Current Topics in Biophysics 2002, 26(1), 175-189

APPLICATION OF THE ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY TO MODERN BIOTECHNOLOGY

PRZEMYSŁAW M. PŁONKA, MARTYNA ELAS

Department of Biophysics, Faculty of Biotechnology,
Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland.

The presentations of the 5th Workshop on EPR Application in Biology and Medicine, as well as selected papers, in which the EPR (electron paramagnetic resonance) spectroscopy technique played a significant role, are reviewed from the point of view of potential biotechnological applications. Selectivity, specificity, non-invasiveness, and other features of the technique make it particularly well suited to meet the high requirements of a tool appropriate for biotechnological research. Several examples of work in molecular biology are summarized, showing that at all levels of genetic information expression, from DNA and RNA to proteome, in all aspects of cellular phenotype, including membranes and sugar residues, EPR is being effectively used to reveal both structural and functional information. Furthermore, in medical biotechnology, EPR is of crucial importance not only in oxidative stress research, but also in the action and transport of drugs, and in medical imaging. Finally, biotechnology in the classical meaning of the term employs EPR in such areas as plant biotechnology and food production and storage, including such a traditional product as beer. Overall, the wide spectrum of presented data demonstrates impressive versatility and extensive usage of EPR in biotechnology, deserving attention of biotechnologists.

INTRODUCTION

The EPR Workshops, which periodically have taken place in Kraków since 1989, and the special EPR issues of Current Topics in Biophysics, have become a specific and unique record of the development of EPR and its biological and biomedical applications. The 5th Workshop on EPR Application in Biology and Medicine was organized as a part of a program of Support for European Centres of Excellence, entitled "Molecular Biotechnology – Integration of Education and Research – BIER" founded by the 5th Framework Programme of EU.

The meeting of various EPR specialists working in diverse fields created a perfect occasion to discuss selected examples of biotechnological applications of the EPR spectroscopy, basing on some presentations of the 5th Workshop, and on papers published elsewhere. This review is addressed mainly to biotechnologists, to make them aware of all the advantages of this method, its unexpected but impressive versatility, and to suggest how the EPR spectroscopy can be used in modern biotechnology, drawing their particular attention to the molecular aspects of the research.

SPECIFIC FEATURES OF EPR IN BIOLOGICAL RESEARCH

Selectivity

Among thousands of types of substances constituting biological systems, only very few, namely – free radicals, including dioxygen (O_2) and nitric oxide (NO), and limited number of transient metal ions reveal paramagnetic properties, and even fewer may be observed without obstacles by the EPR spectroscopy (Abragam & Bleaney, 1970). In some cases exogenous EPR-detectable paramagnetic probes (spin labels) are intentionally introduced into the system. On the one hand, it is a serious limitation of the method, but on the other hand, it often eliminates the necessity to purify biological samples. The few exo- or endogenous paramagnetic species may be observed against an intact diamagnetic background of the system.

Specificity

Many paramagnetic centers reveal EPR signals of a specific shape being in some cases a fingerprint of biologically important or artificially introduced paramagnetic particles (Wertz & Bolton

1986). The centers of a free-radical character, when not in the near vicinity of a magnetic nucleus (in biological systems mainly ^{1}H, ^{14}N, or in artificially introduced isotope-labeled reagents containing ^{15}N), reveal a similar shape and position of their signals, but even then the signal specificity may be elicited by chemical bounding of the radicals, according to the rules of spin-trapping. Usually, the resulting spin adduct reveal a specific, unique EPR signal, which makes it able to follow also these paramagnetic species of a non-specific EPR signal. Spin-trapping becomes indispensable for the measurement of short-living paramagnetic particles (the majority of free radicals), for the particular spin adducts usually live long enough to be observed by EPR (Rosen, Britigan, Halpern & Pou, 1999).

Non-invasiveness

Due to selectivity, satisfactory penetration of living tissues by microwaves (the absorption of which is measured in the EPR spectroscopy) and relatively low levels of energy absorbed by samples during measurements, the technique gives the opportunity to investigate whole living objects, and even to monitor on-line the changes in their paramagnetic properties (e.g. due to the metabolism of paramagnetic labels introduced into the system) without any considerable interference with the native processes. This is a unique property of the EPR spectroscopy, compared e.g. with the spectroscopy UV-VIS working in the spectrum of electromagnetic radiation, for which most of the tissues are optically dense. Nowadays, EPR has been evolving particularly profoundly towards improving on its *in vivo* applicability.

Sensitivity

This aspect of the technique cannot match the sensitivity of numerous techniques of molecular biology (hybridizations, PCR, microarrays etc.) making it able to detect even a single particle in a sample (in the diagnostic praxis – even a single particle per a set of diagnosed samples). The phenomenon of resonant absorption of microwaves reveals a stochastic character and in principle it may be observed only in a population of spins (observation of the spin of a single electron, however, has recently become a spectacular challenge for the emerging spintronics). For the standard EPR spectroscopy, the theoretical, absolute threshold of detectability is of about 10^{11} spins in a sample. In practice, particularly *in vivo*, this value must be multiplied by tens (Wertz & Bolton, 1986). This fact serves as an effective stimulus to develop the ways of improving the sensitivity of detection, as well as to create the conditions for accumulation of a paramagnetic species (e.g. in the form of long-living spin adducts) on EPR-detectable levels. Even though in many particular cases the sensitivity is still non-satisfactory, the other advantages of the method outweigh this drawback.

Well-established physical and mathematical background

The technique was invented and developed by physicists and the way of investigating nature by this branch of sciences takes for granted solid theoretical foundations for interpretation of the observable and experimentally verifiable phenomena. Consequently, almost all features of the EPR spectra, the position of signals, their spectroscopic width and broadening, the asymmetry and Gaussian or Lorentzial character of lines, the number and separation of the lines of hyperfine structure etc., can be interpreted in a strict, quantitative way in terms of the type of interactions of the unpaired electron with the surrounding, character of the orbital occupied by the electron, or the nature of molecular movement. This way, this physical phenomenon delivers rich information not only on the centers themselves, but also on their interactions with the whole system into which they were artificially or naturally introduced. The EPR spectroscopy is obviously a powerful technique – it enables to measure and identify the paramagnetic centers themselves, their interaction with the paramagnetic surrounding, and sometimes even to conclude on the properties of the diamagnetic background of a biological system.

HOW EPR IS APPLIED IN BIOTECHNOLOGY

The main effort of modern molecular biotechnology is to obtain a biotechnological product — usually a recombinant protein, on a satisfactory scale, which is often preceded by genetic manipulation in order to achieve the efficient expression of particular genes in a chosen expression system. EPR may be used on every stage of a biotechnological procedure, giving valuable insights at all levels of the expression of the employed genetic information, from genome, through transcryptome, to proteome and biological membranes.

Other view of modern biotechnology delineates two main areas, namely production of food (classical biotechnology) and treatment of diseases (medical biotechnology), which creates a research space for the EPR spectroscopy, as well.

The major field of EPR application to biological research is the phenomenon of oxidative stress, always associated with the production of large amounts of free radicals. The impact exerted on the biological system is usually negative. Looking for the examples of EPR in biotechnology one can found primarily the study on the oxidative stress, its reasons, progress, consequences, the diseases brought about by the stress along with the means of their prevention, as well as damage to biotechnological products (Naskalski & Bartosz, 2000).

Another aspect of EPR research in biotechnology concerns structural study on macromolecules and macromolecule-like systems (biomembranes). It includes either the structure and dynamics of nucleic acids or their constituents, interactions within nucleic acids or between them and proteins, as well as functioning of proteins – either native, recombinant or immobilized enzymes. Such phenomena like drug transport across membranes or action of some antibiotics and antimicrobial peptides are also conditioned by molecular dynamics of proteins, their interactions with membranes and nucleic acids. The EPR spectroscopy is able to follow some structural aspects of the listed problems, and to deliver information on both statics and dynamics of investigated systems. As neither aminoacids, phospholipids, nor saccharides are paramagnetic, spin-labeling is the predominant technique in the structural EPR research.

