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JSCS–4147 *Review*

REVIEW

The role of EPR spectroscopy in studies of the oxidative status of biological systems and the antioxidative properties of various compounds

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Abstract: In this era of intense study of free radicals and antioxidants, electron paramagnetic resonance (EPR) is arguably the best-suited technique for such research, particularly when considering biochemical and biological systems. No attempt was made to cover all the topics of EPR application but instead attention was restricted to two areas that are both novel and received less attention in previous reviews. In the first section, the application of EPR in assessing the oxidative status of various biological systems, using endogenous stabile paramagnetic species, such as the ascorbyl radical, semiquinone, melanin, and oxidized pigments, is addressed. The second section covers the use of EPR in the emerging field of antioxidant development, using EPR spin-trapping and spin-probing techniques. In both sections, in addition to giving an overview of the available literature, examples (mostly from the authors' recent work) are also presented in sufficient detail to illustrate how to explore the full potential of EPR. This review aims at encouraging biologists, chemists and pharmacologists interested in the redox metabolism of living systems, free radical chemistry or antioxidative properties of new drugs and natural products to take advantage of this technique for their investigations.

Keywords: EPR spectroscopy; oxidative status; antioxidants; spin-probes; spin--traps.

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1. INTRODUCTION

The delicate balance between the advantageous and detrimental effects of free radicals is clearly an important aspect of life. Although reactive oxygen species (ROS) have been labelled as "villains" for a long time,[1,2] they also have been found to regulate the activity of a number of enzymes *via* oxidation in receptor-mediated signalling pathways, a process known as redox signalling.[3–8] This signalling is involved in the regulation of vascular tone, oxygen tension, activity of the immune system, growth in plants, and some other physiological processes.[9] On the other hand, uncontrolled generation of radicals is highly related to many pathophysiological events, such as neurodegenerative diseases (Alzheimer's disease, amyotrophic lateral sclerosis, Down's syndrome, *etc*),[10–13] malignancy,[14] diabetes mellitus,[15] sepsis[16] and atherosclerosis,[17] and also seems to play an important role in the aging process.[1,2,18,19] Hence, a certain level of oxidation performed by free radicals is mandatory in biosystems, but increased oxidation may jeopardize normal functioning and lead to pathophysiological conditions. Therefore, knowledge of the relative level of oxidation in a biosystem, known as oxidative status, clearly represents an imperative in studies of the mechanisms of (patho)physiological processes.[20]

Antioxidant supplements may be of great benefit in treating conditions related to a disturbed oxidative status. However, since free radicals are involved in signalling pathways, biosystems have developed a refractory response against the excessive presence of antioxidants,[21,22] in order to maintain a flexible intracellular redox poise.[19] This could explain why megadoses of ascorbate and some other antioxidants do not prolong life in humans with a balanced diet.[23,24] Although there is a myriad of compounds and foods that possess good antioxidative characteristics, new antioxidants that could be able to overcome the refractory response of the body, such as fructose and its phosphorylated forms,[25] should be investigated. However, the evaluation of antioxidative properties still represents the first step in determining whether a particular food or its compounds could be of any use in health problems related to oxidative stress.

A broad spectrum of techniques has been applied in redox research. Most of them, however, determine only the total antioxidative status or antioxidative capacity and do not provide details on specific reactive species. On the other hand, those which are able to provide more specific data require the use of tedious laboratory procedures or suffer from low sensitivity or artefacts. Electron Paramagnetic Resonance (EPR) spectroscopy stands out from other methods because of its unique ability to detect either short or long lived radicals with high specificity and sensitivity (*e.g.*, EPR spin-trapping detection of the superoxide radical is 40 times more sensitive than spectrophotometric analysis with cytochrome *c* [26,27]). EPR is also capable of directly detecting a number of specific markers of the oxidative status, such as the ascorbyl radical,[28,29] the tocopherol radical,[30,31] melanin,[32,33] semiquinone,[34] and plant pigments,[35,36] in a variety of biosystems – human and animal tissues and fluids, whole insects, plant tissues and others. In an antioxidant investigation, EPR can also be applied to determine the capacity of the selected compound, extract or food to remove specific reactive species, such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), organic radicals, lipid peroxides, or to sequester iron.[37–42] In addition, EPR is, in principle, a non-destructive technique, which is a clear advantage over chemical procedures when dealing with biological systems. This paper represents an overview of recent applications of EPR spectroscopy in determining the oxidative status of biosystems and antioxidative properties of compounds, extracts, and foods. The full capacity of a variety of EPR techniques in redox studies is yet to be explored; hence, the aim was to encourage scientists to apply EPR in their studies and to develop new EPR techniques. As a matter of convenience, most of the examples and illustrations are from a series of the authors' recent studies, with the aim of presenting the main principles of the application of EPR in redox research.

2. EVALUATION OF OXIDATIVE STATUS

2.1. EPR in comparison to other methods

A variety of spectrophotometric assays is available for investigating the oxidative status of biochemical systems. Widely applied is the ABTS (2,2-azino--bis(3-ethylbenzothiazoline-6-sulphonic acid) assay, in which ABTS is added to a system, oxidized by horseradish peroxidase/H_2O_2 to the radical cation $ABTS^+$ which absorbs light at 660, 734 and 820 nm. Results obtained using the ABTS assay are expressed as equivalents of ascorbic acid, indicating the total antioxidant activity of some tissue homogenate, cells (*e.g.*, cultured cells) or liquids (*e.g.*, serum, CSF, *etc*.).[43,44] Increased total antioxidant activity is usually related to activation of the antioxidative system (AOS), indicating a disturbed oxidative status. Useful information on the oxidative status could also be obtained by measuring the total amount of R–SH groups by the method of Ellman,[45] which can provide information on both pro-oxidative and pro-reductive modifications of the

oxidative status. R–SH groups are present in glutathione (GSH), a very important component of the AOS, as well as in proteins.[46] A total amount of R–SH groups less than normal usually means that some GSH has been oxidized to GSSG *via* ROS reduction. In addition, it could be a consequence of an increased production of reactive nitrogen species (RNS), which leads to the formation of R–SNO groups.[47] On the other hand, an increased level of the total amount of R–SH groups indicates a decrease in the production of superoxide, which leads to a decrease of peroxynitrite production and R–SH nitosylation.[48]

The ROS/RNS-system and the AOS are just two intertwined components present within the global physiological mechanisms of homeostasis.[49] Changes in the oxidative status modify the AOS activity, its composition and structure, striving to provide the best possible protection and preservation of cellular homeostasis.[33] Therefore, the activity of a battery of AOS enzymes (catalase, GSH peroxidase and reductase, MnSOD, and CuZnSOD) represent excellent marker of the oxidative status and may provide information on the specific mechanisms of oxidative processes.[29,50] For example, CuZnSOD is inactivated by H_2O_2;[51] hence a decrease in its activity in some biosystem indicates the development of H_2O_2-mediated oxidative stress.

