

Nitroxides: 170 Years of History in Biology and Biomedicine

Gertz I. Likhtenshtein^{1*}

¹Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Author's contribution

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ABSTRACT

It is known that there are delicate links and fine parallels between an art and science. Both these spheres of human endeavor involve a unique combination of professional skill and creative search. Sometimes an intuitive line of a great poet or philosopher may be likened to the opening of a new horizon in science. Thus, the composer Maurice Ravel in his famous "Bolero" allegorically depicts the process of birth and development of an epochal discovery that gives rise to many advantages. At first, a musical tune arises whose sound is so weak, so feeble, that it can be easily drowned out by the surroundings. In the second movement, the music is repeated with the same melody, but now with an additional hue. The process repeats itself, again and again, until eventually the most powerful strains of bold, majestic music are then performed by the symphonic orchestra. Like that opening musical movement, the initial publications on the chemistry and application of a novel class of stable radicals, nitroxides, were first met with skepticism, and even strong criticism, from qualified and very professional members of the scientific community. But, later, more and more young enthusiasts joined the ranks of scientists applying this new tool in their research, and ever increasing reports of nitroxides were published in the various fields of chemistry, physics, biology, material science, and biomedicine. The theoretical and experimental data presented in this review clearly demonstrate both history and the current progress within the nitroxide "empire."

*Corresponding author: E-mail: gertz@bgumail.bgu.ac.il;

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1. LONG PREAMBLE OF A TALE (1845-1960)

As an each way, a long road of the 170 years history of stable radicals bearing a >N-O group has started with the first step. And this step has been made in 1845 by Edmond Frémy (1814–1894), a director the Muséum national d'histoire naturelle, who was one of understanding chemists of 19 century [1]. Edmond Frémy published numerous articles in

the Annales de Chimie et de Physique, 7 volumes of *Traité de chimie générale* and 10 volumes *Encyclopédie Chimique*, in collaboration with several other scientists. But his main advantage, which left memories in the chemical history was a synthesis and characterization of disodium nitrosodisulfonate (potassium nitrosodisulfonate, Frémy's salt, FS) (Fig 1A), a precursor of a family of numerous nitroxide radicals (Fig. 1E, Fig. 2).

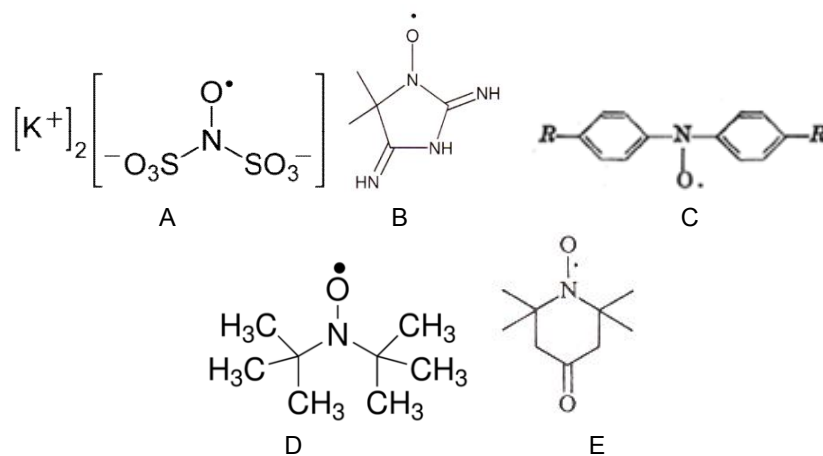


Fig. 1. Structures of pioneering nitroxides

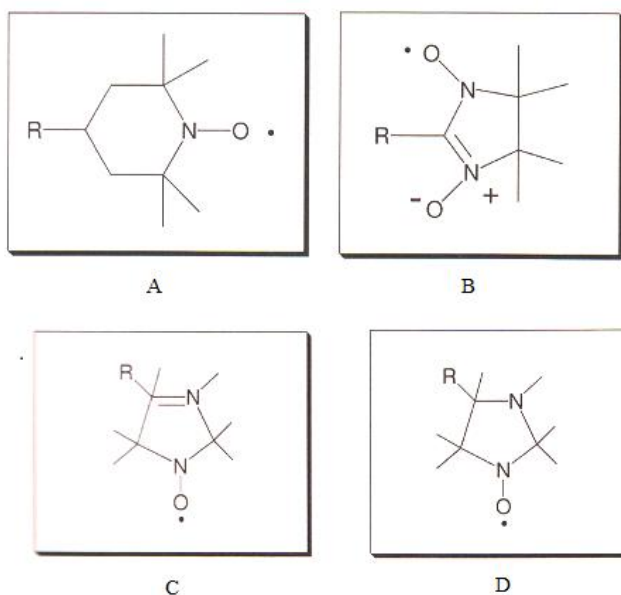
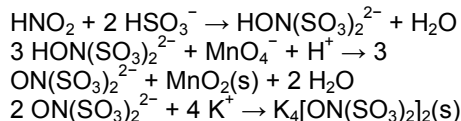


