exception for Fremy's salt that was dissolved in 50 mM K_2CO_3), were taken as controls.

As expected, and confirmed by literature data, AsA (at 3 mM) induces a reduction of 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 decreased the EPR signal intensity by 38% and 70% for 3-maleimido-PROXYL and TEMPO, respectively (as shown in fig. 4.1.2.1 and fig. 4.1.2.2). The time-dependence of this effect showed that the EPR signal intensity of 3-maleimido-PROXYL treated with AsA was almost constant for more than 6 h and decreased progressively over a period of days, ending on the 4th day when the signal could not be further detected (see fig. 4.1.2.1).

It was also observed that AsA induced a smaller reduction effect on the EPR intensity of 3-maleimido-PROXYL than SNAP, but their cumulative effect enhanced the reduction of the nitroxide group.

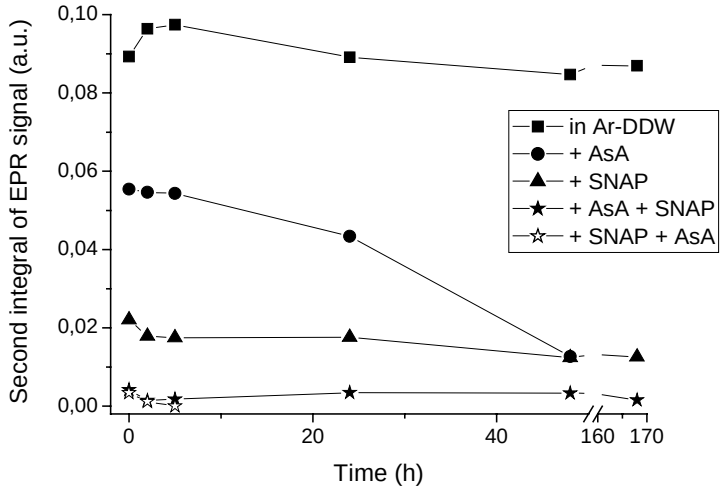


Fig. 4.1.2.1. Time-dependence of EPR signal intensity of 3-maleimido-PROXYL under reductive (+ AsA.) and SNAP addition effects. [3-maleimido-PROXYL] = 0.3 mM, [AsA.] = 3 mM and [SNAP] = 1 mM. Spectra were recorded at RT under anaerobic conditions.

In the case of TEMPO, the EPR signal disappeared much faster, after 2 h (data not shown), than in the case of 3-maleimido-PROXYL. It has been also 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, as shown in fig. 4.1.2.2. 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.

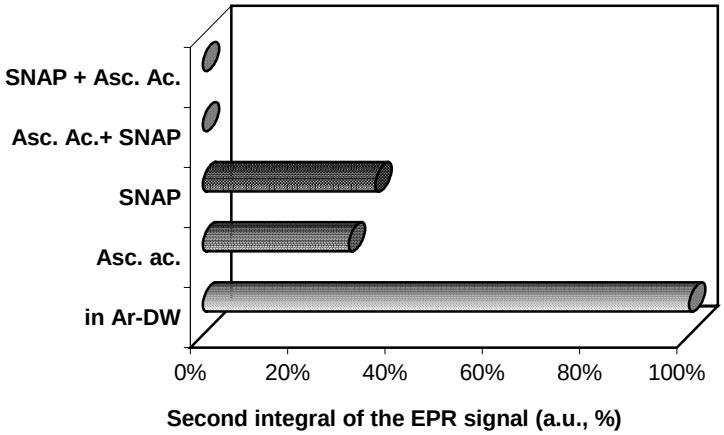


Fig. 4.1.2.2. Decay of EPR signal intensity of TEMPO under reductive (+ AsA) and SNAP addition effects. [TEMPO] = 0.3 mM, [AsA] = 3 mM and [SNAP] = 1 mM. Spectra were recorded at RT under anaerobic conditions.

These results are in agreement with data presented in the literature for cyclic nitroxides, reporting that the piperidine (six-member ring) nitroxides (as TEMPO) are metabolised much faster than the pyrrolidine (five-member ring) nitroxides in biological systems [Komarov et al., 1994; Kuppusamy et al., 1995b].

The literature data also assert that TEMPO is reversibly reduced by the cytosolic ascorbate/glutathione redox cycle of the skin, depending on the O_2 concentration and availability of reductive compounds [Fuchs et al., 1997]. So, we wanted to analyse the effect of SNAP and AsA under aerobic conditions for all three-model nitroxide SLs used.

IV.1.2.2. Effect of AsA on SNAP-treated nitroxide SLs probes under aerobic conditions

The solution of nitroxide SLs treated with SNAP at a molar ratio of SL:SNAP = 0.3:1 have been flushed with O_2 gas either before or after addition of SNAP. In another set of experiments, AsA, 3mM, has been added to the samples either before or after the previous treatment. Again, cyclic nitroxides presented similar effect induced under these conditions, as shown in fig. 4.1.2.3 and fig. 4.1.2.4. Both in the case of 3-maleimido-PROXYL and TEMPO, intensive oxidation of samples treated with SNAP decays the EPR triplet signal of nitroxide group contained by SLs close to zero (3% and 10% for 3-maleimido-PROXYL and TEMPO, respectively). Addition of AsA after SNAP and O_2, but not before, seems to prevent this effect bringing up the intensity of the EPR signal (37% and 22% for 3-maleimido-PROXYL and TEMPO, respectively).