Confocal fluorescent microscopy represents a powerful tool for redox studies on cell cultures and *ex vivo* tissues. With the increased interest in this area of research, a variety of labels have become available that can be used to investigate the oxidative status of cells under different conditions. For example, the intensity of fluorescence of cells stained with MitoTracker Orange is affected by the intracellular level of hydrogen peroxide (H_2O_2) and the oxidative status.[25,52] Some other dyes, the fluorescence of which is dependent on oxidation, such as carboxy-H_2-DCFDA and others, can also be used.[53]

Although these techniques are useful, they do not provide direct information on the reactive species that participate in oxidative processes; hence, EPR spectroscopy should be used. There are two distinct EPR approaches for the study of the oxidative status of biosystems: EPR spin-trapping of short lived radicals and EPR spectroscopy of stabile, paramagnetic biomolecules.[54] EPR spin-trapping represents a technique with a special place in oxidative studies because of its unique ability to identify and quantify relative changes in the level of any specific short-lived free radical involved in oxidative stress, including even $^{\bullet}OH$ with a lifetime of $\approx 10^{-9}$ s or $O_2^{\bullet-}$ ($\approx 10^{-6}$ s).[55] The application of EPR spin-trapping in the evaluation of oxidative status has been covered in recent reviews.[56,57] Hence, in this section, an alternative manner of application of EPR in investigations of the oxidative status is illustrated. Physiologically active molecules can react with a number of reactive species within cells and therefore propagate or attenuate free radical processes and serve as antioxidants and possible biochemical and/or physiological switches. Some endogenous biomolecules, such as as-

corbate, tocopherol, melanin, or plant pigment system P700, can be modified by ROS to stabile organic radicals with a very long lifetime, allowing their direct detection by EPR spectroscopy.[58] Due to the non-destructive nature of EPR, this can be realised without any interference with biochemical processes, which is not the case with any other method.[59] By detecting and discriminating these paramagnetic molecules, the level of which represents a marker of oxidative status, EPR provides essential information on redox mechanisms in biosystems. However, in spite of the unique capabilities of EPR, it should be emphasized that, in our experience, the most complete insight into redox processes can be achieved by complementing EPR with other methods, such as analysis of the AOS or SH groups (see the next section).

The detection of different paramagnetic biomolecules requires the explanation of some technical details. Usually their signals can be detected by means of a conventional X-band EPR spectrometer operating at a resonant frequency of around 9.5 GHz. The measurements are generally performed at physiological or room temperature, but in some cases lower temperatures (liquid N_2) are required to obtain a better S/N ratio, which requires minor technical adjustments. When dealing with oxygen consuming systems or oxygen sensitive processes, it is essential to control the gas environment in the sample. This can be easily achieved by placing samples in gas-permeable Teflon tubes or holders and flowing the required gas mixture over the sample.[60] In a such manner, a constant level of O_2 can be supplied to the system,[56,60] or if the radical is sensitive to oxygen (*e.g.*, the tocopheroxyl radical, $^{\bullet}$TO), to deprive it by using pure N_2 or Ar.[54] An important component in analyzing any EPR spectrum is to perform spectral simulations of each detected signal, to identify species and to determine their signal intensities (in our studies, Brukers' WINEPR SimFonia was used but there are many other software available).[28,60–62] The following sections illustrate different applications of EPR spectroscopy in detecting the most common stabile paramagnetic species in biosystems.

2.2. EPR spectroscopy of the ascorbyl radical

Ascorbate is thermodynamically at the bottom of the pecking order of oxidizing free radicals.[63] That is, all oxidizing species with higher redox potentials, which include $^{\bullet}$OH, alkyl peroxyl radical ($^{\bullet}$ROO), lipid peroxyl radicals ($^{\bullet}$LOO), tocopheroxyl radical ($^{\bullet}$TO),[63] peroxynitrite, [64] and others, can be repaired by ascorbate, leading to the generation of the ascorbyl radical ($^{\bullet}$Asc). This property makes $^{\bullet}$Asc probably the best endogenous marker of the oxidative status in biosystems. EPR can easily detect the ascorbyl radical in almost any system since it gives a characteristic doublet (Figs. 1a, 1b and 1c). Another important generator of $^{\bullet}$Asc in the biological setup are catalytically active transition metals (*e.g.*, iron or copper), which can provoke oxidation of ascorbate: *i*) directly: Fe^{3+}

(Cu^{2+}) + Asc^- → Fe^{2+} (Cu^{1+}) + $^{\bullet}Asc$, *ii*) in cooperation with H_2O_2: $Fe^{2+}(Cu^{1+})\cdots Asc + H_2O_2 \rightarrow Fe^{3+}(Cu^{2+}) + {}^{\bullet}Asc + OH^- + H_2O$ or *iii*) *via* $^{\bullet}OH$, generated in a metal involving the Fenton system: $^{\bullet}OH + Asc^- \rightarrow {}^{\bullet}Asc + OH^-$.[65–67] Obviously, the level of $^{\bullet}Asc$ can indicate how much redox active metals there are in a system,[65,66] but the level of $^{\bullet}Asc$ also depends on the production of free radicals in reactions unrelated to the presence of transition metals and these should be discriminated. It is suggested that the influence of catalytically active metals could be emphasized by the addition of H_2O_2, which should promote reactions *ii* and *iii* depending on the level of the catalytically active metals. On the other hand, it should be stressed that commercial buffers inevitably contain metal impurities which may represent a source of artefacts in biochemical studies by increasing the basic level of $^{\bullet}Asc$.[65] This potential problem can be eliminated by the addition of strong chelating agents, such as DTPA, which sequester transition metals and diminish their oxidative capacity.[67]

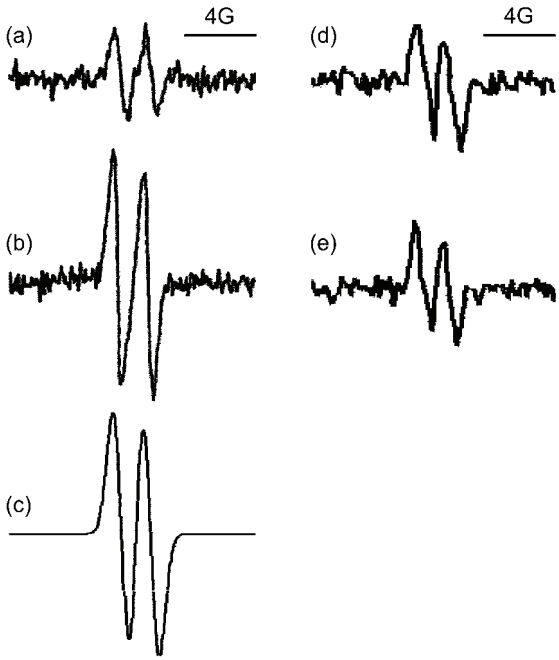

Fig. 1. Left: EPR spectra of the ascorbyl radical ($^{\bullet}Asc$). EPR detects the single unpaired electron of $^{\bullet}Asc$, which has a spin S = ½. However, the electron interacts with surrounding nuclei that also possess spin (here ^1H with a nuclear spin of I = = ½), which leads to hyperfine splitting; hence, two lines emerge in the spectrum. An example of $^{\bullet}Asc$ in human plasma: a) untreated serum;[28] b) serum treated with 0.5 mM peroxynitrite;[64] c) spectral simulation of $^{\bullet}Asc$ EPR signal proving that the signal from serum emerges from this radical, enabling quantification of the signal intensity. Characteristic EPR spectra of $^{\bullet}Asc$ in amnion fluid of d) normal and e) thrombophilic pregnancies[29] (showing no differences between the two samples).

The level of $^{\bullet}Asc$ detected by EPR spectroscopy as an indicator of prooxidative changes was first introduced by Buettner and Jurkiewicz in 1993.[28] Experiments were performed on a phosphate buffer supplemented with different ascorbate concentrations and on human plasma. Using the $^{\bullet}OH$ and $O_2^{\bullet-}$ generating systems, the authors showed high correlations between the production of these radicals (determined by EPR spin-trapping) and the level of $^{\bullet}Asc$. Since

then, •Asc has been detected by EPR in a variety of biological samples: plasma,[68,69] cerebrospinal fluid (CSF),[69] skin,[70,71] extracellular fluid,[72] plant leaves[73] and the midgut fluid of insects.[74] Increase of the steady-state concentration of •Asc was reported in several conditions related to pro-oxidative changes, such as ischemia/reperfusion, sepsis,[75] brain injuries,[76] paraquat poisoning, iron overload, gastric cancerogenesis,[77] pre-eclampsia,[78] and others. Recently, EPR spectroscopy of •Asc was employed to investigate the oxidative status of amnion fluid (Figs. 1d and 1e). The similar level of •Asc in the amnion fluid of control and thrombophilic subjects indicates that the disturbed oxidative status of the placenta which was observed by AOS assays,[29] was not provoked by an increased generation of reactive species in amnion fluid,[29] thus enabling a potential mechanisms of thrombophilia to be suggested. There are many other interesting examples. Menditto *et al.*[79] used EPR spectroscopy of •Asc to study the association between the oxidative status of seminal fluid and the iron and copper content. A very innovative and elegant experimental design was developed by Sharma *et al.*,[80] enabling *in vivo* EPR measurements of the level of •Asc, as a real-time quantitative marker of changes of the oxidative status in dog myocardium during ischemia/reperfusion.