Fig. 2. Nitroxide derivatives: Piperidine A, imidasoline B, imidasolidine C, and nitronyl D

A synthesis of potassium nitrodisulfonate, having a bright yellowish-brown color in the solid phase and bright violet in aqueous solutions and occurring by the Scheme 1

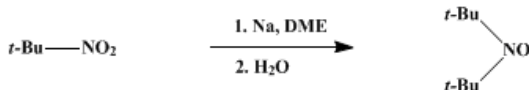


Scheme 1. A synthesis of potassium nitrodisulfonate

now can be readily performed in a student chemical laboratory.

Fremy's salt is long-lived radical in water and other solvents in anaerobic conditions. Nevertheless, a rapid and highly exothermic decomposition of this compound occurs spontaneously in air, that is a serious limitation for its applications. Nowadays FS, as a strong oxidant, finds a number chemical applications. The first physical application Fremy salt gives in solution an intensive signal of ESR of three narrow lines typical for other nitroxides (Fig. 3) and can serve as a standard.

Next step in area was done by O. Piloty and B.G. Schwerin in 1901 [2], who prepared the first organic radical containing >N-O and was named as "porphyrexide" (Fig. 1.B). Remarkable advance, reported by Heinrich Wieland and Moriz Offenbacher in 1914, was the synthesis of diarylnitroxides (I), in which the >N-O group is attached to two conjugated systems [3]. At the first step of the synthesis, Ph_2NOH was obtained in greater yield and purer form by at once decomposing the reaction mixture of PhMgBr and PhNO . Oxidation of Ph_2NOH by Ag_2O led to formation of diphenylnitric oxide, Ph_2NO , as deep red needles (Fig. 1.C). Stable di-tert-butyl nitroxide (Fig. 1.D) was synthesized by Kentaro Hoffmann and Audrey T. Henderson in 1961 [4] using the Scheme 2:



Scheme 2. Synthesis of di-tert-butyl nitroxide

About 60 years after, in the frame of G. A. Razuvaev chemical school, Lebedev et al. prepared 2,2,6,6-tetramethyl-1-piperidinyloxi

from acetone and ammonia. prepared 2,2,6,6-tetramethyl-1-piperidinyloxi from acetone and ammonia (Fig. 1E) [5].

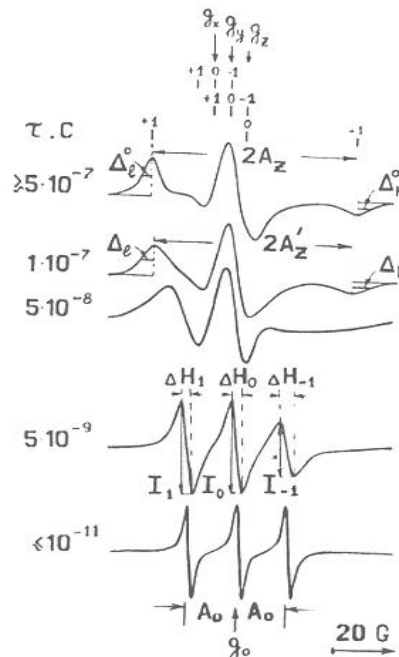


Fig. 3. X-band ESR spectra with different correlation time

Fig. 4 shows portraits of the first discoverers of nitroxide chemistry.

2. "GOLDEN" DECADE (1962 - 1972)

For period 1962 – 1972 practically all cornerstone ideas in the area of nitroxides were exposed. These ideas were sustained by fundamental theoretical and experimental investigations in chemistry and physics of nitroxides. First nitroxides, presented in Fig. 1, though their principle importance, did not find wide application and not be able to serve as a basis for synthesis of new paramagnetic compounds in a broad scale.