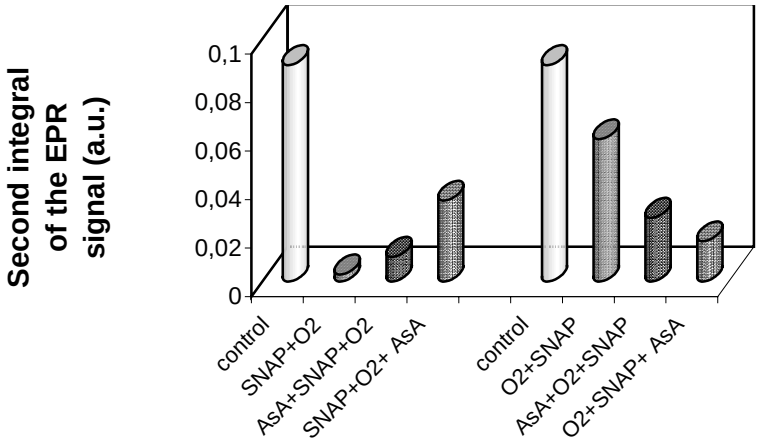


Fig. 4.1.2.3. Effect of AsA on 3-maleimido-PROXYL EPR signal intensity before and after addition of SNAP and O_2. Control bars represent the SL under anaerobic condition (in Ar-DDW). [SL] = 0.3 mM, [SNAP] = 1 mM, [AsA] = 3 mM, O_2 gas flushed for 30 min in the order indicated on the X-axis. Spectra were recorded at RT.

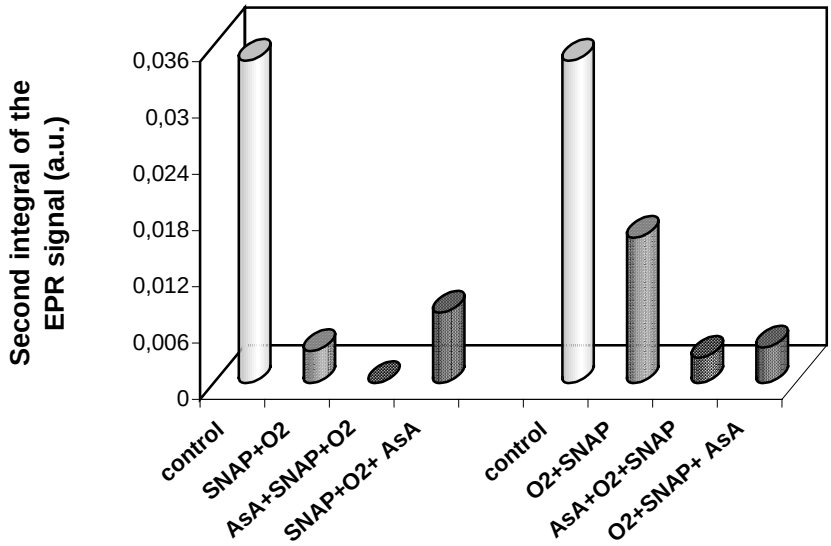


Fig. 4.1.2.4. Effect of AsA on the EPR signal intensity of TEMPO, before and after addition of SNAP and O_2. Control bars represent the SL under anaerobic condition (in Ar-DDW). [SL] = 0.3 mM, [SNAP] = 1 mM, [AsA] = 3 mM, O_2 gas flushed for 30 min in the order indicated on the X-axis. Spectra were recorded at RT.

It can be also observed that addition of SNAP to an aerobic (already flushed with O_2 gas) SL solution induces a smaller effect, leading to a decay of the EPR signal intensity of 66% and 45%, for 3-maleimido-PROXYL and TEMPO, respectively.

In this case, AsA added either before or after this treatment does not prevent the decreasing effect on nitroxide group, it even enhances it. Formation of NO_2^- was determined spectrophotometrically by Griess reaction and concentrations detected were of 36-40 μM for SNAP under aerobic conditions and decreased to 1-2 μM when AsA was added either before or after.

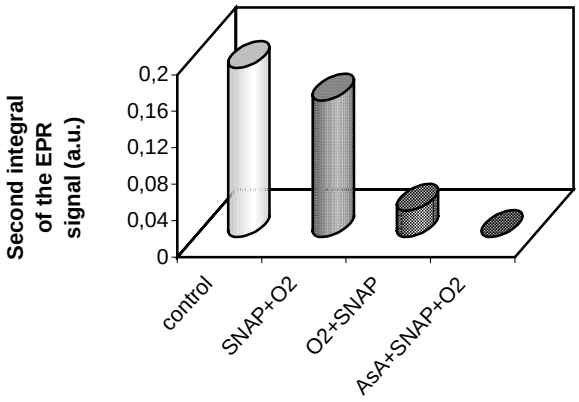


Fig. 4.1.2.5. Effect of AsA on Fremy's salt EPR signal intensity before and after addition of SNAP and O_2. Control bars represent the SL under anaerobic condition (in Ar-DDW). [SL] = 0.3 mM, [SNAP] = 1 mM, [AsA] = 3 mM, O_2 gas flushed for 30 min in the order indicated on the X-axis. Spectra were recorded at RT

Fremy's salt has lost its EPR signal with addition of AsA. The intensity of Fremy's EPR signal decreased to 80% and 16% by flushing with O_2 gas before and after the addition of SNAP, respectively, as shown in fig. 4.1.2.5. The addition of AsA to any combination of SNAP and O_2 led to the lost of the EPR signal.