EPR IN MOLECULAR BIOTECHNOLOGY

DNA

The first level of a biotechnological process aimed at obtaining the expression of a gene construct (production of recombinant proteins), is the level of genetic information coded in DNA. EPR is a convenient technique to investigate the nucleotide-centered free radicals in DNA, either produced by irradiation, or indirectly – by other free radicals. Low-temperature studies revealed that the level of such paramagnetic species, namely guanine and thymine cations, as well as cytosine anions, produced by a local single-electron oxidation or reduction of DNA, corresponded to the severity of DNA damage and might be followed by EPR. The extent of DNA damage depended not only on the intensity of mutagenic action, but also on the presence and level of antioxidants, like cysteamine, which itself may turn to an EPR-detectable free radical, and which is able to decrease the number of potent mutations, i.e. the free-radical products of DNA radiolysis. Cysteamine is an organic cation revealing pronounced affinity to the negatively charged DNA (Kornacka & Ambroz, 2001). Similar action was exerted by iron (III) cations revealing properties of an electron scavenger and binding to DNA. Iron (III) effectively lowered the level of 5-thymyl radicals in B-DNA (Ambroz, Kemp, Kornacka & Przybytniak, 2001). EPR was successfully applied to the analysis of DNA hydration, and the process of the hole or electron transfer from the hydration layer to DNA due to water ionization (Debije, Strickler & Bernhard, 2000), and to the analysis of DNA repair by DNA photolyase, by detection of flavin radical formation (Kim, Sancar, Essenmacher & Babcock, 1993).

Other fields of the EPR usage in the DNA research include molecular dynamics of shorter or longer oligonucleotides, as well as interactions either between the particular DNA strands, or between DNA and proteins. The EPR spectrum of two- or five-athom-tethered spin label residues incorporated into the DNA duplex were sensitive to the length of the duplex, as well as to the B-Z conformational transition. Variations in DNA conformation caused by interactions between the strands of a heteroduplex, and between DNA and proteins could be therefore detected with satisfactory sensitivity (Keyes, Bobst, Cao & Bobst, 1997). The sequence-dependence of the submicrosecond bending dynamics of spin-labeled DNA duplexes were considered the determinants of fundamental DNA features like chromatin packaging and protein recognition (Okonogi, Alley, Reese, Hopkins & Robinson, 2000). The latter may be exemplified by the study on the mobility of the spin-label residues placed 6, 9 or 11 nucleotides up- or downstream to the EcoR1 hexamer binding site in the DNA oligomers. In the presence or absence of EcoR1 protein, low structural distortion propagated along the helix as a result of protein binding was demonstrated (Keyes, Cao, Bobst, Rosenberg & Bobst, 1996). The EPR spectroscopy turned out useful in the analysis of reverse transcriptase (RT) inhibition by polynucleotides (Warwick-Koochaki, Hakan & Bobst, 1983), and, as early as in 1980, in the analysis of RT enzymatic activity (Warwick, Hakam, Bobst & Bobst, 1980). The same group performed detailed EPR study on DNA hybridization, reporting the possibility to detect 1 pmol of spin-labeled 15-mer in a loop-gap resonator (Strobel, Kryak, Bobst & Bobst, 1991). They also compared the base dynamics of Z- and B-DNA (Strobel, Keyes & Bobst, 1990), and the local base dynamics of RNA and DNA duplexes, showing slower motion of the spin probes in base-paired duplexes, than non-base-paired duplexes or single-stranded nucleic

acid. RNA duplexes exhibited also slower motion than respective DNA particles (Kao, Bobst, Pauly & Bobst, 1985). Complementary results of similar consequences, showing differences between the EPR spectra of Watson-Crick paired spin-labeled DNA duplexes, and fragments of oligonucleotides of other structures, were obtained by Spaltenstein *et al.* (Spaltenstein, Robinson & Hopkins, 1989).

Along with NMR, EPR was used to investigate the electrostatic potential of DNA quadruplex, via analysis of Mn (II) binding, showing the possibility to distinguish between different quadruplex structures (Wang, Gerena, Swaminathan & Bolton, 1995).

RNA

Similarly to DNA, spin-labeled nucleotides may serve as convenient spin probes for the research on structure and interactions of various forms of RNA, including structures as large as ribosomes (Macosko, Pio, Tinoco & Shin, 1999). Recently, EPR spectroscopy was employed to analyze structure-dependent molecular dynamics of trans activator responsive (TAR) RNA of a human immunodeficiency virus (HIV) -1 (Edwards, Okonogi, Robinson & Sigurdsson, 2001). This report was followed by a detailed EPR study on the interactions of TAR RNA with peptides derived from a transcriptional activator (Tat) protein coded in the genome of HIV-1, as well as with sodium and calcium cations (Edwards, Okonogi & Sigurdsson, 2002). The particular importance of this study can be supported by the fact that the formation of stable ribonucleoprotein complex may be a key step in regulating other cellular processes leading to viral replication, gene expression and pathogenesis (Richter, Ping & Rana, 2002). This phenomenon possesses also a general biological aspect, as transcription factors are proteins revealing affinity rather to DNA than to RNA. Tat-TAR RNA interactions may represent an ancient type of interaction dating from early stages of biogenesis, being a relic from "RNA-world".

EPR was also used to determine the map of protein–RNA interactions between RNA and ribonuclease P from *E. coli*, as a spin label covalently attached to any protein enzyme at a particular site is sensitive to structural changes in its environment. The study (Gopalan, Kuhne, Biswas, Li, Brudvig & Altman, 1999) revealed immobilization of the residue when attached at position 16, 44, 54, 66, and 113 of the enzyme interacting with M1 RNA as a substrate, but not with the deletion derivative of the RNA, which was catalytically inactive. In contrast, attachment to position 21 revealed an increased mobility, when bound to M1 RNA. Dynamic interaction RNA. Dynamic interaction between RNA, DNA and apoprotein of RT characteristic for its enzymatic activity was also the subject of EPR analysis (Kensch, Restle, Wohrl, Goody & Steinhoff, 2000).

The major interactions responsible for the functions of RNA are the interactions with metal ions, which may be easily followed with EPR. Paramagnetic manganese (II) is often used as a model ion for interactions between RNA and the most abundant divalent ion of physiological interest, diamagnetic Mg (II) (Hoogstraten & Britt, 2002). Similarly, the changes in the EPR spectrum of Mn (II) ions allowed to determine the role of a whole set of metal ions (Mn(II), Co(II), Zn(II), Cd(II), Mg(II)) responsible for the phosphodiesterase-like activity of RNA particles in hammerhead ribozymes (Hunsicker & DeRose, 2000).

Numerous EPR studies were devoted to oxidative damage to RNA, e.g. the study on damage to ribo- and deoxyribonucleotides, as well as polynucleotides, caused by myeloperoxidase and H_2O_2-generated hypochlorite during the oxidative burst of monocytes and neutrophils (Hawkins & Davies, 2002).

In general, the impact of EPR on RNA research seems less powerful than in the case of DNA, apparently because of the (unjustified) lower interest of the researchers in RNA studies. Nevertheless, these several examples prove EPR being a convenient and surprisingly versatile method in DNA and RNA analysis, including not only the investigation of the free-radical damage, but also the mechanisms of genetic information expression.

Protein structure and dynamics

The action of free radicals may be observed on the level of proteome — molecular phenotype of the cell. The free-radical damage of proteins is a field of research still waiting for the complete exploration. One of the crucial problems of oxidative stress in the cell is the generation and action of cell-derived chloramines and bromamines. They arise as the products of oxidation of chlorate or bromate by hydrogen peroxide. Subsequent action of their intermediates — hypochlorite or hypobromite on amine residues of proteins and aminoacids can be deleterious. It turned out that various cells produced a range of cell-derived radicals, in a different way and yield, in the presence of chloramine or bromamine under non-lytic and lytic concentrations. Chloramines and bromamines decomposed then spontaneously leaving a free-radical, nitrogen-centered product reacting with other cellular targets: nucleic acids, membranes, other proteins, and initiating a whole chain of

further oxidation reactions. The free-radical products and intermediates were trapped by DMPO or other spin traps and selectively monitored by EPR (Hawkins & Davies, 2002; 2001; 1999; Davies, 2001).

A separate group of papers suggested a potential scenario of oxidative stress in the nucleosome – the basic structure of chromatin. The EPR study focused on the interactions between the most abundant eucaryotic nuclear proteins — histones, DNA, RNA, and products of their degradation by free radicals, revealing transfer of damage from the protein to the associated nucleic acid or free nucleotides (Luxford, Dean & Davies, 2000; Ho, Gilbert & Davies, 1997).

Production of paramagnetic muteins called site-directed spin labeling (SDSL) introduced in 1989 by Hubbell (Todd, Cong, Levinthal, Levinthal & Hubbell, 1989) has developed into a basic method of investigating structure of proteins. SDSL combines site-directed mutagenesis and nitroxide spin-labeling. Due to a point mutation, a particular aminoacid may be replaced with cysteine, which then binds a sulfhydryl-specific spin label nitroxyl residue. The position of the spin label residue varies according to the location of the mutation. The EPR spectra of the whole set of muteins – derivatives of an investigated wild-type protein, depend on the direct environment of the spin label placed in different regions of the molecule. Interaction of several spin label residues may provide information on the distance between the particular points where mutations took place, in the folded (usually native) protein. Paramagnetic metal ions (Fe(II), Co(II), Mn(II)) constituting the prosthetic group of some metaloenzymes may also influence the signal of the nitroxyl labeling the active site. An interesting example of such a phenomenon was analyzed in the study on interactions between cytochrome *c* and spin-labeled cytochrome *c* oxidase – the respiratory chain complex IV, containing both hemes and copper complexes (Pyka, Osyczka, Turyna & Froncisz, 2001b). This, perhaps the most important of the respiratory enzymes, is the target for many inhibitors of respiration (e.g. cyanide), including new potent drugs and poisons. The cited work took advantage of the presence of a free SH group of a 102 cysteine, but functional muteins containing cysteines in other positions were also generated and employed. The structural changes of the cytochrome *c* oxidase after cytochrome *c* binding could be followed, and the amount of the bound ligand estimated (Pyka, J, Osyczka, Turyna, Blicharski & Froncisz, 2001a; Pyka, J. *et al.*, 2001b).