Presence of the ketone group in the radical E (Fig 1) opened, in principle, a way for further modification. Nevertheless in that time, according to commonly accepted paradigm, the most chemically reactive portion of a radical can be a group bearing spin electron. This paradigm was broken by M.B Neiman and E. G. Rozanzev, who introduced nitroxide reactions without direct involvement of the spin center [6,7]. In parallel, sensitivity of ESR spectra of nitroxide to

molecular dynamic in liquids of different viscosity was demonstrated by A. L. Buchachenko and S.S Troizkaya [8].

As a consequence, rapid and extensive progress in the area, a real burst of works on synthesis and application of nitroxide in chemistry, physics, biology and even in medicine have been broken. These pioneering works have laid a chemical basis for method of nitroxide spin labeling, in particular.

At the end of 1960th new classes of stable radicals, α -Nitronyl nitroxides Fig. 2 D [9] and Doxyl nitroxides, Fig. 7 [10], possessing great potential for further wide applications, were synthesized.

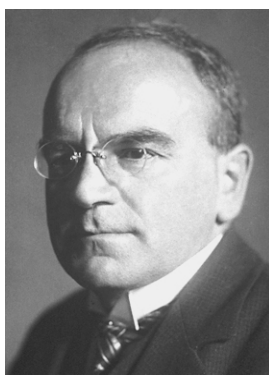
A number of problems of structure and dynamics of complex molecular systems including biological ones can be solved by a modification of chosen portion of object under interest by a physical label (stable radical, paramagnetic complex,

luminophore) following by monitoring by corresponding physical methods.

Decisive breakthrough in the spin labeling area was occurred owing to a whole cascade of pioneering works of Harden McConnell and his collaborators. In the first publication in this series [11], a spin label, 2, 2, 6, 5-tetramethyl-3-isocyanatopyrrolidine-1-oxyl, was covalently tethered to bovine serum albumin and to poly-L-lysine. The ESR spectra indicated sensitivity of the nitroxide segment rotation to molecular motion of the labeled compound. Binding of a spin labeled hapten, the 2,4-dinitrophenyl hydrazone of 2,2,6,6-tetramethyl 4-piperidone nitrogen oxide to antidinitrophenyl antibody was proved by the estimation of the rotational relaxation time of the bound hapten using analysis of the nitroxide ESR spectra [12]. Portraits of the first discoverers of nitroxide chemistry and pioneers of its application are shown in Figs. 4-8.



Edmond Frémy



Heinrich Otto Wieland



Grigory A. Razuvaev

Fig. 4. Portraits of the first discoverers of nitroxide chemistry



Fig. 5. Professor Moisey B. Neiman (1889–1967)



Fig. 6. Gertz I. Likhtenshtein, Anatoly L. Buchachenko, Eduard G. Rozanzev, and Nikolay N. Semenov

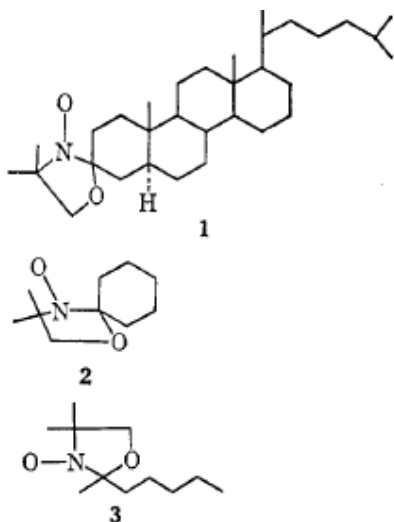


Fig. 7. Doxynitroxides



Fig. 8. Professor Harden McConnell

Powerful potential capacity of spin labeling method for study conformational transitions in

proteins, which are necessary for its functional activity, was illustrated by the McConnell group on example of hemoglobin. The preparation and detection of the Electron Spin Resonance of crystals of horse oxyhemoglobin (HbO_2), spin-labeled with the nitroxidemaleimide [13] were preceded the spin-label study of oxygen-linked conformational changes in hemoglobin at different concentrations of O_2 [14]. Two reactive β -93SH groups in horse hemoglobin were modified with N-(1-oxy-2,2,5,5-tetramethyl-3-pyrrolidiny) iodoacetamide. The ESR spectra of the labeled hemoglobin were dependent on degrees of oxygenation. The Hill constant n , a measure of the cooperativity of sigmoidal oxygen binding, was found to be $n = 2.3$ for the labeled hemoglobin, as compared to $n = 3$ for native hemoglobin. These results appeared to be a direct prove of allosteric conformantional mechanism of the "heme-heme" interaction. It was concluded that each of subunit of the protein tetramers undergoes a substantial conformational change when that subunit binds a molecule of oxygen. The pioneering works of Mc Connell and his colleagues on hemoglobin, a protein composed of subunits, however, has not resolved the important question of allosteric interactions in enzymes and proteins not having a quaternary structure. To tackle the problem, in works of G.I. Likhtenshtein group [15], the enzyme lysozyme was spin labeled by the hystidine-15 group located at the distance 15 Å from the substrate binding center. Addition of specific inhibitors NAG and NAG-NAG induces distinct changes in ESR spectra, which were in a good quantitative agreement with the extent of the substrate binding. Similar trans-globular effect was detected in spin labeled myoglobin.