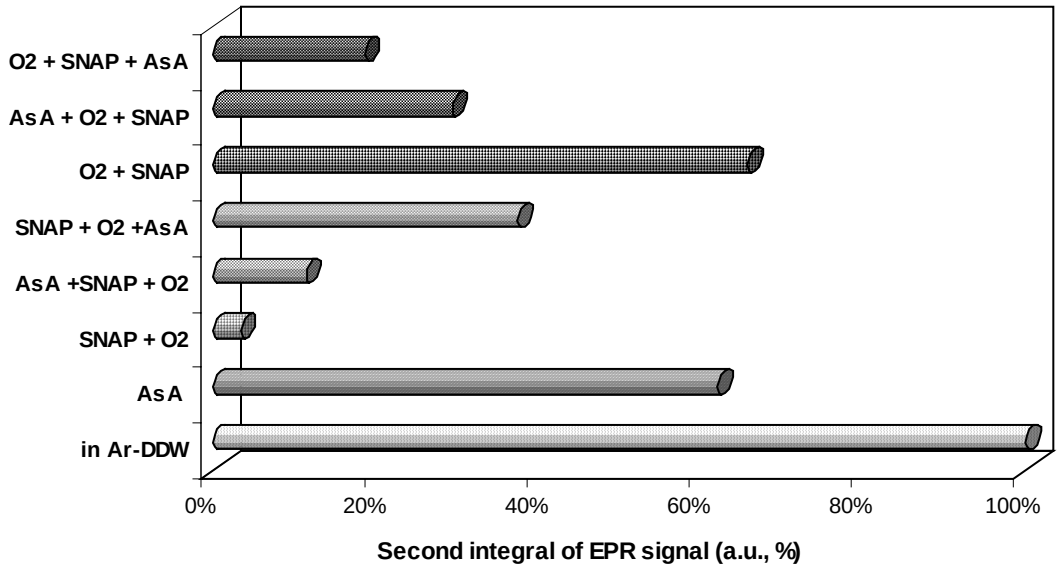


Fig. 4.1.2.5. The decay of the EPR signal intensity of 3-maleimido-PROXYL, before and after addition of SNAP and O_2 and AsA. [SL] = 0.3 mM, [SNAP] = 1 mM, [AsA] = 3 mM, O_2 gas flushed for 30 min in the order indicated on the X-axis. Spectra were recorded at RT.

These results are confirmed by data from the literature, which claim that AsA is very effective at reducing Fremy's salt, resulting in the irreversible formation of its diamagnetic hydroxylamine derivative [Colacicchi et al., 2000], as shown by UV/vis

and EPR methods. Also in the case of Fremy's salt, samples treated with AsA have shown a doublet EPR signal (as seen in Fig. 4.1.1.1., (D)), probably attributed to ascorbyl radical and thus confirming its formation. AsA can be autooxidised in the presence of molecular O_2. Interestingly, AsA seems to regenerate during the reduction of nitroxide, since sustained reduction of nitroxides equivalents was observed in *in vivo* experiments even when most of the intracellular AsA was oxidized [Mehlhorn, 1991].

All these results lead to the conclusion that nitroxides 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 under oxidative conditions appear to act by a complex redox mechanism on the nitroxide group of soluble SLs. This process was simplified by creating a model system, but at cytoplasmic and cytosolic levels, where more compounds are competing for the reactive site, it can became more complex.

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

In the introduction, some of possible NO effects on its various targets associated with biological membranes were summarised. Both, NO and O_2, are hydrophobic and hence biomembranes and other hydrophobic structures, such as low-density lipoproteins or hydrophobic regions of proteins, are potential sinks for them.

We investigated the penetration of NO into lipid membranes by using a potent NO donor, SNAP. This NO donor is a hydrophobic compound and is expected to release NO in membranes during *in vivo* applications, but little is known about its exact location in the membrane bilayers. The spin-label EPR method was used to study the localization of the SNAP in the membranes of DMPC and to determine the transmembrane profiles at high vertical resolution of NO released from SNAP, using spin-labeled positional analogues of the host lipid that bear the nitroxide group at each position from C4 to C14 of the phosphatidylcholine *sn*-2 chain [Nedeianu et al., in press].

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 (Fig. 4.2.1). The control spectra with deoxygenated samples, show relatively broad lines in the gel phase (at 10 ^{0}C, Fig.

4.2.1A), 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, Fig. 4.2.1B), 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.

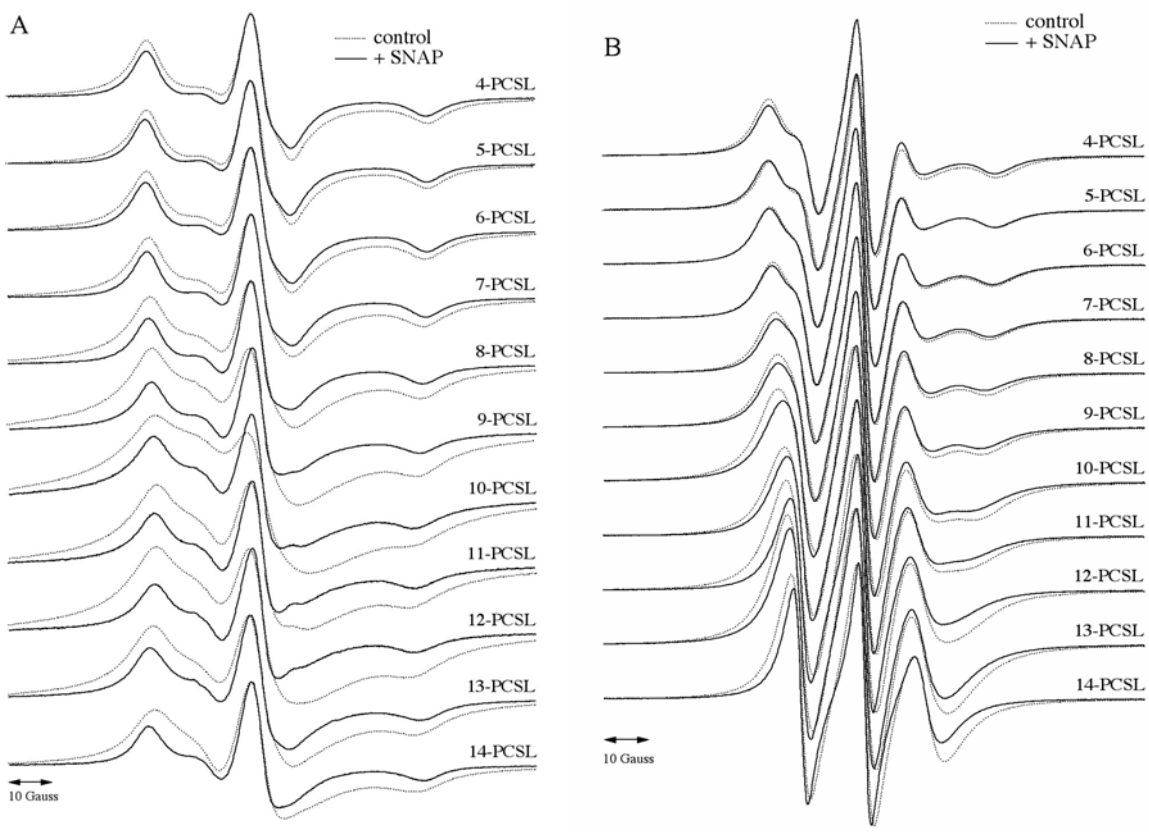


Fig. 4.2.1. EPR spectra of n-PCSL in DMPC membranes at 10^{0}C (panel A) and at 30^{0}C (panel B) in the absence (---) and presence (—) of saturating aqueous concentration of NO donor, SNAP. The spin label: lipid molar ratio is 4:100. Spectra were recorded at a microwave power of 5 mW and are scaled at maximum amplitude.