Another example of the EPR investigations of the interactions between ligands and target proteins is the study on the iron-siderophore complex and its binding to site-directed spin labeled ferric enterobactin receptor responsible for iron uptake by enterobacteria. Its conformational change after binding of siderophores suggested a transmembrane signaling mechanism initiating the iron-enterobactin transport through the bacterial membrane (Feix, 2001). The conformational changes of spin-labeled visual arrestin dimerizing in solution were also followed by EPR (Klug, Gurevich & Hubbell, 2001). Arrestin is a protein blocking the interaction between rhodopsin and transducin, shutting off the photoreceptor activity. This work represents the main current of the progression of SDSL, as historically first applications of the method were reported from the research of bacteriorhodopsin and its conformational changes due to photoexcitation (Altenbach, Marti, Khorana & Hubbell, 1990; Altenbach, Flitsch, Khorana & Hubbell, 1989).

The above examples of the EPR application in biochemistry and molecular biology of proteins, basic products of molecular biotechnology, illustrate the feedback between EPR research and biotechnology. EPR research delivers information on the structure, dynamics, and consequently — functioning of proteins, whereas production of site-directed spin-labeled muteins is itself a biotechnological challenge. The interactions between EPR and biotechnology are clearly reciprocal.

Activity of enzymes

The structural research on proteins aims at better understanding their dynamics and activity. In the future it will also include controlling their activity and, perhaps, designing "artificial" proteins better than the natural ones. Bennett (Bennet, 2002; 2001) used EPR to investigate the activity of some metaloaminopeptidases, belonging to the group of enzymes important for tumor growth, angiogenesis, and HIV infectivity. Some proteinase inhibitors (bestatin, fumagillian or ovalicin) effectively inhibited these pathological processes and can be developed into drugs. EPR was employed to fast screening potential inhibitors effectively interacting with the enzymes, by observing the interactions between the pairs of paramagnetic ions in the active sites, usually substituted, as the native enzymes are isolated as diamagnetic di-Zn(II) proteins. Some paramagnetic variants of proteins (being not real muteins, as the substitution is not due to the change in genetic information), e.g. the Co (II)- variants, revealed the structural similarity

to the native zinc-enzymes, whereas Cu (II)- variants did not (Bennet, 2002).

Nitric oxide, one of the main factors of oxidative stress, had served as a spin probe for EPR studies on hemoglobin structure long before its diverse physiological and pathological effects attracted the attention of researchers (Kon, 1968; Sancier, Freeman & Mills, 1962). EPR, in turn, was applied e.g. to show that the origin of the nitrogen atom of NO is the guanidyl group of L-arginine (Lancaster & Hibbs, 1990). Nowadays, EPR has been still one of the main techniques in the analysis of enzymatic activity of nitric oxide synthases (NOS), the main enzymes delivering NO in biological systems. For example, Rosen *et al.* investigated the mechanisms of electron transport, nitric oxide and superoxide generation by NOS, demonstrating the role of perferryl complex in the radical generation (Porasu-phatana, Tsai, Pou & Rosen, 2002; 2001; Margolis, Porasuphatana & Rosen, 2000). Bastian *et al.* (Bastian, Foster, Ballantyne & Lu, 2002; Bastian, Foster & Lu, 2001) suggested that NOS2 might produce both NO, and NO^-, which would be a compromising solution in the on-going discussion on the real product of nitric oxide synthase. Detailed study of the influence of tetrahydrobiopterin (BH_4) on the activity of NOS3 (Vásquez-Vivar, Joseph, Hogg, Whitsett, Martásek & Kalyanaraman, 2001) exhibited that this co-factor of NO production at the same time effectively inhibited generation of superoxide, revealing this way the positive influence of the reduced BH_4 on the condition of endothelium.

Detailed study was devoted also to cytochrome *c* nitrite reductase, a multiheme enzyme engaged in the process of dissimilative denitrification, and effectively reducing nitrite or nitric oxide to ammonia (Kroneck, 2001). Cytochrome *c* nitrite reductase, together with other multiheme enzymes possessing a set of peculiarities, may serve as a prototype of nanomachines, the era of which is approaching modern biotechnology.

Membranes

The existence of phospholipid bilayers in biological systems, varying in their lipid composition according to particular organelles or cell types, is confusing from the point of view of evolutionary biology, as there is no genetic information on the structure of membranes given explicitely in any genome, as it is in the case of the primary structure of almost all cellular peptides, proteins, and RNA molecules. What is even more puzzling, no place of *de novo* membrane assemblage have been found in any biological system – the new phospholipids are apparently incorporated into the structure of already existing membranes (Vander, Sherman & Luciano, 1990). However, membranes belong to the fundamental constituents of all cell types without exception. Being the products of enzymatic activity, and containing the extremely important proteins of various kinds, they must be included in the molecular phenotype of the cell.

Structure, dynamics and functions of biological membranes created a traditional field of biological EPR, which has been explored for at least 30 years (Subczynski & Wisniewska, 2000; Marsh & Horvath, 1998), its achievements finding various applications in drug design and action (Wisniewska & Subczynski, 1996), cosmetics and dermatology (Fuchs & Packer, 1990), as well as in the study on photosynthetic membrane properties (Wasniowska, Subczynski & Tikhonov, 2002; Khomutov, Gilmiyarova & Tikhonov, 1996). The recent EPR evidence of the existence of structural domains stabilized by membrane proteins in the form of the "rafts" has changed the common view of the architecture of membranes (Šentjurc M., Štrancar & Koklič, 2002; Šentjurc, 2001; Subczynski, 2001, 2002). The model of fluid mosaic, taking into account more or less homogenous distribution of the bilayer components, appears too simple to satisfactorily represent the details of membrane structure, and the respective functions.

Glycobiology

Spin-labeled sugars, sugar residues, and spin-labeled components interacting with sugars are applied in two basic fields of carbohydrate research: sugar metabolism (degradation, transport), as well as structural biochemistry of glycoproteins and membranes. As in the latter case, the structure of oligo- and polysaccharides is not explicitly encoded in the genome. Nevertheless sugars, being an important part of glycoproteins and glycolipids and resulting from precise interactions of various enzymatic reactions, clearly belong to the molecular phenotype of the cell, as well.

EPR spectroscopy was successfully employed to analyze the process of sugar transport in bacteria: nitroxide spin-labeled galactopyranosides interacting with *E. coli* lactose permease revealed higher affinity to the protein than glucopyranosides (Zhao, Kalai, Hideg, Altenbach, Hubbell & Kaback, 2000). This technique was also used to the direct analysis of sugar metabolism – e.g. to monitor the action of alpha-amylase, proving that succinylated amylopectin is the substrate for the enzyme (Marcazzan, Vianello, Scarpa & Rigo, 1999). EPR was applied to the biotechnologically important problem of cellulose degradation, providing detailed analysis of the geometry of binding

spin-labeled cellooligosaccharides by N-terminal cellulose-binding domains of *Cellulomonas fimi* cellulase (Johnson, Brun, MacKenzie, Withers & McIntosh, 1999). The metabolism of sugars was also investigated on the level of a whole organism, revealing, by means of spatiotemporal EPR imaging, slower liver metabolism of spin-labeled dextran than spin-labeled hydroxyethylstarch, suggesting the resistance of the former polymer to hydrolysis (Saito, Yoshioka, Ito, Kazama, Tanizawa, Lin, Watanabe, Ogata & Kamada, 1997). This study may be important from the biomedical point of view in designing artificial blood substitutes and serves as an example of EPR study in medical biotechnology. Another study possessing a clear pharmacological aspect concerns TIMERx, a novel polysaccharide-based controlled release system. The study took advantage of the interactions between TIMERx and low molecular weight spin-labeled models for drugs. The paper showed applicability of the EPR spectroscopy to analyze interactions between pharmacologically active substances and excipients (Tobyn, Maher, Challinor & Staniforth, 1996).