The first work on the use spin labeling in enzyme catalysis has been published by L. J. Berliner, H. M. McConnell in 1966 [16]. It was shown that the nitroxide spin-labeled substrate, DL-2,2,5,5-tetramethyl-3-carboxypyrrolidine-p-nitrophenyl ester can be used to study the activity of the proteolytic enzyme α -chymotrypsin. Broad ESR resonance lines, which relates to the paramagnetic acyl group immobilized at the active site, were transformed to the three narrow lines arising from free nitroxide. Next a whole series of reagents for active serine group in proteolytic enzymes based on phosphate and nitrobenzene derivatives was proposed. Using these derivatives, researchers were able to compare of active centers of various proteases with the active center of α -chymotrypsin, whose structure is known. The most conclusive work in this area was carried out by J. S. Hsia, D.I. Kosman and J. S. Piette [17].

One of remarkable achievement of the spin-labeling methods has been quantitative characterization of flexibility of model and biological membranes. Incorporation of hydrophobic nitroxide probe 2,2,4,4-tetramethyl-1,2,3,4-tetrahydro- γ -carboline-3-oxyl into the sodium dodecyl sulfate (NADS) decreased in the rate of tumbling of the probe, which is quantitatively described by a rotational correlation time, τ_c [18]. The experimental values of τ_c in the NaDSmi cells was found to be 18 time higher than in dodecane.

A progress in investigation of membranes was provided by synthesis of new class of steroid and lipid nitroxide spin probes (Fig. 7) performed in the J. F. W., Keana group [10]. Application of steroid and lipid nitroxide spin probes in sonicated phospholipid dispersions, the walking-leg nerve fibers of *Homarus americanus* and in erythrocytes [19,20] oriented by hydrodynamic shear has revealed the probe motion, with rotational diffusion frequencies of the order of 10^7 to 10^8 s⁻¹ that was considered to lend plausibility to models of membrane transport involving rotations and/or translations of carriers with in the hydrophobic region of the membrane.

The first approach for quantitative investigation of lateral diffusion in membranes with the use of spin labels was developed by McConnell and McFarland [21]. In a typical experiment, small drops of a lipid spin probe were inserted to films of oriented multilayers of lecithin. Because radical diffusion, the probe ESR spectrum change from a singlet, which is characteristic of large local

concentration, to a triplet from for diluted radicals. Analysis of the process kinetics allowed to calculate the coefficient of translational lateral diffusion.

The application of nitroxide radicals to covalent modification and the study of the structure, dynamic and conformational changes of nucleic acids pioneered by [21] was based on the principles established for proteins. Covalent modification of poly rA, poly rU, and poly rG was carried out by reaction of 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinoxyl, with poly rA, poly rU, and poly rG [22]. The possibilities of noncovalently bound nitroxide probes were first demonstrated in [23].

As early as in 1968th Vasserman, A. M.; Buchachenko, A. L.; Kovarskii, A. L.; Neiman, M. B. have shown powerful potentiality of nitroxide spin label methods in investigation molecular dynamics and micro structure of high molecular mass compounds [24]. The first observation of effect of nitroxide on radical polymerization, that is, inhibition with nitroxide mono- and biradicals, was made in 1966 by the M. B. Neiman group [25]. The inhibiting effect of the nitroxides on styrene polymerization at 50°C, initiated by azodiisobutyronitrile, was interpreted as a recombination of nitroxides and polymer radicals which led to the process termination.

Synthesis and investigation of the chelate complex Cu⁺² Schiff bases ligand derivative of TEMPO, which appeared to be the first transition metal complexes with paramagnetic ligands, was carried out in [26].