The absolute •Asc concentration which can be detected is as low as ≈5 nM by measuring the intensity of the EPR signal using spectral simulation (Fig. 1) and calibration. However, the basal •Asc level in tissues and fluids can vary significantly between subjects or populations,[64,68] and is highly dependent on the level of ascorbate in the system.[72] For instance, due to different diets and life styles, the basal level of ascorbate and, consequently, ascorbyl radical in the plasma of the Brazilian population (65 nM)[64] is different from that of the European one (100 nM).[68] Galleano *et al.*[81] developed an approach that could overcome this potential obstacle in oxidative studies. By combining EPR measurements of the •Asc concentration and HPLC analysis for the total ascorbate level, they were able to calculate the ascorbyl radical/ascorbate (•Asc/Asc) ratio, which represents an indicator of oxidative stress independent of individual and population variations in the ascorbate level. This represents an example of the effectiveness of EPR in combination with other techniques, as emphasized before.

2.3. EPR spectroscopy of the tocopheroxyl radical

Tocopherols (vitamin E) are the main lipid-soluble antioxidants of cellular membranes and blood plasma.[30] They react with oxidizing species, such as •LOO (lipid peroxyl radical),[82] •OH, and $•O_2^-$, but also with NO[83] and $ONOO^-$,[84] to produce •TO, which can be detected by EPR spectroscopy (Fig. 2). However, the generation of •TO in biosystems is thermodynamically interlinked with the metabolism of ascorbate, *via* the reaction: •TO + Asc → TOH + •Asc.[87] For example, it was demonstrated that in human plasma exposed to oxidative stress, the EPR

signal of •TO emerges (Fig. 2b) only after the virtual disappearance of the ascorbyl radical.[31] Therefore, the EPR spectroscopy of the tocopheroxyl radical could be used in investigations of oxidative status, emphasizing an occurrence of intense oxidative stress capable of depletion of the antioxidative capacity of the ascorbate in the system.

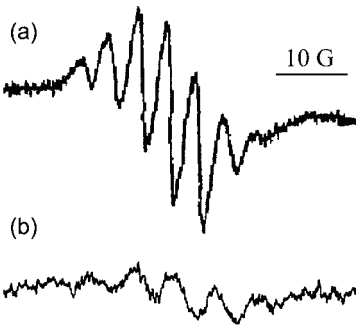

Fig. 2. Characteristic seven-line EPR signal of •TO. a) Generated by purging the suspension with 0.1 mM tocopherol and 100 mM SDS with NO gas;[86] b) in human plasma exposed to $O_2^{\bullet-}$ generated by the hypoxanthine/xanthine oxidase system.[31] The interactions which provoke hyperfine splitting resulting in a complex seven-line signal and the spectral parameters were described in detail by Matsuo *et al.*[87]

The detection of •TO in biosystems *ex vivo* is complicated by the fact that •TO can react with oxygen to produce TO and superoxide (an EPR silent species), which can occur during sample collection and storage, as well as during EPR measurements, hence even EPR measurements have to be performed in an inert atmosphere (N_2). For this reason, most of the EPR studies of •TO have been performed on model systems *in vitro.* Naužil *et al.*[30] applied EPR spectroscopy of •TO to study the inhibitory effects of *α*-tocopherol against radical-initiated oxidation of low-density lipoproteins (LDL) lipids. Zhou *et al.*[88] used a similar approach to investigate the regeneration of *α*-tocopherol by green tea polyphenolics in phospholipid micelle exposed to oxidative stress. The metabolism of TO can also be explored using the more stabile and hydrosoluble vitamin E derivative – Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). For example, Opländer *et al.*[89] applied EPR to investigate the effects of the Trolox against UV-A induced cell death of human skin fibroblasts. It was determined that the production of the Trolox radical is increased in the presence of nitrite, indicating that the EPR spectroscopy of •TO could be useful for studying not only ROS, but also for nitrogen reactive species.

2.4. EPR spectroscopy of melanin

Melanins represent a broad class of biopolymers without a unique structure, composed of 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, and their various oxidized forms (Fig. 3).[90] Melanins are divided into three groups: eumelanins and pheomelanins in animals, and allomelanins in plants. Melanins react with ROS, organic radicals and oxidize transition metals.[91] In these reactions, DHI is converted to a stabile SQ radical and other paramagnetic species,[90]

which can be detected by EPR. These properties make the intensity of the EPR signal of melanin an excellent marker signifying the oxidative status of specific melanin-containing systems, such as mammalian skin[92] and eyes,[93,94] some plants,[95] fungi,[96,97] insects,[33,98] and others. Nevertheless, it should be stressed that although EPR spectroscopy can distinguish between different types of melanins, it is virtually impossible to connect the recorded spectrum to a specific eumelanin structure by any spectral analysis.

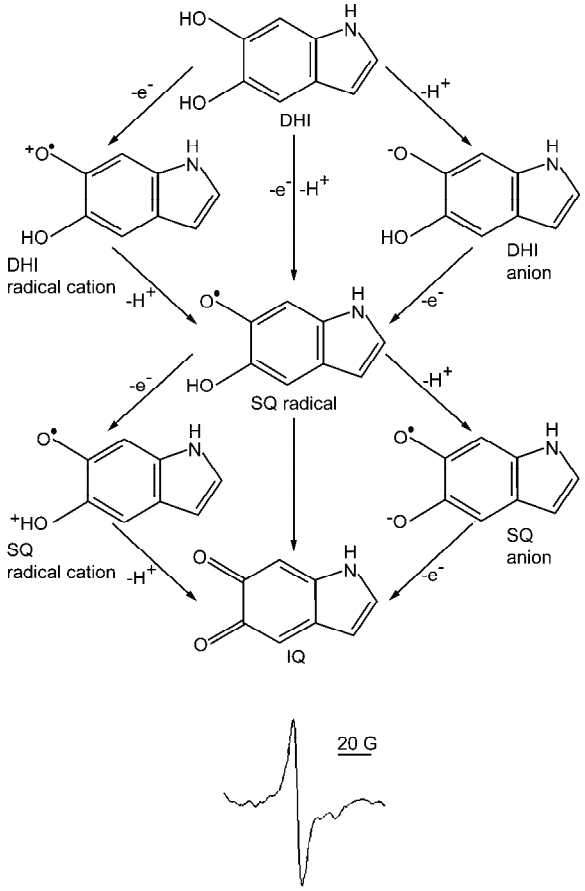

Fig. 3. Redox chemistry (a) and characteristic EPR signal of eumelanin (b). The key monomeric building blocks of eumelanin are: DHI (5,6-dihydroxyindole), SQ (semiquinone) and IQ (5,6-indolequinone). Spin–spin interactions, fast electron transfer reactions and anisotropy related to low mobility of paramagnetic species inside the melanin macromolecules make only one broad line in the melanin EPR spectrum emerge.

Neuromelanin, a pigment found in deep brain regions, such as *substantia nigra*, has drawn much attention because of its role in Parkinson's disease (PD).