It has been observed that SNAP significantly increased the membrane fluidity and decreased the rigidity of erythrocytes, suggesting that NO might improve the microviscosity of erythrocyte membranes and play a role in the regulation of physicochemical properties of blood membranes [Tsuda et al, 2000]. But the role, if any, of SNAP as a hydrophobic molecule inside the membrane has not been studied so far. We studied, for the first time, the direct effect of SNAP onlipids in a membrane model. The effect of SNAP on lipid chain dynamics was quantitated by measuring the

outer hyperfine splitting of the *n*-PCSL, in the presence and absence of the NO donor. Fig. 4.2.2 shows the difference, at 10 ^{0}C and 30 ^{0}C, in the outer splitting as a function of the position, *n*, of the doxyl group in the acyl chain of the *n*-PCSL. 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 (30 ^{0}C), 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.

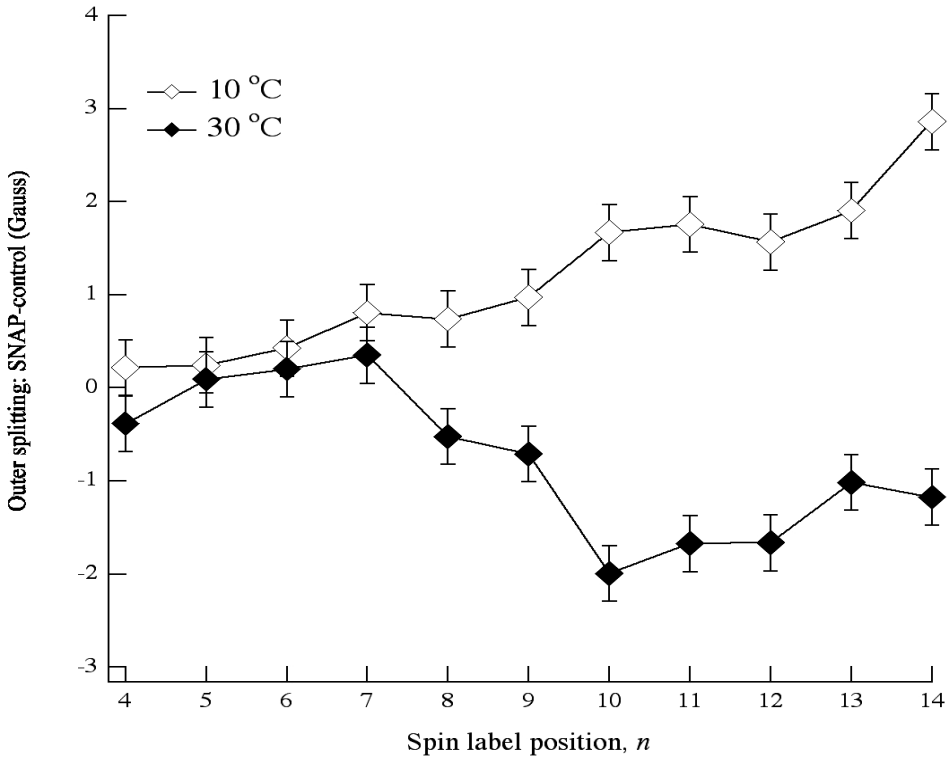


Fig. 4.2.2. Changes in the outer hyperfine splitting of spin-labelled PC, bearing the doxyl group at different positions along the *sn*-2 acyl chain (given in the x-axis), induced by the presence of saturating aqueous concentration (~9 mM) of the NO donor, SNAP. The spin label:lipid ratio is 4:100, the temperature is 10^{0}C and 30^{0}C for gel- and fluid-phase membranes, respectively.

The spectral effects observed in the gel phase of DMPC with ~9 mM SNAP are due to the molecule itself and not to the released NO, since NO is known to enhance, rather than to decrease, spin relaxation of spin labels in membranes [Singh et al., 1994]. In addition, we observed similar effects with N-acetylpenicillamine (NAP), an analogue of SNAP that does not contain the NO-group, at the C14 position (data not shown). These results demonstrate that SNAP incorporates massively in DMPC and certainly other membranes, where it is situated in the hydrophobic core of the bilayer. 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. Typical CW power saturation curves for 4-PCSL at 10 ^{0}C and 30 ^{0}C and for 14-PCSL at 10 ^{0}C are shown in Fig. 4.2.3.