Method of double spin labeling, invented by Likhtenshtein in 1968 [27], was based on specific modification of chosen groups of object of interest by two or several spin labels, nitroxides or complexes of paramagnetic metal, followed by analysis of effects of the spin-spin interactions on the label ESR spectra. This approach allows to estimate distance between the paramagnetic centers up to 2.5 nm [28-31]. In parallel, effects of spin exchange interaction on the labels ESR spectra were used for establishment of structure of systems under investigation, such as iron-sulphur clusters in nitrogenase, ferredoxins and non-heme protein [32].

The first version of method of spin label-spin probe method (SLSPM) proposed by Likhtenshtein and coworkers [33-36] was based on a dynamic exchange spin-spin interaction of a

stable radical, mostly nitroxide, attached to molecular object of interest with a spin probe, which are chemically inert paramagnetic species capable of diffusing freely in solution. Because for a particular pair of paramagnetic species, the value of the dynamic exchange rate constant k_{ex} depends on microviscosity, steric hindrances, and distribution of electrostatic charges the method was intensively employed for investigating microstructure of object under interest, proteins, in particular, in the vicinity of the spin label.

One of principle advantage of nitroxides is a possibility of measurement of their molecular motion. In immobilized state a radical ESR spectra are overlap of individual spectra (spin packets) and each of them depends on the molecule orientation relative of the magnetic field. The radical motion leads to averaging of spin electron spin nuclear interaction and to a drastic change of the ESR spectra. Correlation time for isotropic and anisotropic rotation τ_c for a nitroxide on a homogeneous media in the area of fast ($\tau_c = 10^{-9} - 10^{-10}$ s) and slow ($\tau_c = 10^{-7} - 10^{-8}$ s), can be estimated in the frame of theory developed by D. Kivels on [37] and JH. Freed [38].

Principles of application of nitroxide spin label method as a tool for experimental investigation of proteins molecular dynamics ("breathing") have been formulated at the end of 1960th by Likhtenstein [39] and then were implemented in collaborative works.

A suggested approach relied on the well known finding that the rotation of a molecule in a condensed phase are modulated in the great extend by the molecular dynamics of the surrounding molecules. Therefore, parameters of motion of a nitroxide may serve as characteristics of surrounding media dynamics. For example, the monitoring motion of hydrophobic nitroxide spin probe in binding site of human serum albumen revealed low amplitude wobbling of the probe with the correlation time $\tau_c \approx 10^{-8}$ s modulated by the binding site dynamics [40]

Protective antitumor activity of nitroxide in animals was demonstrated in pioneering work of N. P. Konovalova, M. B. Neiman, E. G. Rozanzenov, and Emanuel N. M. in 1964 [41]. Later in 1970 G. Sosnovsky, M. Konieczny have performed syntheses anticancer drugs belonging

to the class of alkylating agents containing aminoxyl radicals [42].

In period described in this section, the commonly used method of nitroxide investigations of nitroxides was continuous wave Electron Spin Resonance (CW ESR). Nevertheless, techniques such as Electron nuclear double resonance (ENDOR) introduced by George Feher [43] and electron-electron double resonance (CW ELDOR) was independently reported in 1968 by the Hyde and Freed [44] and Bendersky and Blumenfeld [45] groups. In the frame of these techniques, interactions which are not accessible in the CW EPR spectrum could be resolved. ENDOR spectra contain information on the type of nuclei in the vicinity of the unpaired electron, on the distances between nuclei and on the spin density distribution and on the electric field gradient at the nuclei. The ELDOR spectra were shown to be sensitive to very slow rotations which may provide unique information on the details of molecular dynamics and can be used for distance estimation between centers bearing spin electron.

Thus, chemical and physical contributions within the "golden" decade have formed the basis for subsequent progress in the area.

3. FURTHER DEVELOPMENTS

In subsequent decades, fundamentals and tendencies laid out in the "golden" period were intensively developed and number of publications in the area accelerated almost in a geometric progression. The process includes the expanding of objects of spin labeling, synthesis of new piperidine, pyrrolidine and doxyl spin labels, specific modification multiple functional groups of objects of interest and fitting computation ESR spectra to theoretical ones.

Synthesis of imidazoline nitroxides (Figs. 2B and 2C) markedly expand ability nitroxides for applications as spin labels, as ligands for materials with ferromagnetic properties, as inhibitors in polymer processing, as initiators for "living" radical polymerization [46-47].

Interglobular allosteric effects were found in myosin, myoglobin, aspartate amino transferase, serum albumin, antibodies, serin proteases, myosin, ribonuclease A, glyceraldehyde 3-phosphate dehydrogenase, rhodopsin, etc. [48-51] and references therein).