Numerous studies investigate the influence of sugar residues on the conformation, structure and dynamics of proteins and membranes. The study on trans interactions between two major constituents of multilayered myelin sheath: galactosyl ceramide and its sulfated form, cerebroside sulfate, showed a strong interaction between their microstructures. Both components interact via a trans interaction across apposed bilayers, which most probably leads to dehydration of their headgroup and interface region, and in consequence – to the stabilization of the myelin sheath (Boggs, Menikh & Rangaraj, 2000). By means of EPR, Hajela *et al.* (Hajela, Kayestha & Sumati, 1996) demonstrated a dramatic decrease in membrane fluidity due to binding of various mono-, di- and trisaccharides to membrane lectins, and synergy of the action of carbohydrate ligands. The structural investigations often reveal medical aspects – EPR was applied to the analysis of the influence of diabetes on the properties of erythrocytes showing the decrease in erythrocyte deformability due to the non-enzymatic glycation of hemoglobin. Such a phenomenon may contribute to the handicapped release of oxygen in tissues under hyperglycemic conditions (Watala, Golanski, Witas, Gurbiel, Gwozdzinski & Trojanowski, 1996). Finally, the phenomenon of sucrose-induced protein stabilization, important from biochemical, biotechnological and pharmacological point of view, was analysed for recombinant interleukin 1 receptor agonist spin-labeled on cysteine 116. The presence of sucrose resulted in burying the residue in the protein interior and lowering its hydrodynamic diameter, suggesting a shift of the protein conformation towards higher compactness (Kendrick, Chang, Arakawa, Peterson, Randolph, Manning & Carpenter, 1997).

From this chapter one can conclude that all main aspects of molecular biotechnology may become an opportunity to use EPR as an independent method of research, starting from the expression of genetic information, through structural and functional analysis of the proteome, to all the constituents of the molecular cell phenotype.

EPR IN MEDICAL BIOTECHNOLOGY

Action and transport of drugs

Several pharmacological investigations taking advantage of the EPR spectroscopy focused on interactions between DNA-binding drugs and DNA. For example, Ireland *et al.* demonstrated two modes of binding daunomycin to spin-labeled nucleic acid duplexes (Ireland, Pauly, Bobst & Bobst, 1986). Another study was devoted to bleomycin, used in anti-tumor treatment. Bleomycin may degrade DNA, creating an activated iron-oxygen complex of a characteristic EPR spectrum (Sam & Peisach, 1993). The study conclusively proved the lack of oxygen exchange with the solvent, which earlier had been disputable. An original study on the action of potential drugs was carried out on temporins – antimicrobial peptides from the skin of the European red frog (*Rana temporaria*). Their bactericidal activity turned out to depend on the ability to destabilize membrane bilayer organization on a local scale, leading to the small, EPR-detectable increase in hydrocarbon chain mobility of model nitroxide-labeled liposomes (Rinaldi, Di Giulio, Liberi, Gualtieri, Oratore, Bozzi, Schinina & Simmaco, 2001).

EPR may be also used to characterize some herb-derived products of potential importance for biotechnology, which act by increasing the level of free radicals and other reactive species produced during light-induced oxidative stress of the cell. Berberine, along with berberastine and hydrastine are products of the root of Goldenseal (*Hydrastis canadensis*), a representative of *Ranunculaceae.* The lotions and washes produced from the root of Goldenseal had been long used to treat various skin and eye diseases. The investigated substances turned out to produce EPR-detectable spin adducts with DMPO, as well as singlet oxygen, which, in the presence of UVA, decreased viability of cells *in vitro* in the concentration-dependent manner,

and caused DNA damage detectable by the comet assay (Inbaraj, Kukielczak, Bilski, Sandvik & Chignell, 2001).

The study on photosensitizing properties of the Goldenseal leads to a more general aspect of EPR application in dermatology and cosmetics. The EPR spectroscopy is used to study either photosensitization or photoprotection of skin. Damiani *et al.* attempted to employ indolinic nitroxide, TEMPO, and TEMPOL radicals in the presence of dibenzoylmethane and a related product Parsol 1789 as a sunscreen to prevent light-induced DNA strand breakes. They suggested that perhaps the active substance of the sunscreen is responsible for the occurrence of carbon-centered radicals in naked, illuminated DNA (Damiani, Greci, Parsons & Knowland, 1999).

Many papers focused on the means and mechanisms of skin drug penetration and delivery: *in vivo* EPR experiments revealed that multilamellar liposomes enhanced the topical delivery of hydrophilic compounds (Honzak, Šentjurc & Swartz, 2000), and clindamycin, a drug used to treat *acne vulgaris*, turned out to be more effective when applied in liposomes than in solution (Honzak & Šentjurc, 2000). The penetration behavior of liposomes in skin was shown to depend on the phospholipid composition and cholesterol amount (Coderch, Fonollosa, De Pera, Estelrich, De La Maza & Parra, 2000). EPR was also successfully applied to the *in vitro* screening of the putative antivesicant topical skin protectants (Arroyo & Janny, 1995).

Imaging

A separate biomedical and physico-medical problem is detection of free radicals in living tissues and organisms. As it was shown during the Workshop, EPR imaging is an excellent non-invasive means delivering physiologically relevant information *in vivo*. Oxygen level estimation in tissues, redox mapping, as well as spin trapping of free radicals can be performed using various EPR techniques. What is particularly interesting, advantages of Nuclear Magnetic Resonance (NMR) and NMR Imaging (MRI) are now being often used for information complementary to that gained by EPR spectroscopy or imaging.

Tumors growing in the leg of living mice revealed a good correlation between BOLD (Blood Oxygen Level Dependent) MRI and the EPRI data on the level of oxygenation (Elas, Williams, Parasca, Mailer, Pelizzari, Lewis, Rivers, Karczmar, Barth & Halpern, 2002). Great heterogeneity in the oxygen distribution was found in tumor tissue. Oxygen partial pressure of poorly vascularized regions was found to be approximately 0-10 torr. This quantitation of oxygen concentration was performed with 3-4 torr resolution and 0.6 mm^3 of spatial resolution, which is more than adequate to provide information useful for medical purposes (Elas *et al.*, 2002; Elas, Mailer, Williams, Parasca, Barth, Rinard, Quine, Eaton, Eaton & Halpern, 2001; Halpern, Chandramouli, Barth, Williams & Galtsev, 1999; Halpern, Yu, Peric, Barth, Bowman, Grdina & Teicher, 1994).

Another approach to estimate oxygen level in living tissues utilizes OMRI — Overhauser-enhanced MRI (Krishna, English, Yamada, Yoo, Murugesan, Devasahayam, Cook, Golman, Ardenkjaer-Larsen, Subramanian & Mitchell, 2002; Golman, Petersson, Ardenkjaer-Larsen, Leunbach, Wikstrom, Ehnholm & Liu, 2000). OMRI is a double resonance technique that couples the sensitivity of EPR with good anatomical resolution of MRI. Again, spatial heterogeneity of oxygen distribution was revealed in the tumor tissue, with regions of hypoxia. The pO_2 measurements from OMRI were in agreement with those obtained by independent polarographic measurements using a pO_2 Eppendorf electrode. OMRI allowed for spatial resolution of approximately 1 mm^3, and temporal resolution of 2 minutes only.

Both OMRI and pulsed EPR spectroscopy are also being explored for *in vivo* imaging of free radicals (Lurie, Li, Petryakov & Zweier, 2002; Youngdee, Lurie & Foster, 2002; Yamada, Murugesan, Devasahayam, Cook, Mitchell, Subramanian & Krishna, 2002).

NMR spectroscopy can be used for spin-trapping, which provides complementary information to spin-trapping by EPR. The electron resonance allows to perform identification of free radical spin adduct and to follow its pharmacokinetics, whereas the nuclear resonance, e.g. of ^{19}F substituted spin traps, helps to identify other metabolites of spin trap and/or adduct (Berliner, Khramtsov, Clanton & Fujii, 2002).

EPR imaging is a valuable tool for spatially resolved redox mapping of living tissues. Redox status of tumor tissues is significant for understanding tumor physiology, and for determining the effects of chemotherapy and radiation. Non-invasive measurements of tumor redox status were performed with spatial resolution of $0.2 \times 0.2 \times 5$ mm and redox map were taken every 1.5 minutes. Some heterogeneity of redox status was detected in a RIF tumor, and GSH depleting agents brought about a dramatic change in the tumor redox status (Kuppusamy & Krishna, 2002; Kuppusamy & Krishna, 2001; Kuppusamy, Wang, Shankar, Ma, Trimble, Hsia & Zweier, 1998).

All the results create a picture of EPR imaging as a powerful modality to understand tumor physiology, focused also on the physiological activity of other normal and pathological tissues. Their further development from the point of view of medical biotechnology should most probably concentrate on-, and help to solve the crucial question of what really happens in a tissue during the process of treatment, re-modelling, regeneration, as well as, perhaps, what really happens with a piece of biomaterial introduced to the living tissue.