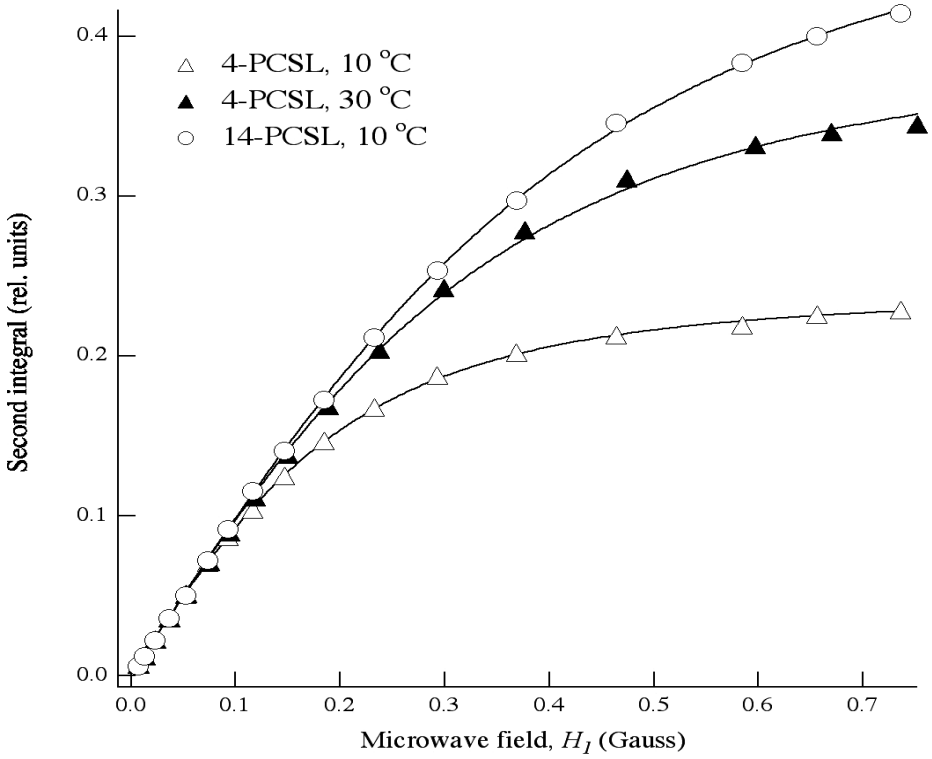


Fig. 4.2.3. CW saturation curves for double-integrated spectral intensity, as a function of microwave field (H_1) incident on samples of PCSL (4- and 14-PCSL) incorporated into DMPC vesicles at a label:lipid molar ratio of 1:800, measured at the temperatures indicated. The second integrals are scaled to the same starting slope to aid comparision. The experimental values were least-squares fitted according to Eq. 1 (solid lines).

The dependence of the integrated intensities (I) on the microwave field strength (H_1) are normalised to the same initial slope $(dI/dH_1)_0$ and are fitted according to eq. 3:

$$I = \frac{(dI/dH_1)_0 H_1}{\sqrt{1+\gamma_e^2 T_1 T_2 H_1^2}} \qquad (3)$$

where I is the second integral, $(dI/dH_1)_0$ is the linear slope at low power, γ_e is the electron gyromagnetic ratio and T_1T_2 is the effective spin relaxation time product [Páli et al., 1993].

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 effective spin relaxation time product (T_1T_2) obtained from such CW progressive saturation experiments, is presented in Fig. 4.2.4 as a function of the spin label position, *n*, of the PC spin label in DMPC membrane, at different temperatures as indicated.

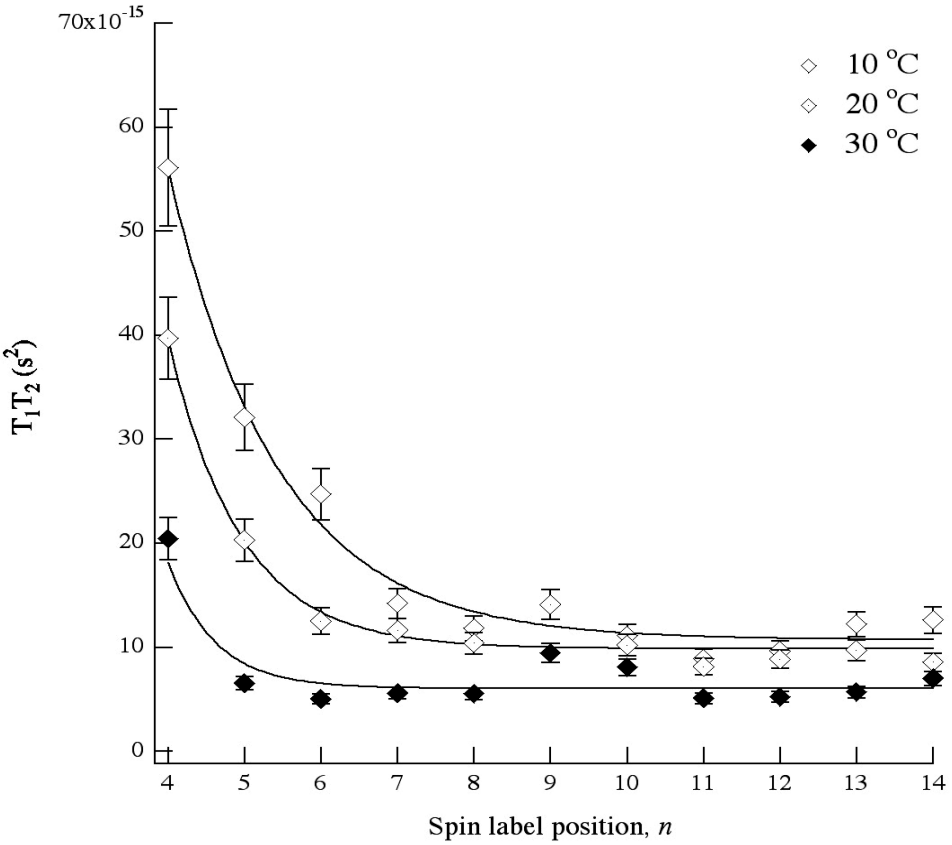


Fig. 4.2.4. Effective spin relaxation time product (T_1T_2) as a function of the doxyl group along the *sn*-2 chain of *n*-PCSL in DMPC membrane vesicles, at a label:lipid molar ratio of 1:800 and the temperatures indicated. Both the hydrating media and the samples were flushed extensively with Ar.

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.

The intrinsic values of T_1T_2 serve as references for SNAP- and O_2-treated samples. Any type of spin-spin interaction decreases the value of T_1T_2. At a label:lipid molar ratio of 1:800, spin-spin interaction between spin labels have negligible effects on T_1T_2. Fig. 4.2.5 gives the relaxation enhancement parameter, $R = \Delta(1/\Delta H_{pp}T_1T_2)$, for NO and O_2, relative to corresponding Ar-flushed samples, as a function of the spin

label position, *n*, of the *n*-PCSL probes, in both gel-and fluid-phase DMPC membranes.