A general survey was carried out on the theoretical grounds for methods of spin, luminescence and Mössbauer labels, as well as their application in the study of protein intramolecular dynamics [49,51]. When combined, these methods allow the protein dynamics to be investigated within a wide range of correlation times ($\tau_c = 10^2$ – 10^{-10} s) and amplitudes. The experiments indicated correlations between the local segmental mobility of protein globules in a nanosecond temporal scale and biochemical reactions, such as long-distance electron transfer, hydrolysis and photoreactions.

Measurement of distance between spin labels by analysis of line shape of ESR spectra using new computation methods were carried out for double and multiple labeled proteins and enzymes [52]. Labeling by conformationally constrained nitroxide amino acid, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid, TOAC, have improved the distance estimation precision [53]. Advance simulation of nitroxide ESR spectra and synthesis of ^{15}N - and deuterium-substituted, spin-labeled analogues also essentially improve efficiency of the method [54].

In the biomembrane and its models area, researches on location of labels, water and oxygen in membranes, membranes microstructure, molecular dynamics and phase transitions using CW-ESR supported by new computational programs were performed (marsh). Various aspects of spin-label magnetic resonance studies on lipid-lipid and lipid-protein interactions with integral proteins were reviewed [55-57].

Works of 1970th-1990th on chemical modification of DNA with nitroxide derivatives and ESR examination of structure and dynamics of the labeled biopolymer have laid the basis for further detail investigations in this extremely important area of molecular biology [58,59].

After the first publication on labeling of linear polymers such as of cotton, silk, and wool by trichlorotriazine-based nitroxide [60,61], a series works on investigation of cotton fibres and cellulose were reported by Marupov and Likhtenshtein groups [50] and references therein).

In the investigation of chemical polymers molecular dynamics, a set dynamic parameters

such as rotational correlation time, rotation energy and entropy activation and its distribution, spin relaxation times are commonly measured. As an example, molecular mobility in regular and irregular polycarbosylan dendritic macromolecules of various generations was studied by the spin probe technique [62].

A new impact in nitroxide mediated polymerization has started when P. G. Griffiths, P. G., E. Rizzardo, and D. H. Solomon, showed that it was possible to prepare well-controlled and living (homo-, co) polymer by radical polymerization in the presence of nitroxyl radical as controlling agent [63].

Hundreds complexes transition metals with nitroxide ligands were synthesized and investigated (for reviews see [64,65].

Further development of the spin probe –spin label method was described in [66].

In the frame of CW ESR, new methods of investigation of structure and molecular dynamics were proposed and realized. The great sensitivity of power saturation curves of a nitroxide radical to dipole-dipole interactions between the radical and paramagnetic ions made it possible to expand values of distance between spins to be measured maximum up to 80 Å [67].

The CW ESR saturation techniques was employed for the investigation of depth of immersions of nitroxide spin probes radicals up to 40 Å in aqueous and lipid phases of biomembranes [68,69] and references therein). High sensitivity of spin-lattice relaxation parameters of nitroxide was also taken in consideration at development of theory of very slow motion ($\tau_c = 10^{-3}$ – 10^{-6} s) and the theory application [70].

Fig. 9 Shows leaders of groups involved in the nitroxide spin labeling till 1979

4. NEW ERA: MULTIFREQUENCY AND PULSE ESR, SITE DIRECTED SPIN LABELING

Starting from the pioneering works of professor Ya. S. Lebedev and his colleagues [71,72], problems of poor resolution of the 3 cm ESR have been solved by the use of the high-field high frequency (148 GHz), high resolution 2-mm EPR spectroscopy. Significant progress in the high field-high frequency (HFHF) ESR has been

achieved with the use of millimeter-wave quasi-optic technique, permitting the construction of a 9-Tesla, 250-GHz (1.2mm) spectrometer and application of multifrequency technology in spin labeling [73]. The HFHF ESR have found numerous and effective application in various areas of physics, chemistry and biophysics [74-77].

In the past decades a real burst of development of new ESR pulse methods has been broken [78-80]. Among methods used for study of nitroxide labeling objects are three-pulse electron-electron double resonance (PELDOR or pulse DEER) and its development [79-81], pulse saturation recovery ESR (SR ESR) [82], two-dimensional ELDOR [83], High Frequency Pulsed ENDOR/EPR [84], double quantum coherence pulsed ESR (DQC ESR) [85], and ESR Spectra Hole Burning [86]. Application these methods provided expanded possibility for study structure and dynamics of complex biological objects.