EPR IN CLASSICAL BIOTECHNOLOGY

Plant biotechnology

The Nobel Prize for the discovery of radiation-induced mutagenesis was conferred on H. J. Muller already in 1946. Since then, irradiation has been often used to produce new mutants of plants possessing unique biological features valuable in agriculture, as well as in scientific research. The effects of irradiation may be observed on the level of whole organs of plants, namely seeds**.** The level of EPR-measurable free radical damages caused by irradiation usually decreases rapidly, and irradiated seeds do not differ in their paramagnetic properties from the non-irradiated ones. A long-lasting effect of irradiation is the loss of ability to absorb water by the plant embryo due to damage of membranes. The loss of water in irradiated embryos is quicker than in control, which may be easily followed by EPR upon use of an exogenous spin label, 4-hydroxy TEMPO (TANON). The high-field line of its hyperfine structure is sharply resolved into water and lipid (polar and non-polar) parts. The signal of extracellular label is broadened by potassium ferrocyanide, and this way the proportion of intracellular water- and lipid fraction of TANONE can be estimated (Sünnetçíoğlu, Dadaylipaktas & Ercan, 2002; Sünnetçíoğlu, Dadaylipaktas & Ercan, 2001). The decrease of the water fraction after incubation of embryos with the water solution of TANONE was quicker after seed irradiation and the effect was dose-dependent, lasting for months (Sünnetçíoğlu, Dadayli, Celik & Koksel, 1998). This way, the main physiological condition of seed germination and its dependence upon irradiation can be monitored by EPR.

Resistance to some pathogens or improved photosynthetic yield and productivity are the two main features being improved in cultivated plants either by the stochastic irradiation of whole seeds, or by intentional action of genetic engineers. Photosynthesis may be concisely described as a process of photoelectrolytic water oxidation coupled with the reduction of carbon. Such an intricate redox system generates diverse EPR-detectable products, either photosystem I-related free radicals (Webber & Lubitz, 2001), or various forms of manganese related to oxygen-releasing photosystem II with a water-oxidizing complex (Peloquin & Britt, 2001). The production of ATP in chloroplasts, photosynthetic phosphorylation, itself is a non-redox process. Nevertheless, coupled with the transport of protons across thylakoid membrane and generation of a transmembrane gradient of pH, it can be followed by EPR. A method of pH-sensitive spin-labeling using TEMPOAMINE revealed that a relatively small pH gradient (1.4-2.6) was enough to elicit ATP synthesis in thylakoids (Tikhonov, 2001). The EPR spectroscopy turned out to be helpful even at developing artificial photosynthesis (leading, according to some evolutionists, to the transformation of *Homo sapiens* into an autotrophic species), being unquestionably the biggest biotechnological challenge for the Mankind (Hammarstrom, Sun, Akermark & Styring, 2001; Levanon & Mobius, 1997).

Food production and storage

The most biotechnological of all applications would be no doubt brewery, as the oldest biotechnological product is beer. A commercial EPR system is now available to analyze shelf life of beer (Barr, Heiss, Kamlowski, Maier, Erstling & Meling, 2001). It is based on studies showing that free radicals generated in beer due to the action of light, or spontaneously during the process of storage, contribute to the degradation and flavor changes of the product (Burns, Heyerick, De Keukeleire & Forbes, 2001). The level of free radicals would strongly depend on antioxidants present in the solution. Therefore, antioxidant capacities of beer help to predict its stability (Stasko, Rapta & Malik, 2000; Laane, de Roo, van den Ban, Sjauw-En-Wa, Duyvis, Hagen, van Berkel, Hilhorst, Schmedding & Evans, 1999).

Similar approach was applied to other food products, such as oils or milk. Storage, handling and stress conditions of the oils were shown to influence significantly the radical concentration. Mimicking these conditions in fresh oil samples and estimating its antioxidant capacities may help to predict its stability (Ottaviani, Spallaci, Cangiotti, Bacchiocca & Ninfali, 2001). EPR measurements revealed also photosensitizing action of the important milk ingredient – vitamin B_2, which may affect quality of the product (Berliner, Bradley, Meinholtz, Min & Ogata, 1994).

In the same way, studies on wine focused on determining the redox properties of wine constitu-

ents and mechanisms underlying its antioxidant capacities, as wine polyphenoles were shown to reduce the risk of atherosclerosis and coronary heart diseases (Andriambeloson, Kleschyov, Muller, Beretz, Stoclet & Andriantsitochaina, 1997). EPR was also employed to study free radical generation and antioxidant activity of whole wine samples. Certain antioxidants present in wine, such as polyphenols, gallate esthers and flavon(ol)s were studied to estimate their role in wine stability and its antioxidant properties (Bors, Michel & Stettmaier, 2000; Stasko, Brezova, Liptakova & Savel, 2000; Urizzi, Monje, Souchard, Abella, Chalas, Lindenbaum, Vergnes, Labidalle & Nepveu, 1999). Finally, EPR was used to demonstrate the protective role of resveratrol, a natural product present in grapes and wine (Karlsson, Emgard, Brundin & Burkitt, 2000). Recently, a review (Troup & Hunter, 2002) summed up the EPR application in wine production, predicting its further development.

Another field of EPR application in food science is hydratation, water diffusion, and small molecule mobility in food systems, or sugar-water systems used to model much more complicated food systems. Rotational mobility of nitroxide spin-labeled molecules studied by saturation transfer EPR brings information on the presence of molecular cavities, solvent interactions and arrangement of the hydrogen-bonded network in the matrix. Spin-labeling of cysteine residues in proteins was applied to determine bread-making quality of wheat flour. It depends on the ability to form a protein network, called gluten, from water-insoluble glutenins and gladins. Gluten generation is based on hydratation processes and information on resulting protein network structure can be provided by EPR (Capocchi, Cinollo, Galleschi, Saviozzi, Calucci, Pinzino & Zandomeneghi, 2000; Pinzino, Capocchi, Galleschi, Saviozzi, Nanni & Zandomeneghi, 1999).

The EPR spectroscopy turns out to be useful and versatile in the so-called "classical" biotechnology and in the future one can expect also industrial applications of the method. In this context symptomatic appears the fact that the extensively examined non-heme iron complexes of nitric oxide were discovered in the oldest biotechnological organism – yeast (Vanin & Nalbandyan, 1965). This last example shows the depth of mutual influence of EPR and biotechnology.

CONCLUSIONS

Modern biotechnology is very deeply rooted in molecular biology, genetics and biochemistry, being in fact their applied branch. No wonder that the majority of the examples presented here might be qualified as basic research and illustrate as well biological or biochemical applications of EPR. However, going from general aspects of biotechnology towards the basic biological or chemical foundations of the problem, a researcher can come across the opportunity to use EPR as an independent method. Not only to follow the effects of oxidative stress that may affect any biotechnological products – food, recombinant proteins, modified cells of animals or plants, as well as whole organisms: even examples such "exotic" for a traditional EPR research as mechanisms of the expression of genetic information, structure and dynamics of nucleic acids, and their interactions with transcription factors can be found in the literature. Not to mention the structural protein research based on SDSL, so important in the modern engineering of molecules, finding its practical expression in drug design, or in applied enzymology.

Due to selectivity and non-invasiveness of the technique, the same phenomenon can be observed at the level of molecules, cells, tissues and whole organs, using one and the same method. Here lies the attractiveness of the method, as an EPR experiment can be performed both on "naked" irradiated DNA and on a whole irradiated organism, both on an isolated chemically purified substance and on a sample of a real biotechnological product like beer or red wine. It is a matter of the researcher's imagination and creativity how to use the EPR spectroscopy.

Clearly, the intention of the authors was not to give any exhaustive review of all the works where the EPR spectroscopy is used in biotechnology, but rather to show the perspective of the immense diversity of this usage. EPR turns out applicable in all branches of both classical and molecular biotechnology. The particular studies are sometimes impressively innovative and original, which should encourage other researchers to include EPR in the repertoire of their favorite methods.

Acknowledgements

This paper was written as a deliverable of the project "Molecular Biotechnology – Integration of Education and Research – BIER" (Support for

Centres of Excellence, Contract No ICA1-CT-2000-70012) awarded to IBMB UJ from the resources of the 5 FP EU in 2000. The authors wish to thank Professors Aleksander Koj and Tadeusz Sarna for their generous support and encouragement and particularly Professor Gerald Rosen for his comments and helpful suggestions.

REFERENCES

Abragam A. & Bleaney B. (1970). *Electron Paramagnetic Resonance of Transition Ions*. Clarendon Press Oxford.