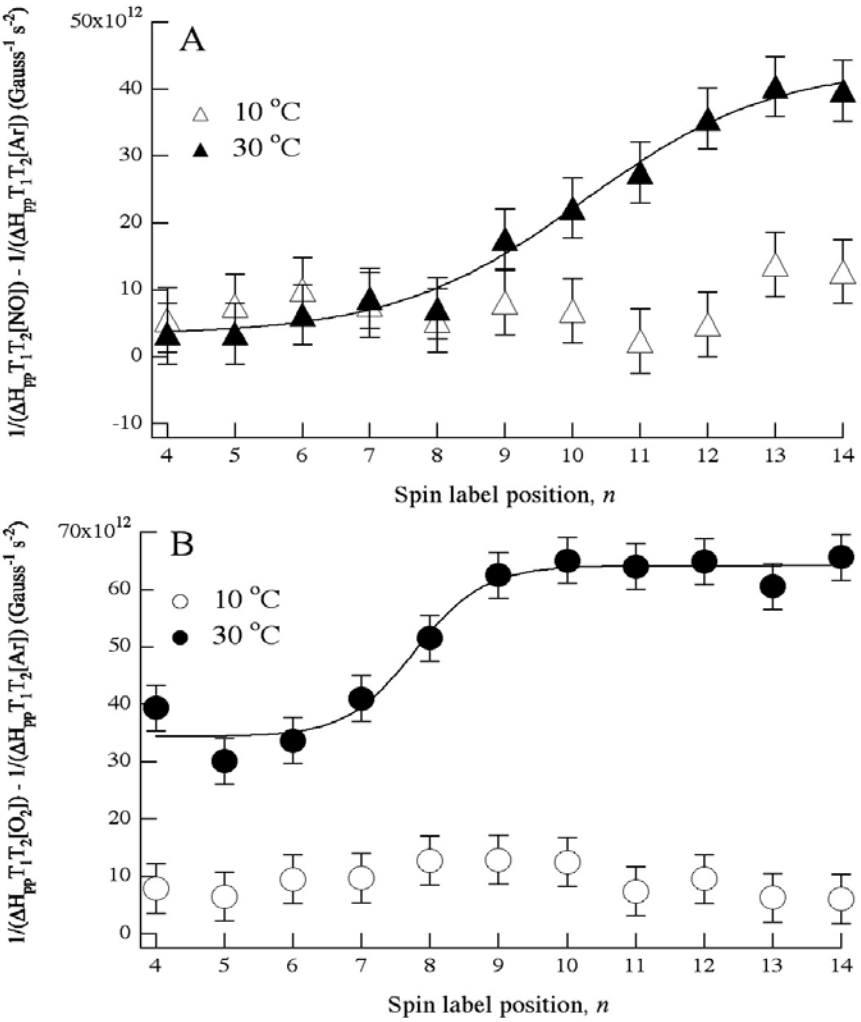


Fig. 4.2.5. Spin relaxation enhancement, R = $\Delta(1/\Delta H_{pp}T_1T_2)$, by NO (panel A) and by O_2 (panel B), of the positional spin label isomers (*n*-PCSL) in DMPC membranes in the gel (10^0C) and fluid (30^0C) phases. The label:lipid molar ratio is 1:800. The solid lines are least-squares fits of Eq. 5 with parameters n_0 = 10.2±0.3, λ = 1.4±0.3 and n_0 = 7.8±0.2, λ = 0.5±0.2 for NO and O_2 data, respectively.

Because the diffusion of NO is fast compared with its release by SNAP [Megson et al., 1997], the size of the membrane [Lancaster, 1994] and the time-scale of our experiment, the transmembrane profile is proportional to the local steady-state concentration-diffusion product of NO [Subczynski, et al., 1989].

The fact that we see no relaxation enhancement in the rigid gel-phase supports the earlier observation that the spin-spin interactions between the spin labels and the paramagnetic gases NO and O_2 are exclusively of the Heisenberg spin-exchange type [Dzikovski et al., 2003; Singh et al., 1994], in which the relaxant

comes into direct contact with the spin label, so that the spin orbitals overlap. The enhancement in spin-lattice relaxation rate, $1/T_1$, is proportional to the local concentration-diffusion product of the relaxant [Subczynski and Hyde, 1981; Livshits et al., 2001] as presented in eq. 4:

$$\frac{1}{T_1} = \frac{1}{T_{1,0}} + k_0 D_R(z) c_R(z) \tag{4}$$

where $T_{1,0}$ is the spin-lattice relaxation time in the absence of relaxant, k_0 is a constant that depends on the structure of interacting molecules and $D_R(z)$ and $c_R(z)$ are the diffusion constant and local concentration of the relaxant, respectively, at position z along the membrane. A similar equation applies to T_2, but because T_2 is about 2 orders of magnitude smaller than T_1 for nitroxide spin labels, the relative enhancement in $1/T_2$ is negligible compared with that in $1/T_1$ [Livshits et al., 1998]. The relaxation enhancement parameter,$1/T_1T_2$, was corrected with the peak-to-peak width ΔH_{pp} of the central line, in order to reduce differences between various spin labels and to minimize the potential effect of SNAP on the lineshape [Altenbach et al., 1994].

The permeability profiles of NO and O_2, shown in Fig. 4.2.5, are similar, but are in the opposite sense to the polarity profile of DMPC bilayers [Marsh, 2001]. The relaxation enhancement, R, of NO and O_2 observed at low concentration of n-PCSL (with n = 4-14) in fluid DMPC membranes were fitted with a sigmoidal function according to ref. [Marsh, 2001]:

$$\Delta\left(\frac{1}{\Delta H_{pp} T_1 T_2}\right) = \frac{R_1 - R_2}{1 + \exp[(n - n_0)/\lambda]} + R_2 \tag{5}$$

where R_1 and R_2 are the base and maximum values of the sigmoid, n_0 is the point of the maximum gradient and λ is the decay constant. While O_2 is non-polar, with zero dipole moment, that for NO is 0.159 Debye [Lide, 1990], which also may be responsible for the flatter profile and lower partitioning of NO compared to O_2.