Due to the complex chemistry of neuromelanin in the brain, EPR measurements do not provide a straightforward evaluation of the oxidative status, but studies on neuromelanin provide a valuable insight into the mechanisms of neurodegeneration in PD.[99–101] The EPR signal of melanin has been used to study the pro-oxidative effects of UV irradiation in skin and eyes. Wood *et al*.[32] used the melanin signal as an indicator of the oxidative status of melanin-containing skin cells from a genetically melanoma-susceptible cross of *Xiphophorus* fish (a model system for human melanoma research) exposed to UV irradiation of different wavelengths and intensities. A strong correlation between the intensity of the melanin signal and melanoma induction was observed, indicating that pro-oxidative changes represent an important causative step in melanoma development.[32] Pertinent to this, some attempts have been made to develop an *in vivo* EPR technique in the diagnostics of melanoma in humans. In a study performed by Vahidi *et al*.,[102] the EPR spectra of melanin in frog skin were recorded *in situ* using a surface EPR coil and 2D imaging, and the intensity of the melanin signal was observed to depend on the level of oxygenation, which is one of the parameters defining the oxidative status. It was proposed that 2D EPR spectroscopy could potentially be useful in early melanoma detection. Seagle *et al*.[93] employed EPR of melanin to study the effects of UV radiation on human retinal pigment epithelium with different coloured pigments and to compare the observed changes to the modifications in synthetic melanin. Samples were placed in an EPR "flat cell" and irradiated inside the cavity. As expected, the melanin signal from the cells increased with the power of irradiation, but not in two distinguishable portions as observed for synthetic melanin, indicating that *in vivo* radical photogeneration is different from photogeneration in a chemical system. This shows that chemical systems with synthetic melanin do not represent an adequate model of the structure and metabolism of melanin in biosystems.

Melanin represents an important constituent of the oxidative metabolism of insects. Barbehenn *et al*.,[98] applied EPR spectroscopy of melanin to investigate the pro-oxidative defence of plants against herbivorous insects. It was determined that melanin-like reactive substances are formed in the midgut fluid of insects, from the tannic acid present in leaves. In this way, plants disturb the oxidative status of the digestive system of insects and indirectly protect themselves.[103,104] On the other hand, some insects generate melanin to use its ROS scavenging ability in the regulation of their oxidative status. For example, it was recently shown using biochemical assays and EPR of melanin that exposure of the European corn borer (*Ostrinia nubilalis*) to low temperatures leads to an overproduction of H_2O_2 followed by an increase of the melanin signal (Fig. 4).[33] Hydrogen peroxide is an important player in the freeze tolerance development of insects, but as was shown, it can disturb the oxidative status of insect tissues.[33]

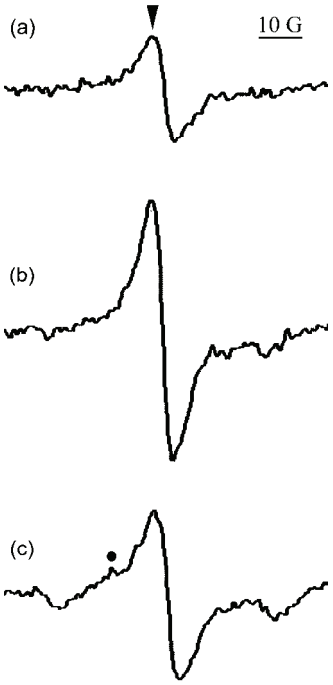

Fig. 4. EPR spectra of melanin in larvae maintained at: a) 5, b) –3 and c) –16 °C. The downward triangle (▼) marks the characteristic eumelanin-related EPR signal. The signal of pheomelanin (circle) can be observed on the left side of the eumelanin signal in panel c). It can be observed that the underlying signal of pheomelanin slightly shifts the eumelanin signal. The measurements were performed at liquid N_2 temperature in order to obtain a higher S/N ratio than at room temperature.[33]

Thus, in this case, insects use melanin to protect their cells from the oxidative burst involved in the adaptation process.

The presented examples illustrate the vast potential of EPR in studying various organic radicals,[105] which should be further utilized in redox studies of biosystems. For example, EPR is able to detect redox-active amino acid radicals: tyrosyl, tryptophanyl, glycyl, and histidyl radical.[106] The EPR of these stabile radicals was previously employed to study enzyme activity and protein damage,[57,107] but, potentially, it may also find applications in investigations of the oxidative status.[108]

2.5. The oxidative status of plants

Although previously unappreciated, the application of EPR spectroscopy is becoming more and more important in plant studies. It was previously illustrated that the EPR spin-trapping technique with advanced spin-traps (*e.g.* DEPMPO) is able to provide crucial data about plant oxidative processes,[34,56,60,109] but when it comes to endogenous paramagnetic species, the great diversity in plants opens even more possibilities for redox research. Although, there are many similarities between evaluating the oxidative status in plants and studies on human and animal systems, it was decided to dedicate a separate section to plants, in order to provide a compact overview and to emphasize the potential of the EPR methods. In a recent review of techniques applicable in the investigation of plant redox

processes, Shulaev and Oliver[27] concluded that, at present, measurements of oxidative stress in plants are limited and there are no truly non-invasive methods. Although they considered the EPR spin-trapping technique, the application of EPR spectroscopy of stabile organic radicals in plants was not taken into account. Since it enables the acquisition of signals of paramagnetic species in plant cells, parts or even whole plants without any interference with the metabolism, EPR spectroscopy could be the missing 'truly non-invasive' technique in plant research. It should be emphasized that the measurements can be performed under selected temperature, atmosphere, and light regimes, which can be varied inside the EPR spectrometer cavity during the course of the experiment.

As in animal tissues and fluids, the ascorbyl radical can be used to determine the oxidative status of plants. Malanga *et al.*[110] used the endogenous signal of the ascorbyl radical in an algal culture (*Chlorella vulgaris*) and intact soybean leaves to study UV-provoked oxidative stress. Puntarulo *et al.*[111] applied EPR measurements of $^{\bullet}$Asc to investigate the effects of NO on the oxidative status of soybean chloroplasts, proposing an antioxidative role of NO based on the decreased level of $^{\bullet}$Asc after treatment with NO.

The photosystem I (PSI), which contains an EPR active specie – oxidized pigment – $P700^+$, could also be used as a marker of the oxidative status.[35,36] The increased generation of reactive species leads to photoinhibition of the PSI and a related decrease in the $P700^+$ level.[112] Thus, in contrast to previous illustrations, a decrease in the EPR signal signifies pro-oxidative changes of the oxidative status. This approach was employed to investigate the oxidative stress in intact pea leaves exposed to chilling conditions.[36] The light inducible EPR signal, which was recognized to reflect the oxidized form of the PSI pigment P700 ($P700^+$), is shown in Fig. 5. The level of $P700^+$ was lower in plants exposed to cold, showing that the chilling conditions led to pro-oxidative changes of the status in leaves. A similar non-invasive approach could be used to investigate the oxidative status of plants exposed to some other stressors.