The first pioneering step in this direction was invention of Echo-detected ELDOR (three-pulse ELDOR, PELDOR, double electron-electron resonance DEER) by Milov, Salikhov and

Tsvetkov [78]. This method allows to measure spin-spin distances up to 80 Å and investigate spin relaxation dynamics and was further widely and successively applied by Zvetkov and other groups [81-84,87-90]. After the PELDOR discovery, a series works with an aim to improve and expand the pulse techniques. The four pulse technique allows to avoid a number artifacts in the spin-spin distance measurements [88,89].

The novel five-pulse DEER method introduced in [91] possesses considerable potential to develop into a widely useful technique to study nanoscale systems, wherein long or more accurate distances need to be measured. This technique also could help to considerably shorten data measurements by offering greater sensitivity than could be achieved using standard DEER methods. Detail theory and experimental data on this novel the six-pulse ESR technique for distance measurement, based on the detection of double quantum coherence (DQC), which yields high quality dipolar spectra, as well as its applications, were reported by Freed with coworkers [85]. The DQC significantly extends the range of measurable distances.



Fig. 9. Leaders of groups involved in the nitroxide spin labeling till 1979. From right to left: rector of the Pech University, Leonid B. Volodarsky, Larry Berliner, Gertz I. Likhtenshtein, R. Rassat, John Keena, George Sosnovsky, Kalman Hideg

The use site-directed mutagenesis in combination with modern ESR spectroscopy gave new breath to the nitroxide spin labeling method. Site-directed spin-labeling (SDSL) invented in W. Hubbell group [92] is the substitution of a selected amino acid for cysteine via the site directed mutagenesis technique following by chemical modification with a sulfhydryl reactive nitroxide radical, or 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (1-Oxy-2,2,5,5-tetramethyl-3-pyrroline-3 methyl) methanethiosulfonate (MTSL).

The efficiency of combination of the site-directed spin-labeling with the advance pulse techniques can be illustrated by several examples. Eight T4 lysozyme (T4L) mutants, 61/135, 65/135, 135/80, 61/86, 65/86, 61/80, 65/80, 65/76 have been prepared and doubly labeled with the methanethiosulfonate spin-label followed by determination of the distances between labels by double-quantum coherence ESR [93]: A distance r was measured at the nanometer scale via the electron dipole-dipole interaction between two 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid spin labels incorporated at positions 1 and 16 a linear peptide antibiotic Alamethicin of 19 aminoacids in hydrated egg-yolk phosphatidylcholine (ePC) vesicles at 77 K. using PELDOR conformational analysis [94]. Spin labeling of mini c TAR DNA and mini TAR RNA for the measurement spin-spin distances by pulse dipolar EPR spectroscopy were reported [95]. Pulse ESR techniques have been also applied to study structure and dynamics of phospholipid membranes [96,97].

5. MISCELLANEOUS APPLICATION

Spin oximetry, first reported by Subczynski and Hide et al. [98] is a version of spin label –spin probe method in which molecular oxygen plays role of spin probe. A method invented for measurement the oxygen diffusion-concentration product was based on the dependence of the spin-lattice relaxation time T_1 of the spin label detected by using saturation-recovery (SR) on the bimolecular collision rate with oxygen. Various aspects of the spin oximetry applications have been reviewed [99-101].

As was pioneered in [10], EPR spectra of stable nitroxides of the imidazoline and imidazolidine types are sensitive to pH and can served as spin pH probes. Data on synthesis and application of a wide set of pH-sensitive nitroxides of different

sensitivity, stability to reduction, lipophilicity and its covalent binding macromolecules have been reported [102-104].

Nitroxide radicals have found various applications in the field of materials science. The landmark was the discovery by M. Kinoshita group et al. in 1991 [105], who prepared the first purely organic ferromagnet ($T_c = 0.6$ K) with respect to one of several polymorphs of a nitronyl nitroxide, 2-(4-nitrophenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Since then, stable NR structures have been used as the spin source and building block for the elaboration of organic or molecule-based magnetic materials [106,107].