Hyde et al [Subczynski et al, 1996], measuring the permeability of NO through lipid bilayer membrane, found that the profile of NO was similar to that of O_2, which is in agreement with fluorescence data [Denicola et al, 1996], and data that cholesterol increased the maximum in the profile of NO permeability and shifted deeper into the

hydrophobic core of the membrane. SNAP itself is quite hydrophobic and has a molecular mass (220 Da) smaller than that of cholesterol (387 Da), but SNAP can also dimerise via S-S bridges after releasing NO [Wang et al, 2002]. Therefore, a perturbing effect of SNAP on the profile of NO can be anticipated. It is clear from fig. 4.2.2 that SNAP perturbs the mobility of the lipid chains, although in a different way from that of cholesterol. It has been observed that SNAP significantly increased the membrane fluidity of erythrocyte, suggesting that NO might improve the microviscosity of erythrocyte membrane and play a role in the regulation of their physicochemical properties [Tsuda et al., 2000]. Part of the shifted and broadened profile of NO, relative to that of O_2, may therefore be attributed to the incorporation of the NO donor (SNAP) into the hydrophobic interior of the membrane. In particular, because perturbation by the donor reaches a plateau at the positions n = 10-11 in the fluid-phase membrane, it is very likely to influence and possibly broaden the profile of NO relative to that of O_2 for which no donor was used.

The relaxation-rate enhancements, $R = \Delta(1/\Delta H_{pp}T_1T_2)$, for the nitroxides at position n, given in fig. 4.2.5, are directly proportional to the product $K_R(n)D_R(n)$ of the local partition and diffusion coefficients of the relaxant in the membrane. Because of their comparable molecular size, the diffusion coefficients of NO and O_2 are likely to be similar. In water at 20 ^{0}C, D_w = 2.35 and 2.00x10^{-5} cm^{2}s^{-1} for NO and O_2, respectively [Lide, 1990]. For O_2, the partition-diffusion product found at the interface of the membrane is $K_1D_1 \approx$5x10^{-5} cm^{2}s^{-1} [Dzikovski et al, 2003]. Considering data from fig. 4.2.5B, the partition-diffusion product of O_2 at the centre of the membrane then becomes $K_2D_2 = K_1D_1x(R_2/R_1) \approx 10^{-5}$ cm^{2}s^{-1} (cf. eq.). Thus, the partition-diffusion product for NO in the centre of the membrane is $K_2D_2 \approx$ 7x10^{-4} cm^{2}s^{-1}, under steady-state NO-released from SNAP. This determines the rate of reactions of NO within the membrane. Comparison with the diffusion coefficient of NO in water shows that NO reaction rates in the membrane are potentially higher than those in the aqueous phase. Considering the same value for the diffusion coefficient of NO in water and taking $K_1D_1 \approx D_w$ from the spin-label line-broadening results of Subczynski [Subczynski et al., 1996], yields an effective permeability coefficient P = 160 cm s^{-1} for NO in fluid DMPC bilayers.

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 investigating the effect of this important NO donor (SNAP) on a phospholipid membrane.

Concluding remarks

One of the smallest molecules, nitric oxide (NO), has been more known previously as a toxic compound (being 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) until it was discovered in mammalian body where it can function as: an endogenous vasodilator influencing cardiovascular tone, a neurotransmitter, an inhibitor of platelet aggregation, a modulator of inflammation and immunity, being implicated in numerous diseases, by the inhibition of key enzymes in DNA synthesis, mitochondrial respiration, iron and calcium metabolism, to mention just some of its effects. Having so many implications in various metabolic processes, it is demanding to have a sensitive and specific method to be able to detect and to measure quantitatively formation of NO under *in vivo* conditions and in real time in whole animal bodies. In the present study we demonstrated that EPR spectroscopy and especially spin trapping and spin-labelling EPR are very good tools for detection and characterization of NO effects in different media.

The spin trapping EPR assay together with the $Fe(DTC)_2$ complexes provide a very sensitive and specific method for detection of NO. The triplet EPR signal characteristic for the nitrosyl-adduct can be recorded practically at any temperature, from the physiological (37 ^{0}C) to cryogenic temperatures (77 K and below). Using a water-soluble ($Fe(MGD)_2$) and a lipid-soluble ($Fe(DETC)_2$) spin trap, we analysed the triplet signals of the nitrosyl adducts ($NO\text{-}Fe(DTC)_2$) formed and characterise their magnetical parameters. CW-saturation measurements combinated with spin trapping technique were performed and the characteristic relaxation times (T_1T_2) were calculated for the first time for these types of complexes in various media and at different temperatures [Nedeianu and Páli, 2002]. The presence of reducing agents improved the stability of the nitrosyl-adduct formed, as well as prevented the oxidation effects. The sensitivity of the spin trapping EPR method with the above $Fe(DTC)_2$ complexes was tested by the lower limit of detection for NO (about 0.4 nM NO, under anaerobic conditions). We found that a relatively low concentration of Fe necessary for formation of the spin trapping complex.

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). In the latter case, the characteristic triplet EPR signal was very strong and its intensity decreased by less than 20% after the first 5-8 days then it sustained at an almost constant level for more than 30 days. This method was used previously for other NO specific spin traps, PTIO [Woldman et al., 1994]. This approach, the encapsulation into liposome vesicles, can be used not only to avoid oxidation, but also to improve NO quantitation and to reduce the effect of NO_2, a second product of the reaction. This effect can be used for potentially clinical purposes, since 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 to *in vivo* organisms. For example, the Fe(DETC)$_2$-MLVs can be used to trap the excess of NO produced under various pathophysiological conditions, when high concentration of NO constitute a disadvantage in the biological systems. NO-MLVs, loaded with a NO donor, can be useful by releasing controlled concentrations of NO when the presence of NO would help to prevent, ameliorate or cure in diverse diseases. Of course, further experiments are necessary to attempt for the preparation and characterization of lipid unilamellar vesicles, to investigate the tolerance, their transport inside the animal bodies and delivery to specific targets, as well as the clearance time of these complexes.