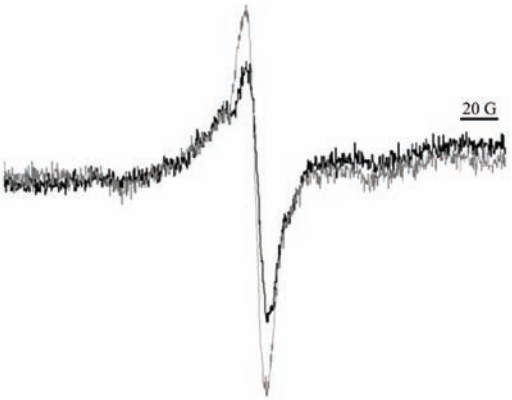

Fig. 5. Spectra of light-induced EPR active species – oxidized PSI pigment ($P700^+$) in pea leaves. Pale trace – samples from leaves of control plants exposed to 22 °C/70 μmol m^{-2} s temperature/light conditions; dark trace – samples of leaves of peas under a 2 °C/70 μmol m^{-2} s^{-1} regime. The spectra were recorded at –100 °C.[36]

To study paramagnetic species not present in sufficient concentrations in plants to enable non-invasive detection, plant isolates or extracts could be used in EPR studies. For example, the EPR signal of quinhydrone was identified in isolated cell walls from pea roots, which could not be detected in the whole plant parts. The intensity of the EPR signal of quinhydrone was highly correlated with the concentration of H_2O_2 supplemented to the cell wall and with $^{\bullet}OH$ production evaluated by the EPR spin-trapping technique, showing itself to be a useful marker of oxidative status.[34] Pedersen[113–115] published several comprehensive studies on alcohol plant extracts showing that hundreds of species from the families Lamiaceae and Gesneriaceae contain quinone related paramagnetic species easily detectable by EPR, and potentially valuable as markers of oxidative status. Examples of EPR signals of such species in extracts of *Peltodon radicans, Salvia hispanica* and *Monarda clinopodia* are illustrated in Fig. 6.

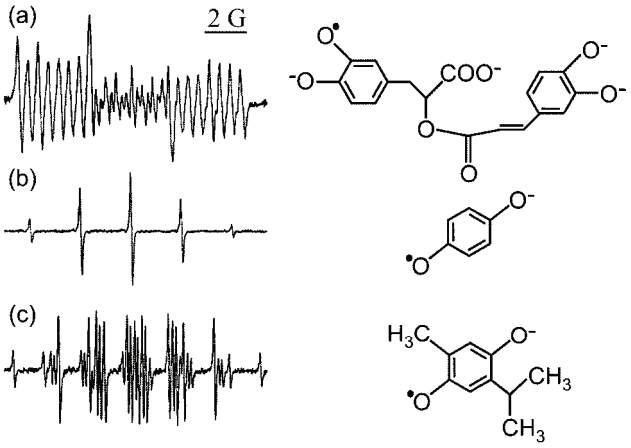

Fig. 6. The EPR semiquinone spectra and chemical structures of a) rosmarinic acid from *Peltodon radicans*, b) hydroquinone from *Salvia hispanica* and c) thymohydroquinone from *Monarda clinopodia* all obtained from crude alcoholic leaf extracts. The signals were identified using the EPR spectra of the corresponding chemicals.[115]

Based on these examples, it is proposed that any plant part or extract that has not been investigated previously by EPR should be first examined using EPR with a wide scan range. If a signal is observed, the recording parameters should be optimized to obtain a good S/N ratio and detailed hyperfine structure of the spectrum. The signal should then be compared with signals of paramagnetic species that are available in the literature. This is usually sufficient to identify the EPR active compound(s). If not, then the chemical composition of the sample should be analysed, in order to narrow the list of compounds with paramagnetic properties that may be present. After this, each "suspect" should be examined separately using EPR spectroscopy and compared to the signal from the sample.

Spectral simulations should be used to compare the levels of paramagnetic species in different samples, and to determine whether the signal of the sample is composed of EPR spectra of more than one paramagnetic species.

3. EVALUATION OF ANTIOXIDATIVE ACTIVITY

3.1. EPR spectroscopy – a technique of choice for investigating the antioxidative properties of compounds, extracts and foods

Overproduction or inefficient removal of reactive species by scavenging enzymes, as well as an increase of the level of catalytically active metals (*e.g.*, iron or copper) related to the generation of the notorious $^{\bullet}$OH radical, have been shown to lead to damage of biomolecules and cellular membranes in a process known as oxidative stress,[9,116] which has been proposed to be a hallmark of a variety of pathophysiological conditions.[9,10–17,29,54] Under such circumstances, the supplement of antioxidants aimed at re-balancing the disturbed oxidative status could be a very beneficial component of a treatment or a diet.[19,20,24,25] To establish whether some food or a compound could be useful in health problems related to oxidative stress, it is necessary to establish their antioxidative properties. A broad range of methods available for the evaluation of the so-called "total antioxidative capacity" of some compounds, plant extracts or foodstuffs were reviewed previously.[20,117,118] These assays (such as ABTS, ORAC, TRAP, and DPPH assays[118]) are easy to perform; hence, they are frequently employed in studies pointing to an antioxidative capacity of various foods or plant extracts. However, the majority of studies were performed in chemical systems not taking into account the specific properties of target biosystems and metabolic processes that can occur *in vivo*. Although such studies are a prerequisite for further investigations of the antioxidant effectiveness of a certain compound, several other points should be taken into consideration before it should be recommended as a potential cure: task #1: to determine the activity against specific radicals; #2: to determine the distribution of active compounds in both principal environments present in biosystems – hydrophilic and lipophilic; #3: to determine whether the antioxidant acts against radicals in cells, extracellular milieu, or both; #4: to take into account metabolic changes of the investigated compound(s) depending on the route of administration; #5: to determine which specific compound(s) present in the metabolized extract or food is (are) active against free radicals; #6: to determine whether the active compounds could somehow overcome the refractory response. In the following sections, it will be illustrated that the majority of these tasks can be performed by EPR or by combination of EPR with other methods in a carefully planned experimental setup.

EPR spectroscopy can be solely used to perform tasks #1–#3. Two EPR techniques can be applied in antioxidant studies: spin trapping and spin probing. The general concept of the application of EPR spin-trapping for these three tasks

is to generate a specific radical by a selected chemical system and to quantify the inhibiting effects of a compound or extract against radical production (#1). On the other hand, EPR spin-probing can be employed to determine the distribution of potential antioxidants in a hydrophilic, lipophilic, or extracellular environment (#2 and #3). The application of spin-probing in antioxidant research is based on the measurement of the ability of studied compound(s) to reduce synthetic long lived radicals (spin-probes) to EPR-silent hydroxylamines. The spin-probing approach in the study of antioxidants has a long history and has been extensively documented in the literature; in comparison, spin-traps represent an emerging field in EPR spectroscopy.

Tasks #4 and #5 also include the EPR approach, but require specific processing or analysis of the studied systems prior to EPR measurements. For example, antioxidants are usually applied orally, therefore an *in vitro* digestion model system[119] or a more simple methanolysis[39] can be used to process potential antioxidants to compounds that are absorbed *in vivo* (#4). After establishing the antioxidative properties (#1–#3) and metabolic modifications (#4) of some food or extract, analysis of its chemical composition, using HPLC, GC/MS and other methods, should be preformed, to reveal compound(s) that could be "responsible" for antioxidative activity. Selected compounds can be then separately tested for antioxidative activity using EPR techniques (#5).

It should be stressed that even if an investigated compound or extract appears to be an excellent antioxidant on the account of these five points, it might not be effective *in vivo* if it is unable to overcome the refractory response (#6).[18–20] In order to resolve this, the protective effects of a potential antioxidant in cell cultures and experimental animals exposed to oxidative stress should be determined. Fluorescent microscopy with redox sensitive dyes has shown itself to be very useful in studies of antioxidative actions in cell cultures, due to its high sensitivity and ability to detect sub-cellular changes related to oxidative stress. On the other hand, a non-destructive *in vivo* EPR technique is the only available method to follow up the oxidative status of animals, with the ability to determine the oxidative stress in specific organs.

As an ideal approach covering all six points is rarely seen in one study, it was decided in this review to separately present applications of spin-trapping and spin-probing techniques, indicating each phase of antioxidant research.