Altenbach C., Flitsch S. L., Khorana H. G. & Hubbell W. L. (1989). Structural studies on transmembrane proteins. 2. Spin labeling of bacteriorhodopsin mutants at unique cysteines. *Biochemistry,* **28**, 7806-7812.

Altenbach C., Marti T., Khorana H. G. & Hubbell W. L. (1990). Transmembrane protein structure: spin labeling of bacteriorhodopsin mutants. *Science,* **248**, 1088-1092.

Ambroz H. B., Kemp T. J., Kornacka E. M. & Przybytniak G. K. (2001). Role of iron ions in damage to DNA. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 61.

Andriambeloson E., Kleschyov A. L., Muller B., Beretz A., Stoclet J. C. & Andriantsitochaina R. (1997). Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Brit. J. Pharmacol.*, **120**, 1053-1058.

Arroyo C. M. & Janny S. J. (1995). EPR/spin-label technique as an analytical tool for determining the resistance of reactive topical skin protectants (rTSPs) to the breakthrough of vesicant agents. *J. Pharmacol. Toxicol. Methods,* **33**, 109-112.

Barr D., Heiss A., Kamlowski A., Maier D., Erstling J. & Meling H. (2001). Shelf life analysis of beer using an automated lag-time EPR system. *Spectroscopy,* **16**, 16-19.

Bastian N. R., Foster M. J. P., Ballantyne M. J. & Lu Y. (2002). Nitrogen Oxides, the Good, the Bad, and the Ugly. *Curr. Top. Biophys.,* **26**, 115-127.

Bastian N. R., Foster M. J. P. & Lu Y. (2001). Nitrogen oxides, the good, the bad, and the ugly. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 45.

Bennet B. (2001). Dinuclear metalloaminopeptidases: substituted metal ions as structural and mechanistic probes, *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 41.

Bennet B. (2002). EPR of Co (II) as a Structural and Mechanistic Probe of Metalloprotein Active Sites: Characterisation of Aminopeptidase. *Curr. Top. Biophys.,* **26**, 49-57.

Berliner L., Khramtsov V., Clanton T. & Fujii H. (2002). NMR and MRI Spin Trapping: Using NMR to learn about Free Radical Reactions. *Curr. Top. Biophys.,* **26**, 21-27.

Berliner L. J., Bradley D. G., Meinholtz D. C., Min D. B. & Ogata T. (1994). The detection of riboflavin photosensitized singlet oxygen formation in milk by electron spin resonance. [In:] *Supramolecular Structure and Function*. G. Pifat (eds.). Balaban Publishers, Rehovot, Israel, 67-77.

Boggs J. M., Menikh A. & Rangaraj G. (2000). Trans interactions between galactosylceramide and cerebroside sulfate across apposed bilayers. *Biophys. J.,* **78**, 874-885.

Bors W., Michel C. & Stettmaier K. (2000). Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Arch. Biochem. Biophys.,* **374**, 347-355.

Burns C. S., Heyerick A., De Keukeleire D. & Forbes M. D. E. (2001). Mechanism for formation of the lightstruck flavor in beer revealed by time-resolved electron paramagnetic resonance. *Chem. - Eur. J.,* **7**, 4553-4561.

Capocchi A., Cinollo M., Galleschi L., Saviozzi F., Calucci L., Pinzino C. & Zandomeneghi M. (2000). Degradation of gluten by proteases from dry and germinating wheat (*Triticum durum*) seeds: An in vitro approach to storage protein mobilization. *J. Agric. Food Chem.,* **48**, 6271-6279.

Coderch L., Fonollosa J., De Pera M., Estelrich J., De La Maza A. & Parra J. L. (2000). Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. *J. Control Release,* **68**, 85-95.

Damiani E., Greci L., Parsons R. & Knowland J. (1999). Nitroxide radicals protect DNA from damage when illuminated in vitro in the presence of dibenzoylmethane and a common sunscreen ingredient. *Free Radic. Biol. Med.,* **26**, 809-816.

Davies M. (2001). EPR spin trapping of novel nitrogen-centred radicals in biological systems. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 51.

Debije M., Strickler M. & Bernhard W. (2000). On the efficiency of hole- and electron transfer from the hydration layer to DNA: an EPR study of cristalline DNA X-irradiated at 4K. *Radiat. Res.,* **154**, 163-170.

Edwards T. E., Okonogi T. M., Robinson B. H. & Sigurdsson S. T. (2001). Site-specific incorporation of nitroxide spin-labels into internal sites of the TAR RNA; structure-dependent dynamics of RNA by EPR spectroscopy. *J. Am. Chem. Soc.,* **123**, 1527-1528.

Edwards T. E., Okonogi T. M. & Sigurdsson S. T. (2002). Investigation of RNA-protein and RNA-metal ion interactions by eletron paramagnetic resonance spectroscopy. The HIV TAR-Tat motif. *Chem. Biol.,* **9**, 699-706.

Elas M., Mailer C., Williams B. B., Parasca A., Barth E. D., Rinard G. A., Quine R. W., Eaton G. R., Eaton S. S. & Halpern H. J. (2001). New developments in in vivo EPR imaging at 250 MHz. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 33.

Elas M., Williams B. B., Parasca A., Mailer C., Pelizzari C. A., Lewis M. L., Rivers J., Karczmar G. S., Barth E. B. & Halpern H. J. (2002). Comparison of Quanti-

tative Tumor Oxymetric Images from 4-Dimensional Electron Paramagnetic Resonance Imaging (EPRI) with Blood Oxygen Level Dependent (BOLD) MRI, submitted. *J. Magn. Reson*.

Feix J. B. (2001). Site-directed spin labeling of the bacterial iron transport protein, FepA. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 13.

Fuchs J. & Packer L. (1990). Electron paramagnetic resonance in dermatologic research with particular reference to photodermatology. *Photodermatol. Photoimmunol. Photomed.*, **7**, 229-232.

Golman K., Petersson J., Ardenkjaer-Larsen J., Leunbach I., Wikstrom L., Ehnholm G. & Liu K. (2000). Dynamic in vivo oxymetry using Overhauser enhanced MR Imaging. *J. Magn. Reson. Imaging,* **12**, 929-938.

Gopalan V., Kuhne H., Biswas R., Li H. M., Brudvig G. W. & Altman S. (1999). Mapping RNA-protein interactions in ribonuclease P from *Escherichia coli* using electron paramagnetic resonance spectroscopy. *Biochemistry,* **38**, 1705-1714.

Hajela K., Kayestha R. & Sumati. (1996). Carbohydrate induced modulation of cell membrane. 2. Spin label study of fluidity changes in peripheral blood lymphocyte membrane. *FEBS Lett.,* **380**, 165-168.

Halpern H., Chandramouli G., Barth E., Williams B. & Galtsev V. (1999). Approaches to problems in high resolution *in vivo* spectral spatial imaging with radiofrequency EPRI. *Curr. Top. Biophys.,* **23**, 5-10.

Halpern H. J., Yu C., Peric M., Barth E., Bowman M. K., Grdina D. J. & Teicher B. A. (1994). Oxymetry deep in tissues with low frequency electron paramagnetic resonance. *Proc. Natl. Acad. Sci. U. S. A.,* **1991**, 13047-13051.

Hammarstrom L., Sun L., Akermark B. & Styring S. (2001). A biomimetic approach to artificial photosynthesis: Ru(II)-polypiridine photo-sensitisers linked to tyrosine and manganese electron donors. *Spectrochim. Acta A Mol. Biomol.Spectrosc.*, **57**, 2145-2160.

Hawkins C. & Davies M. (1999). Hypochlorite-induced oxidation of proteins in plasma: formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation. *Biochem. J.,* **340 (Pt 2)**, 539-548.

Hawkins C. & Davies M. (2001). Hypochlorite-induced damage to nucleosides: formation of chloramines and nitrogen-centered radicals. *Chem. Res. Toxicol.,* **14**, 1071-1081.

Hawkins C. & Davies M. (2002). Hypochlorite-induced damage to DNA, RNA, and polynucleotides: Formation of chloramines and nitrogen-centered radicals. C*hem. Res. Toxicol.,* **15**, 83-92.

Ho W. F., Gilbert B. C. & Davies M. J. (1997). EPR spin-trapping studies of radicals generated from the Fe-II-catalysed degradation of nucleobase, nucleoside, RNA and DNA hydroperoxides. *J. Chem. Soc.-Perkin Trans.*, **2***,* 2525-2531.

Honzak L. & Šentjurc M. (2000). Development of liposome encapsulated clindamycin for treatment of *acne vulgaris*. *Pflugers Arch.,* **440**, R44-R45.

Honzak L., Šentjurc M. & Swartz H. M. (2000). In vivo EPR of topical delivery of a hydrophilic substance encapsulated in multilamellar liposomes applied to the skin of hairless and normal mice. *J. Control Release,* **66**, 221-228.