The spin redox probe techniques utilizes ability of nitroxides to be reduced with reducing agent to corresponding hydroxyl amine. CW ESR spectroscopy of the nitroxide radicals (NRO \cdot) are widely used for quantitative characterization of redox processes and protection from radical damage [108-111] and references therein). Nevertheless, the sensitivity of CW ESR technique is not sufficient for real time monitoring picomole redox processes in biological systems. Novel methods of fast and sensitive analysis of antioxidant status of biological systems, spin redox probing and spin trapping, investigation of molecular dynamics, models for studies of photo physical and photochemical processes and construction of new magnetic light sensitive materials are based upon the use of dual chromophore-nitroxide (CN) compounds [112-117].

Stable nitroxide free radicals are used in animal models and human diseases to protect processes of reactive radical formation, reactive oxygen species, ROS ($O_2^{\cdot-}$, H_2O_2 , $\cdot OH$) in particular involving oxidative stress [118-121]. ROS are the normal product of native oxidative metabolism. Nowadays potential clinical application have been realizing step by step. Portraits scientists who significantly contributed in nitroxide chemistry, physics and miscellaneous applications are presented in Fig. 10.

The pioneering works, in which the principle possibility of effectiveness of the spin trapping was demonstrated, came to the light in 1968 [122]. Spin trapping WAS designed for an investigation of processes with participation of short living particles, reactive free radicals, bearing unpaired electron. The technique is based on reaction of with a molecule (spin A Strap) with formation sufficiently more persistent

paramagnetic species, called the spin adduct, most commonly nitroxide. The spin adduct can be detected by EPR spectroscopy. Since then it is customary to use this technique for investigation radical processes in chemistry and biology [121,123] and references therein ESR).

The newly developed immuno-spin trapping technique make it possible to detect and identify free radical during metabolism and to unravel the molecular mechanisms that lead to oxidative stress [124].



Fig. 10. Gallery of scientists who significantly contributed in nitroxide chemistry, physics and miscellaneous applications

Biologically active spin label molecules have been the focus of biophysical, biochemical, and synthetic and medicinal chemical studies [125-127]. For example, nitroxide amino acids are applied to design of novel labeled peptides [126]. A derivative of the polypeptide cardio stimulant anthopleurin-B (AP-B) labeled with the spin label 1-oxy-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl azide has been prepared and characterized [127].

A spin cascade method (SCM) based of photochemical and photophysical reactions between a triplet sensitizer and a fluorescence photochrome probe exhibiting the phenomena of cis-trans photoisomerization, triplet-triplet energy transfer and the triplet excited state quenching by a stable radical has been proposed and developed [128,129]. The sensitized cascade triplet cis-trans photoisomerisation of the excited stilbene includes the triplet sensitizer (Erythrosin B), the photochrome stilbene-derivative probe (4-dimethylamino-4'-aminostilbene) (DMAAS) and nitroxide radicals (5-doxyl stearic acid) quenching the excited triplet state of the sensitizer. In the frame of SCM, the following dynamics parameters of the cascade system components can be experimentally measured: the spin label rotation correlation time and spin relaxation parameters, the fluorescence and phosphorescence polarization correlation times, the singlet and triplet states quenching rate constants, the rate constant of photoisomerization, and the rate constant of the triplet-triplet energy transfer. This set of parameters appears to be a cumulative characteristics of the dynamic state of biomembranes in the wide range of the probes amplitude and characteristic time of eight order of magnitude.

Some aspects of application of dual fluorescence-nitroxide super molecules as model systems for the investigation of mechanism of electron transfer and light energy conversion were discussed in [130].

6. CONCLUDING REMARKS

Nitroxides for 170 years of its history have widely "stretched out its hands" to miscellaneous regions of physics, chemistry and biology. Synthesis of hundreds nitroxides of various chemical and photo reactivity, redox potential, size, hydrophobicity, charge, etc, has paved a way for their use as organic magnetic materials, photo switching systems, spin redox and spin PH probes, antioxidants, anticancer and other medicine preparation, and spin labeling, as a

powerful tool for study structure and molecular dynamics of proteins, enzymes, membranes, nucleic acids and other molecular object, in particular.

Author of this review wrote in the first book on spin labeling (Likhtenshtein G.I).

Spin Labeling Method in Molecular Biology. Moscow. Nauka (In Russian).1974) : "It is thus our hope that spin labeling will continue to be an effective tool for solving various complicated problems in molecular biology". Now after 40 years, it is evident that present-day reality has surpassed all optimistic expectations.

As far as concern outlook for further developments, there are all reasons to believe that slow but permanent progress in the area would continue in the next decades. Nevertheless, who knows, new unexpected bright ideas would be launched and implemented and ensure vigorous success, unexpected today.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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