3.2. Applications of EPR spin-trapping in antioxidant research

EPR spin-trapping can provide data on antioxidative effects against the free radicals that are most relevant in physiology $-O_2^{\bullet -}$ and $^{\bullet}OH$ (#1), as well as many others (carbon-centred, $^{\bullet}LOO$, $^{\bullet}SG$).[56,120–127] As these reactive species cannot be directly detected due to their short life-times, a specific compound (spin-trap) is introduced into the system. Spin-traps react with free radicals, thus

forming stabile paramagnetic species (spin adducts), which are readily detected by EPR spectroscopy (Fig. 7). Each reactive species that has been trapped shows its own specific signature EPR spectrum. The advantage of such an approach over other methods that measure the total antioxidative capacity of the compound lies in the ability of EPR to distinguish antioxidative activity against different free radicals, even when simultaneously present in the system. This can be performed using spectral simulations, which enable the identification of each radical and the quantification of the signal intensity. In addition, recently a number of various hydrophilic and lipophilic spin-traps have evolved[120] enabling antioxidative measurements in any selected medium.

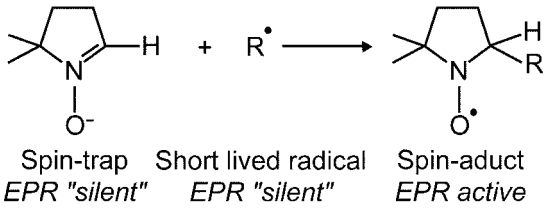

Fig. 7. The basic principles of the EPR spin-trapping technique.

For antioxidative studies, *in vitro* chemical systems are used to generate a specific free radical (*e.g.*, the xanthine/xanthine oxidase system or SOTS1 (di-(4--carboxybenzyl)hyponitrite) for $O_2^{\bullet -}$ or the Fenton system and Haber–Weiss-like reaction for $^{\bullet}OH$). The spin-trap is added to a selected system prior to the start of the reaction, and after a specific incubation time, the EPR signal of the spin adduct is recorded and the intensity of the specific trapped radical determined.[56] The application of an antioxidant to the system (before the initiation of the free radical production) should lead to a decreased generation of the spin adducts, due to radical scavenging or interactions with the reactants of the generating system, which is detected by the lower intensity of the EPR signal of a given radical as compared to the control antioxidant-free system.[37–42] The antioxidative activity (*AA*) is then calculated by comparing the signal intensities obtained in the control setup and in the system with the antioxidant, using the simple equation:

$$AA = (I_0 - I_x)/I_0$$

where I_0 and I_x are the intensities of the EPR spectra obtained in the control and the samples with the antioxidant, respectively. The determined antioxidative activity can then be compared to the *AA* of some antioxidant intrinsically present in metabolism, such as ascorbate or tocopherol.[39] This method enables a comparison of the *AA* of the investigated compound with the antioxidative properties of other previously studied compounds. An alternative approach is to determine the EC_{50} value (mg mL^{-1}) – an effective concentration at which the studied compound or extract shows an *AA* of 0.5.[128–130] The EC_{50} value is determined by

interpolation from the linear regression analysis of several *AA* values obtained for different concentrations of the compound or extract. Although attractive for comparative analysis of data obtained in different studies, this approach suffers from a disadvantage as the EC_{50} value depends on the concentration of reactants used in the radical generating system, which may differ from study to study. Therefore, EC_{50} can be used for comparison of the *AA* only when they were obtained in studies using an identical experimental setup.[129,130]

Antioxidative activity against $O_2^{\bullet-}$ can be determined using the xanthine/xanthine oxidase (X/XO) reaction, as an "$O_2^{\bullet-}$ generating" system which is also present in biological systems. EPR spin-trapping with the X/XO system was used in a number of studies to determine AA ($O_2^{\bullet-}$) of β-carotene,[131] vitamin E,[131] glutathione,[124] aminoguanidine,[123] lazaroids,[132] various tea extracts,[133] fullerenes[42] and others. In antioxidant studies, the spin-trap DMPO (5,5-dimethyl-L-pyrroline-*N*-oxide) was usually applied, because of its wide availability and low price. However, the DMPO adduct with the $O_2^{\bullet-}$ radical (DMPO/OOH) has a short lifetime and it is spontaneously transformed into DMPO/OH (the adduct of the $^{\bullet}$OH radical),[56] which may result in unrealistically high values of *AA* ($O_2^{\bullet-}$). Hence, the application of alternative spin-traps, such as DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide) is strongly recommended since the DEPMPO/OOH adduct undergoes transformation at a much lower rate than DMPO/OOH[56] or BMPO (5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide), the $O_2^{\bullet-}$ adduct of which does not undergo transformation at all. However, DEPMPO is the spin-trap of choice when the identification of different radicals is necessary.[134]

As an example of the scheme outlined in the previous paragraphs, the antioxidative properties of extracts of chestnut (*Castanea sativa* L.), and fructose and its phosphorylated forms were examined in two recent studies.[25,37,39] EPR, with DEPMPO and X/XO and the Fenton system, was applied to investigate the antioxidative properties of extracts of chestnut (*C. sativa* L.) leaves, catkin, and spiny burs in an aqueous medium in comparison to the *AA* of ascorbate (#1 and #2), while spin-probing with lipophilic probes was used to determine the antioxidative activity in membranes (#2; see the following section). The EPR signals of DEPMPO/OOH in the X/XO system without and with the catkin extract are shown in Figs. 8a and 8b, respectively. Interestingly, the extracts did not show significant antioxidative activity against $^{\bullet}$OH. Based on the data present in the literature that chestnut extracts are predominantly composed of tannins, which are not absorbed as such but are metabolized by intestinal flora, methanolysis of the extracts was performed to simulate the degradation of tannins in human intestines (#4). In order to determine which compound(s) in the extracts may be responsible for the observed high *AA* values, chemical analysis of the methanolysates was performed using LC/MS and HPLC/DAD (#5). Among the variety of identified

compounds, ellagic and valoneic acids were recognized previously for their antioxidative and anticancer properties.[135,136] Hence, it was proposed that the high AA ($O_2^{\bullet-}$) of chestnut extracts is most likely based on the antioxidative properties of these two acids and similar compounds that were detected, such as flavogallonic acid. Tasks #3 and #6 were not covered in this study and will be the subject of future research. However, it was shown by others that the derivatives of tannins are present in human plasma[137] and have long persistency in the body upon dietary uptake,[138] indicating that they may be able to overcome the refractory response (#6).

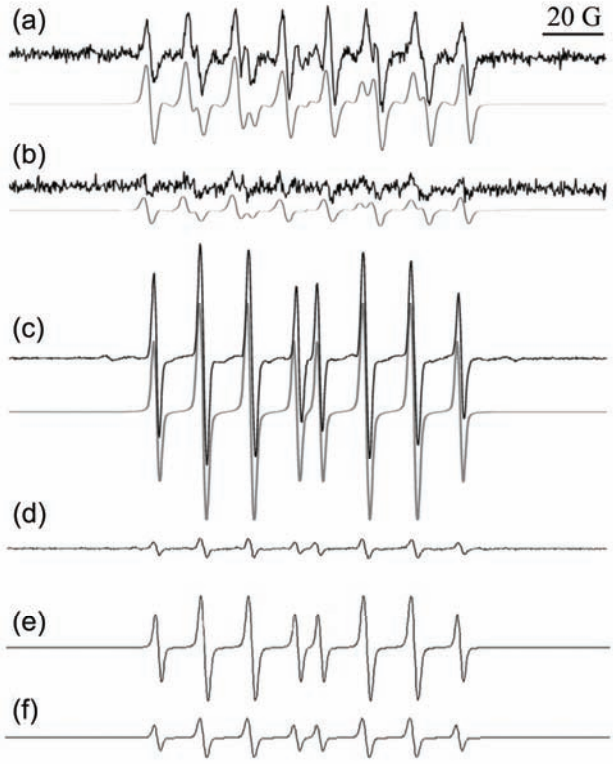

Fig. 8. The characteristic eight-line EPR signals of the DEPMPO/OOH adduct generated in: a) the X/XO system (X 1.6 mM; XO 1.6 IU mL^{-1}); b) X/XO with catkin extract (0.2 mg mL^{-1}). Gray – spectral simulations of the corresponding DEPMPO/OOH signals. Catkin extract *AA*($O_2^{\bullet-}$) = 0.65±0.02; *AA*($O_2^{\bullet-}$) for the same concentration of ascorbate (0.2 mg mL^{-1}) was 0.85±0.04.[39] Characteristic 8-line EPR spectra of the DEPMPO/OH adduct in: c) the Fenton reaction (Fe^{2+} 0.3 mM; H_2O_2 1.2 mM); d) Fenton reaction + 3 mM F16BP, *AA*($^{\bullet}$OH) = = 0.91±0.01. Gray – spectral simulation of the DEPMPO/OH signal.[37] Characteristic EPR signals of the DEPMPO/OH adduct in the metal-free Haber–Weiss-like $^{\bullet}$OH-generating system (KO_2 1.4 mM; H_2O_2 5 mM): e) Haber–Weiss reaction; f) Haber–Weiss reaction + 3 mM F16BP, *AA*($^{\bullet}$OH) = 0.40±0.05.[37]