Since Fe(DETC)$_2$ complex is lipid-soluble and permeable through the membrane, it is suitable especially for detection of intracellular and intramembranous NO in biological systems and animal bodies, *in vivo*. Similarly, the Fe(MGD)$_2$, as water-soluble complex, can scavenge NO in the hydrophilic compartments and extracellular, in biological fluids, such as blood and urine.

The spin labelling EPR method was our approach used to study the effects induced by NO on other free radicals under different redox conditions, in both aqueous media and in membranes.

As water-soluble spin labels, three different compounds were used in order to investigate the nitrosylation (NO), nitration (NO_2), oxidation and reduction effects on the nitroxide group of the SL, which depend on the substituents of the nitroxide group. The chemical structure and properties of the water-soluble nitroxide SLs were different: two are cyclic aromatic nitroxides (TEMPO and 3-maleimido-PROXYL) and the third one is a linear nitroxide compound, Fremy’s salt.

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^-, seemed to induce an enhanced decay of the EPR triplet signal of nitroxide SLs, especially the cyclic nitroxides (TEMPO and 3-maleimido-PROXYL). This decay was not dependent on the concentration, but on time. Using CW power saturation measurements it was found that the relaxation enhancement induced by NO_2^- on nitroxide SLs was a result 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.

AsA, a very common reducing agent in biological systems, induced a potent reductive effect on the nitroxide group of SLs, as observed by the decrease of its EPR triplet signal intensity at RT. AsA affected the three nitroxide SLs, in a different manner, as they were affected by O_2 and/or SNAP. We 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. Both in the case of 3-maleimido-PROXYL and TEMPO, intensive oxidation of samples treated with SNAP decays the EPR triplet signal of nitroxide group contained by SLs, close to zero. Addition of AsA after SNAP and O_2, seems to prevent this effect to some extent. 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.

The membrane penetration profiles of NO, its derived reactive species (RNOS) and its potent hydrophobic donors, are of considerable biological and biochemical relevance. 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. Our results show that the penetration profiles of NO and O_2 are similar, but that of NO is broader and is shifted somewhat deeper in the membrane relative to that of O_2. Both NO and O_2 have maximum concentration in the centre of the membrane. Therefore, the chemical reactions in the membrane involving NO or O_2, or both, will proceed at higher rates in the centre of the bilayer then in the phospholipid headgroup region. We also found that SNAP perturbs the mobility of the lipid chains, suggesting its incorporation into the hydrophobic interior of the membrane. The fact that SNAP also concentrates in the hydrophobic core of the

bilayer is one of the reasons why it is an effective and functional NO donor in *in vivo* experiments.

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.

The present 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.

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Summary

Nitric oxide (NO) is an important second messenger with many implications in various physiological and pathophysiological processes, which take place in microbes, plant and animals. NO is a molecular “double-edged sword”; although excess of NO can cause many organ dysfunctions and diseases, it remains an important modulator of a wide variety of functions in the cardiovascular, central nervous and immune systems. Enzymatic and nonenzymatic pathways for NO synthesis in biological systems are known. NO has many important targets inside biological systems in various locations. It has a high affinity for transitions metals, especially for Fe and therefore 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 peroxinitrite ($ONOO^-$), all these products are usually more reactive than NO itself and can induce various metabolically dysfunctions. 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. This molecule is hydrophobic and can diffuse freely through cellular membranes, travelling from one organ to another *in vivo* conditions. The metabolism of inhaled, injected or endogenously formed NO in biological systems depends on diffusion, convection, distribution and chemical reactions within the different compartments. Its lifetime depends on the cell type and the composition of the environment in which NO exists. NO is a hydrophobic free radical and thus, biological membranes and hydrophobic compartments are important locations for the nitrosative chemistry, which occurs upon NO autooxidation, such as formation of nitrosothiol, nitrosamine and lipid peroxide, DNA damage and genotoxicity.

Direct detection of NO in biological systems is difficult because of its short life time (few seconds under aerobic conditions), high reactivity, low concentration (0.01-10 μM) and its partitioning between aqueous and hydrophobic environments. The most sensitive and selective and most widely used method, both in vivo and in vitro, is spin trapping. Spin traps are compounds that interact with the unstable radicals, producing a more stable adduct which can be detected by electron paramagnetic resonance

(EPR) method. In this study we used spin-trapping compounds with high affinity for NO, the iron dithiocarbamate ($Fe(DTC)_2$) type of complexes, which form stable nitrosyl iron-dithiocarbamate ($NO\text{-}Fe(DTC)_2$) adducts characterised by a three line EPR spectrum. Using a water-soluble ($Fe(MGD)_2$) and a lipid-soluble ($Fe(DETC)_2$) complex as specific spin traps for NO, the nitrosyl-adducts have been monitored both in aqueous and lipid media in order to obtain more information about: their formation and stability in different environments; the influence of different physical and chemical factors, the Fe:DTC stoichiometry and the trapping efficiency. Various NO sources were used for this purpose, either NO gas or a NO donor ($NaNO_2$, SNP or SNAP) with different chemical and physical properties. The stability of the hydrophobic $NO\text{-}Fe(DETC)_2$ 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 label EPR technique, the effect induced by NO on various 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 occur. NO, being also a hydrophobic molecule can freely diffuse through the lipid bilayers of membranes unless it meets some of its specific targets associated with membranes. 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 diffusion and penetration of NO and O_2 were analysed in lipid vesicles at different depths of the bilayer. Also the diffusion and penetration of SNAP, a hydrophobic donor of NO, often used as a relaxing agent by releasing NO, into DMPC membranes were studied at different depths of the lipid bilayer at near atomic resolution. 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.

In conclusion, it is biologically and physiologically important to understand the differences in generation and interaction of NO with different compounds. The EPR technique seems to be the best method providing highest sensitivity and specificity to monitor NO formation and disappearance in both aqueous and lipid environments and under different conditions. 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.).

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-α production and sensitises to lethal shock.” (to be submitted)