Hoogstraten C. G. & Britt R. D. (2002). Water counting: Quantitating the hydration level of paramagnetic metal ions bound to nucleotides and nucleic acids. *RNA- Publ. RNA Soc.,* **8**, 252-260.

Hunsicker L. M. & DeRose V. J. (2000). Activities and relative affinities of divalent metals in unmodified and phosphorothioate-substituted hammerhead ribozymes. *J. Inorg. Biochem.,* **80**, 271-281.

Inbaraj J. J., Kukielczak B. M., Bilski P., Sandvik S. L. & Chignell C. F. (2001). Photochemistry and photocytotoxicity of alkaloids from Goldenseal (*Hydrastis canadensis* L.) 1. Berberine. *Chem. Res. Toxicol.,* **14**, 1529-1534.

Ireland J. C., Pauly G. T., Bobst E. V. & Bobst A. M. (1986). Redox-active daunomycin-spin-labeled nucleic acid complexes. *Biochemistry,* **25**, 6890-6895.

Johnson P. E., Brun E., MacKenzie L. F., Withers S. G. & McIntosh L. P. (1999). The cellulose-binding domains from *Cellulomonas fimi* beta-1,4-glucanase CenC bind nitroxide spin-labeled cellooligosaccharides in multiple orientations. *J. Molec. Biol.,* **287**, 609-625.

Kao S. C., Bobst E. V., Pauly G. T. & Bobst A. M. (1985). Molecular dynamics in protein-single stranded DNA complexes. Two distinct nucleoside mobilities in poly(deoxythymidylic acid)-gene 5 protein complexes. *J. Biomol. Struct. Dyn.,* **3**, 261-268.

Karlsson J., Emgard M., Brundin P. & Burkitt M. J. (2000). Trans-resveratrol protects embryonic mesencephalic cells from tert-butyl hydroperoxide: Electron paramagnetic resonance spin trapping evidence for a radical scavenging mechanism. *J. Neurochem.,* **75**, 141-150.

Kendrick B. S., Chang B. S., Arakawa T., Peterson B., Randolph T. W., Manning M. C. & Carpenter J. F. (1997). Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: Role in restricted conformational mobility and compaction of native state. *Proc Natl. Acad. Sci. U. S. A.,* **94**, 11917-11922.

Kensch O., Restle T., Wohrl B. M., Goody R. S. & Steinhoff H. J. (2000). Temperature-dependent equilibrium between the open and closed conformation of the p66 subunit of HIV-1 reverse transcriptase revealed by site-directed spin labelling. *J. Molec. Biol.,* **301**, 1029-1039.

Keyes R. S., Cao Y. Y., Bobst E. V., Rosenberg J. M. & Bobst A. M. (1996). Spin-labeled nucleotide mobility in the boundary of the EcoRI endonuclease binding site. *J. Biomol. Struct. Dyn.,* **14**, 163-172.

Keyes R. S., Bobst E. V., Cao Y. Y. & Bobst A. M. (1997). Overall and internal dynamics of DNA as monitored by five-atom-tethered spin labels. *Biophys. J.,* **72**, 282-290.

Khomutov G. B., Gilmiyarova S. G. & Tikhonov A. N. (1996). EPR Study on Surface Potential of Thylakoid Membranes. *Curr. Top. Biophys.*, **20**, 31-35.

Kim S., Sancar A., Essenmacher C. & Babcock G. (1993). Time-resolved EPR studies with DNA photolyase: excited state- FADH0 abstracts and electron from Trp-306 to generate FADH-, the catalically active form of the cofactor. *Proc. Natl Acad. Sci. U. S. A.,* **90**, 8023-8027.

Klug C. S., Gurevich V. V. & Hubbell W. L. (2001). Structural investigation of visual arrestin using SDSL EPR spectroscopy. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 15.

Kon H. (1968). Paramagnetic resonance study of nitric oxide hemoglobin. *J. Biol. Chem.,* **243**, 4350-4357.

Kornacka E. M. & Ambroz H. B. (2001). Influence of cysteamine on the protection and repair of radiation damages to DNA. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 63.

Krishna M., English S., Yamada K., Yoo J., Murugesan R., Devasahayam N., Cook J., Golman K., Ardenkjaer-Larsen J., Subramanian S. & Mitchell J. (2002). Overhauser enhanced magnetic resonance imaging for tumor oximetry: coregistration of tumor anatomy and tissue oxygen concentration. *Proc Natl. Acad. Sci. U. S. A.,* **99**, 2216-2221.

Kroneck P. M. H. (2001). Multiheme proteins and enzymes: from spectroscopy to three-dimensional structure. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 39.

Kuppusamy P., Wang P. H., Shankar R. A., Ma L., Trimble C. E., Hsia C. J. C. & Zweier J. L. (1998). In vivo topical EPR spectroscopy and imaging of nitroxide free radicals and polynitroxyl-albumin. *Magn. Reson. Med.,* **40**, 806-811.

Kuppusamy P. & Krishna M. C. (2001). Functional EPR imaging of tumor. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 34.

Kuppusamy P. & Krishna M. (2002). EPR imaging of tissue redox status. *Curr. Top. Biophys.,* **26**, 29-34.

Laane C., de Roo G., van den Ban E., Sjauw-En-Wa M. W., Duyvis M. G., Hagen W. A., van Berkel W. J. H., Hilhorst R., Schmedding D. J. M. & Evans D. J. (1999). The role of riboflavin in beer flavour instability: EPR studies and the application of flavin binding proteins. *J. Inst. Brewing,* **105**, 392-397.

Lancaster J. R. Jr., Hibbs J. B. Jr. (1990). EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc Natl. Acad. Sci. U. S. A.,* **87**, 1223-1227.

Levanon H. & Mobius K. (1997). Advanced EPR spectroscopy on electron transfer processes in photosynthesis and biomimetic model systems. *Annu. Rev. Biophys. Biomol. Struct.*, **26**, 495-540.

Lurie D., Li H., Petryakov S. & Zweier J. (2002). Development of a PEDRI free-radical imager using a 0.38 T clinical MRI system. *Magn. Reson. Med.,* **47**, 181-186.

Luxford C., Dean R. T. & Davies M. J. (2000). Radicals derived from histone hydroperoxides damage nucleobases in RNA and DNA. *Chem. Res. Toxicol.,* **13**, 665-672.

Macosko J. C., Pio M. S., Tinoco I. & Shin Y. K. (1999). A novel 5 ' displacement spin-labeling technique for electron paramagnetic resonance spectroscopy of RNA. *RNA- Publ. RNA Soc.,* **5**, 1158-1166.

Marcazzan M., Vianello F., Scarpa M. & Rigo A. (1999). An ESR assay for alpha-amylase activity toward succinylated starch, amylose and amylopectin. *J. Biochem. Biophys. Methods,* **38**, 191-202.

Margolis A., Porasuphatana S. & Rosen G. (2000). Role of paraquat in the uncoupling of nitric oxide synthase. *Biochim. Biophys. Acta,* **1524**, 253-257.

Marsh D. & Horvath L. I. (1998). Structure, dynamics and composition of the lipid-protein interface. Perspectives from spin-labeling. *Biochem. Biophys. Acta*, **1376**, 267-296.

Naskalski J. & Bartosz G. (2000). Oxidative modifications of protein structures. *Adv. Clin. Chem.,* **35**, 161-253.

Okonogi T. M., Alley S. C., Reese A. W., Hopkins P. B. & Robinson B. H. (2000). Sequence-dependent dynamics in duplex DNA. *Biophys. J.,* **78**, 2560-2571.

Ottaviani M., Spallaci M., Cangiotti M., Bacchiocca M. & Ninfali P. (2001). Electron paramagnetic resonance investigations of free radicals in extra virgin olive oils. *J. Agric. Food Chem.,* **49**, 3691-3696.

Peloquin J. M. & Britt R. D. (2001). EPR/ENDOR characterization of physical and electronic structure of the OEC Mn cluster. *Biochim. Biophys. Acta*, **1503**, 96-111.

Pinzino C., Capocchi A., Galleschi L., Saviozzi F., Nanni B. & Zandomeneghi M. (1999). Aging, free radicals, and antioxidants in wheat seeds. *J. Agric. Food Chem.,* **47**, 1333-1339.

Porasuphatana S., Tsai P., Pou S. & Rosen G. (2001). Involvement of the perferryl complex of nitric oxide synthase in the catalysis of secondary free radical formation. *Biochim. Biophys. Acta,* **1526**, 95-104.

Porasuphatana S., Tsai P., Pou S. & Rosen G. (2002). Perferryl complex of nitric oxide synthase: role in secondary free radical formation. *Biochim. Biophys. Acta,* **1569**, 111-116.