It should be noted that in studies of specific compounds, some of the points in the present scheme could be found in the literature. For example, the antioxidative properties of fructose and its phosphorylated forms (fructose 1,6-bisphosphate, F16BP; fructose 1-phoshate, F1P; and fructose 6-phosphate, F6P), the biodistribution and metabolism were extensively studied previously, were investigated.[25] Their antioxidative activity against $^{\bullet}OH$ was determined using the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$), as the "$^{\bullet}OH$ generating system", which represents a constituent of various pathophysiological processes. EPR spin-trapping with the Fenton reaction is the most frequently used method for studying antioxidative properties. It was applied to determine the *AA* of monosaccharides,[37] fullerenes,[42] polysaccharides,[128] vitamins,[139] extracts of various plants,[140,141] seeds,[142] mushrooms,[129,130] teas,[133] spices[143] and others. It was shown that F16BP represents a very efficient antioxidant (#1; Figs. 8c and 8d), which may be useful as an infusion sugar for the treatment of pathophysiological conditions related to oxidative stress.[37] It is known that charged F16BP is preferentially located in the hydrophilic medium (#2), that it is transported into the cells (#3) and that it is not metabolized outside the cell (#4), so all these points in addition to #5 were not the subject of our study. However, if not able to overcome the refractory response and to protect cells from oxidative stress (#6), the application of F16BP in treatment could be futile.[144] Hence, EPR investigations were complemented by the study of intracellular antioxidative properties of F16BP in a cultured astroglial cell exposed to H_2O_2-mediated oxidative stress using confocal fluorescent microscopy and fluorescent markers of oxidative stress, which showed that F16BP is indeed able to overcome the refractory response and protect the cells by diminishing oxidative stress. Further research was conducted to resolve the mechanisms of the antioxidative effects of F16BP. In principle, antioxidative actions against $^{\bullet}OH$ generation in the Fenton system can occur *via* two mechanisms: direct $^{\bullet}OH$ scavenging and sequestration of a transition metal (iron, copper, manganese). In order to establish the mechanisms of antioxidative actions against $^{\bullet}OH$ production, the *AA*($^{\bullet}OH$) of a studied compound should be measured in two "$^{\bullet}OH$ generating" systems: *i*) the Fenton reaction, which contains metal ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$), and *ii*) the Haber–Weiss-like reaction, which is a metal-free system ($O_2^{\bullet-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2$). The difference between *AA*($^{\bullet}OH$) obtained in the first and the second system represents a measure of the metal sequestration of a certain agent. To the best of our knowledge, we were the first to apply this approach in the study of antioxidative activity of F16BP and some other monosaccharides.[37] F16BP showed significantly higher *AA*($^{\bullet}OH$) in the Fenton system (Figs. 8c and 8d), when compared to the Haber–Weiss-like reaction (Figs. 8e and 8f). Consequently, it was concluded that F16BP performs antioxidative actions in biological systems *via* both iron

sequestration and $^{\bullet}$OH scavenging, whereby the first mechanism is predominant.[37]

The ability of any compound to sequester metals is a very important feature as it enables an antioxidant to prevent progression of Fenton chemistry, which is a more efficient strategy for stopping or slowing down oxidative stress, than attempting to scavenge already produced highly reactive $^{\bullet}$OH. For example, different neurodegenerative conditions, such as Parkinson's disease, Alzheimer's disease, are most likely related to the misbalanced metabolism of redox active metals and with consequential propagation of Fenton chemistry.[145,146] Contemporary attempts to treat these conditions are primarily focused on compounds that are efficient in the sequestration of metals.[146] The potential of the EPR approach presented here can be used to screen various compounds for their potential applicability in the chelation therapy of neurodegenerative diseases and to further examine their *in vivo* effects on experimental models.

The presented examples are aimed at illustrating the principles of the application of EPR spin-trapping in antioxidant research and were focused on the biologically most important free radicals ($O_2^{\bullet-}$ and $^{\bullet}$OH). However, it should be stressed that antioxidative activity against various other radicals can be determined using the corresponding generating system and EPR spin-trapping. In an EPR spin-trapping study of the antioxidative activity of chitosan gallate, Pasanphan and co-workers[128] used UV irradiation of AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) as the generating system of carbon-centred radicals. Schafer *et al.*[131] applied the Photofrin/light/Fe^{2+} system to provoke the generation of lipid radicals in HL-60 cells, and the antioxidative effects of β-carotene, vitamin E and NO against lipid radicals were evaluated using the EPR spin-trapping method. Finally, a recent paper of Šentjurc *et al.*[134] on the antioxidative capacity of leaf extract of the evergreen plant, *Sempervivum tectorum*, superbly illustrates the outstanding possibilities of EPR spin-trapping in antioxidant research. UV irradiation of the liposomal system was used to generate $^{\bullet}$OH, $O_2^{\bullet-}$, and carbon-centred radicals simultaneously, simulating a real biological setup. Using EPR with the spin-trap DEPMPO and spectral simulations, the authors were able to identify specific radicals, quantify their production and determine the antioxidative activities of the extract against each of these three radicals (Fig. 9).

3.3. Applications of EPR spin-probing in antioxidant research

Nitroxyl radicals (or nitroxides) are *N*,*N*-disubstituted >$^{\bullet}$NO radicals that are widely used as spin-probes (or spin labels) in various systems, primarily because of the relatively high chemical stability of the nitroxide moiety (up to 30 min *in vivo*) which enables their detection not only by EPR spectroscopy, but also by NMR spectroscopy. A variety of EPR spin-probes (over few hundreds, available at reasonably low prices) is available for studying various properties and pro-

cesses in biochemical and biological systems. The applications of spin-probes go beyond redox research, since their EPR spectra can depict their mobility and different characteristics of their environment (viscosity, pH, pO_2, temperature, *etc*.).[127,147,148] The three types of rings that are most commonly used for nitroxide spin-probes: piperidine (*e.g.*, Tempone, Cat_1), pyrrolidine (*e.g.*, PCA) and doxyl (doxyl stearates, *e.g*,. 7-DS) are shown in Fig. 10.[127]

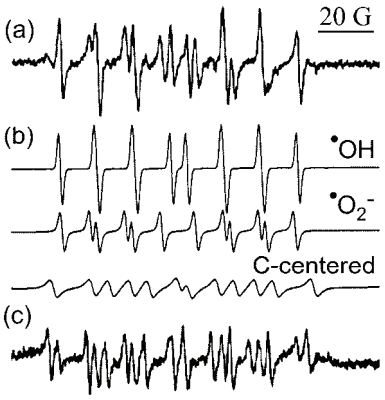

Fig. 9. EPR spectra and spectral simulations of the DEPMPO adducts obtained in liposomes after 2 h irradiation with UV light (λ = 365 nm). a) Control antioxidant-free system; b) computer simulations of the spectra of DEPMPO/OH, DEPMPO/OOH, and carbon-centred radical adduct; c) liposomes + *S. tectorum* (12.5 % v/v) after 2 h irradiation with UV light. *AA*($^{\bullet}$OH) = 0.88; *AA*($^{\bullet}O_2^-$) = 0.95; *AA*(C-centred) = = 0.18.[134]