Pyka J., Osyczka A., Turyna B., Blicharski W. & Froncisz W. (2001a). EPR studies of iso-1-cytochrome *c:* effect of temperature on 2-component spectra of spin label attached to cysteine 102 and 47. *Eur. Biophys. J.,* **30**, 367-373.

Pyka J., Osyczka A., Turyna B. & Froncisz W. (2001b). Interaction of cytochrome *c* oxidase and cytochrome *c* studied by the use of site-directed spin labeling. *5th Workshop on EPR Applications in Biology and Medicine*, Kraków, p. 16.

Richter S., Ping Y. & Rana T. (2002). TAR RNA loop: A scaffold for the assembly of a regulatory switch in HIV replication. *Proc Natl. Acad. Sci. U. S. A.,* **99**, 7928-7933.

Rinaldi A. C., Di Giulio A., Liberi M., Gualtieri G., Oratore A., Bozzi A., Schinina M. E. & Simmaco M. (2001). Effects of temporins on molecular dynamics and membrane permeabilization in lipid vesicles. *J. Pept. Res.,* **58**, 213-220.

Rosen G. M., Britigan B. E., Halpern H. J. & Pou S. (1999). *Free Radicals: Biology and Detection by Spin Trapping*. Oxford University Press, New York.

Saito K., Yoshioka H., Ito N., Kazama S., Tanizawa H., Lin Y. J., Watanabe H., Ogata T. & Kamada H. (1997). Spatiotemporal ESR-CT study on the metabolism of spin-labeled polysaccharide in a mouse. *Biol. Pharm. Bull.,* **20**, 904-909.

Sam J. & Peisach J. (1993). EPR spectroscopic investigation of the lability of oxygen inactivated bleomycin: implications for the mechanism of bleomycin-mediated DNA degradation. *Biochemistry,* **32**, 1488-1491.

Sancier K., Freeman G. & Mills I. (1962). Electron spin resonance of nitric oxide-hemoglobin complexes in solution. *Science,* **137**, 752-754.

Šentjurc M. (2001). Membrane domain alteration under the action of biologically active substances. An ESR study. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 43.

Šentjurc M., Štrancar J. & Koklič T. (2002). Membrane domain alteration under the action of biologically active substances: an EPR study. *Curr. Top. Biophys*, **26**, 65-73.

Spaltenstein A., Robinson B. H. & Hopkins P. B. (1989). Sequence- and structure-dependent DNA base dynamics: synthesis, structure, and dynamics of site and sequence specifically spin-labeled DNA. *Biochemistry,* **28**, 9484-9495.

Stasko A., Brezova V., Liptakova M. & Savel J. (2000). Thermally initiated radical reactions of K2S2O8: EPR spin trapping investigations. *Magn. Reson. Chem.,* **38**, 957-962.

Stasko A., Rapta P. & Malik F. (2000). Characterisation of beer stability by means of spin trapping (an EPR study). *Monatsschrift Fur Brauwissenschaft,* **53**, 4-7.

Strobel O. K., Keyes R. S. & Bobst A. M. (1990). An electron paramagnetic resonance probe to detect local Z-DNA conformations. *Biochem. Biophys. Res. Commun.,* **166**, 1435-1440.

Strobel O. K., Kryak D. D., Bobst E. V. & Bobst A. M. (1991). Preparation and characterization of spin-labeled oligonucleotides for DNA hybridization. *Bioconjug. Chem.,* **2**, 89-95.

Subczynski W. (2001). Pulse EPR detection of membrane domains. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 42.

Subczynski W. K. (2002). Pulse EPR detection of membrane domains. *Curr. Top. Biophys.*, **26**, 59-64.

Subczynski W. K. & Wisniewska A. (2000). Physical properties of lipid bilayer membranes: relevance to membrane biological functions. *Acta Biochimica Polonica*, **47**, 613-625.

Sünnetçíoğlu M., Dadaylipaktas D. & Ercan I. (2002). EPR investigation of irradiated seeds using nitroxide spin labels. *Curr. Top. Biophys.,* **26**, 93-99

Sünnetçíoğlu M. M., Dadayli D., Celik S. & Koksel H. (1998). Application of the electron paramagnetic resonance spin probe technique for detection of irradiated wheat. *Cereal Chem.,* **75**, 875-878.

Sünnetçíoğlu M. M., Dadaylipaktas D. & Ercan I. (2001). EPR investigation of irradiated seeds using nitroxide spin probes. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 21.

Tikhonov A. (2001). Regulation of electron and proton transport in chloroplasts as studied with pH-sensitive spin labels. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 18.

Tobyn M. J., Maher J., Challinor C. L. & Staniforth J. N. (1996). Investigations of the interactions between a novel polysaccharide controlled release matrix and model compounds using ESR. *J. Controlled Release,* **40**, 147-155.

Todd A. P., Cong J., Levinthal F., Levinthal C. & Hubbell W. L. (1989). Site-directed mutagenesis of colicin E1 provides specific attachment sites for spin labels whose spectra are sensitive to local conformation. *Proteins,* **6**, 294-305.

Troup G. & Hunter C. (2002). EPR, free radicals, wine, and the industry: some achievements. *Ann. N. Y. Acad. Sci.,* **957**, 345-347.

Urizzi P., Monje M. C., Souchard J. P., Abella A., Chalas J., Lindenbaum A., Vergnes L., Labidalle S. & Nepveu F. (1999). Antioxidant activity of phenolic acids and esters present in red wine on human low-density lipoproteins. *Journal De Chimie Physique Et De Physico-Chimie Biologique,* **96**, 110-115.

Vander A. J., Sherman J. H. & Luciano D. S. (1990). *Human Physiology. The Mechanism of Body Function*. McGraw-Hill Publishing Company: New York.

Vanin A. F. & Nalbandyan R. M. (1965). Free radicals of a new type in yeast cells. *Biofizika (Rus)*, **11**, 167-168.

Vásquez-Vivar J., Joseph J., Hogg N., Whitsett J., Martásek P. & Kalyanaraman B. (2001). Superoxide formation from endothelial nitric oxide synthase is enhanced by tetrahydrobiopterin analogs. *5th Workshop on EPR Applications in Biology and Medicine*, IMB JU, Kraków, p. 46.

Wang K., Gerena L., Swaminathan S. & Bolton P. (1995). Determination of the number and location of the manganese binding sites of DNA quadruplexes in solution by EPR and NMR. *Nucleic Acids Res.,* **23**, 844-848.

Warwick P. E., Hakam A., Bobst E. V. & Bobst A. M. (1980). Reactivity of reverse transcriptase toward (s4U,U)n copolymers and spin-labeled nucleic acid lattices. *Proc. Natl. Acad. Sci. U. S. A.,* **77**, 4574-4577.

Warwick-Koochaki P. E., Hakan A. & Bobst A. M. (1983). Interaction of polymerases with 2'-deoxyuridine-5'-triphosphate spin-labeled at the 5-position. *FEBS Lett.,* **152**, 157-162.

Wasniowska A., Subczynski W. & Tikhonov A. (2002). Inhibition of ATP synthesis in chloroplasts by lipid soluble nitroxide radical spin label. *Curr. Top. Biophys.,* **26**, 83-91

Watala C., Golanski J., Witas H., Gurbiel R., Gwozdzinski K. & Trojanowski Z. (1996). The effects of in vivo and in vitro non-enzymatic glycosylation and glycoxidation on physico-chemical properties of haemoglobin in control and diabetic patients. *Int. J. Biochem. Cell Biol.,* **28**, 1393-1403.

Webber A. N. & Lubitz W. (2001). P700: the primary electron donor of photosystem I. *Biochim. Biophys. Acta*, **1507**, 61-79.

Wertz J. & Bolton J. (1986). *Electron Spin Resonance. Elementary Theory and Practical Applications*. Chapman and Hall, New York, London.

Wisniewska A. & Subczynski W. K. (1996). Spin-Label Study on Gramicidin -Phosphatidylcholine Interface: Fluidity, Hydrophobicity and Ion Penetration. *Curr. Top. Biophys.,* **20(1)**, 86-92.

Yamada K., Murugesan R., Devasahayam N., Cook J., Mitchell J., Subramanian S. & Krishna M. (2002). Evaluation and comparison of pulsed and continuous wave radiofrequency electron paramagnetic resonance techniques for in vivo detection and imaging of free radicals. *J. Magn. Reson.,* **154**, 287-297.

Youngdee W., Lurie D. & Foster M. (2002). Rapid imaging of free radicals in vivo using hybrid FISP field-cycled PEDRI. *Phys. Med. Biol.,* **47**, 1091-1100.

Zhao M., Kalai T., Hideg K., Altenbach C., Hubbell W. L. & Kaback H. R. (2000). Binding of spin-labeled galactosides to the lactose permease of *Escherichia coli*. *Biochemistry,* **39**, 11381-11388.