The stability of nitroxides is primarily based on steric blocking *via* bulky groups (usually methyl) on the adjacent ring carbons, but is not absolute since they can be reduced to EPR-silent hydroxylamines in reactions with various antioxidants. The great assortment of available nitroxides, which can be more or less stabile (*e.g.*, pyrrolidines are more stabile than piperidines), hydrophilic (tempone) or lipophilic (doxyl stearates), charged (Cat_1) or neutral,[127] enable various applications of the EPR spin-probing technique in redox research. The most frequent approach is to add a spin-probe to some biological system (*ex vivo* or *in vivo*) and to follow the decrease of the pertinent signal over time, in order to evaluate the intrinsic oxidative status of the system.[54,127] Here, the application of EPR spin-probing in the measurement of the antioxidative capacity of a specific compound, extract or food will be illustrated. The basic principle is to combine a spin-probe with a potential antioxidant *in vitro* and to evaluate the total capacity of the studied compound to reduce a spin probe, which could be specifically positioned in an aqueous solution, membranes of liposomes or cells, or in the extracellular space (tasks #2 and #3). It should be noted that this approach is not very specific, since spin-probes only represent models of biological free radicals.

3.4. Evaluation of antioxidative activity with EPR spin-probing

The hydrophilic spin-probes most frequently applied in antioxidant research are tempo (2,2,6,6-tetramethylpiperidine-1-oxyl) and its derivatives tempone and Cat_1. Vilhar *et al.*[149] measured the reduction of tempo in tissues of *in vitro*

grown potato plants exposed to jasmonic acid, in order to evaluate antioxidative and metabolic effects. Kocherginsky and co-workers[150] used EPR monitored reduction of tempo and tempone to show that the "reducing power" of beer decreases with increasing temperature and period of storage. EPR and tempone were also applied in the measurements of the antioxidative activity of various plant extracts.[134,151,152] This approach was also utilised in several studies in which the antioxidative activity of chocolate,[41] wild garlic (*Allium ursinum* L.) volatile oil,[38] and the reducing power of plant plasma membranes were investigated.[153]

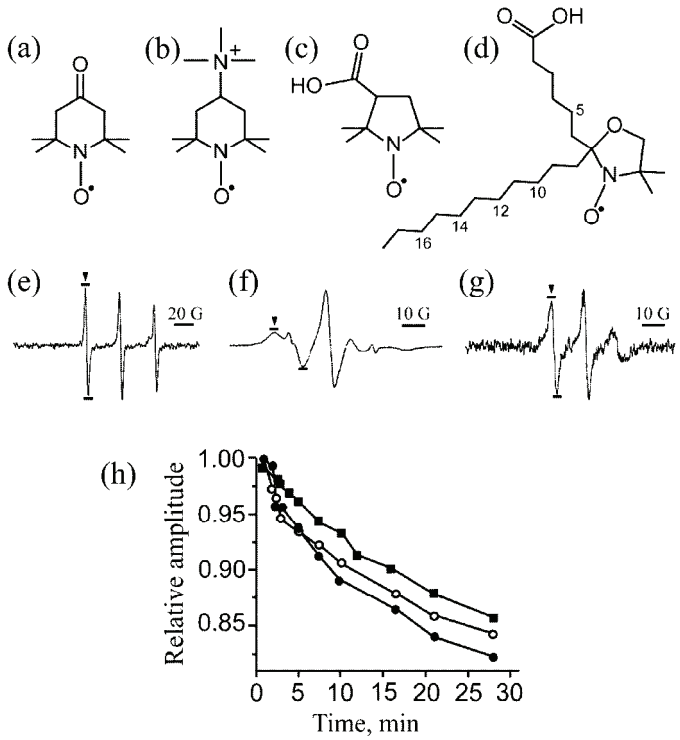

Fig. 10. Chemical structure of spin-probes: a) tempone (4-oxo-2,2,6,6-tetramethylpiperidine--1-oxyl); b) Cat_1 (4-(trimethylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl); c) PCA (3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl); d) 7-DS (2-(5-carboxypentyl)-2-undecyl--4,4-dimethyloxazolidine-3-oxyl). The numbers mark the position of the doxyl group on the fatty acid chain in other frequently used doxyl stearates – 5-DS, 10-DS, 12-DS, 14-DS, and 16-DS. Characteristic EPR spectra of tempone (e) in solution; and 7-DS (f) and 12-DS (g) intercalated into liposomes; h) relative amplitude (compared to the amplitude at the start of incubation) of the EPR signal of tempone (■), 7-DS (○) and 12-DS (●) in the presence of AUVO (4.8 mM). The downward triangles in (e–g) mark the lines the amplitudes of which were measured.[38]

The charged spin-probe Cat_1 cannot pass the membrane of cells. Hence, it can be used to study the antioxidative activity of some compounds or extracts in the extracellular medium. Hochkirch *et al.*[154] used EPR with Cat_1 to measure the antioxidative capacity of extracellular solutions in human skin biopsies exposed to UV irradiation. By evaluating the decrease in the EPR signal, they showed that UV light diminishes the activity of antioxidants in the extracellular milieu. Mehlhorn[155] developed an assay for determining the concentration of ascorbate in plasma and hemolysates, based on following the rate of EPR signal disappearance, provoked by ascorbate-mediated reduction of Cat_1.[64]

EPR with doxyl stearates is used to explore whether a compound or extract component(s) acts as an antioxidant inside cellular membranes. Doxyl stearates inserted in a membrane orient their hydrophilic carboxyl group toward the outer aqueous phase of the lipid bilayer and the fatty acid chain extends toward the core of the membrane. Since nitroxide groups could be placed at different positions on the fatty acid chain, the antioxidative activity at different depths of the membrane could be established by measuring and comparing the kinetics of reduction of specific doxyl stearates. EPR measurements of the rates of reduction of 5-, 7-, 10-, 12-, and 16-DS were applied to evaluate the antioxidative activity of ascorbate in the membrane of unilamellar liposomes, showing that ascorbate does not occupy a specific position in the membrane and that the primary site of antioxidative activity of ascorbate is in the external medium.[156] In a similar study, Schreier-Mucillo *et al.*[157] showed that ascorbate is transported through the membrane by diffusion, which explains the similar antioxidative activities at different depths of the membrane. May and co-workers[158] used 5-DS and 16-DS to study the antioxidative activity of ascorbate 6-palmitate (A6P) in the membrane of erythrocytes. A6P reduced 5-DS more efficiently than 16-DS, indicating that the ascorbyl group of A6P is located superficially, but with access to the hydrophobic membrane interior. Takahashi *et al.*[159] studied the intra-membrane antioxidative activity of tocopherols by measuring the reduction of 5-, 7-, 10-, 12-, and 16-DS. It was demonstrated that tocopherols show a higher antioxidative activity closer to the membrane surface, than deep in the lipid region of the bilayer membrane.

Measurements of the reduction of tempone and two doxyl stearates (7- and 12-DS) incorporated into liposomes were combined, to study the antioxidative properties of wild garlic (*Allium ursinum* L.) volatile oil (AUVO) (Fig. 10).[38] The ability of the oil to reduce tempone in water indicated that AUVO is capable of removing radicals in an aqueous environment of biosystems. However, the rank order of signal decay, 12-DS > 7-DS > tempone, demonstrates that the antioxidant compounds in AUVO are preferentially lipophilic, intercalating and protecting the deeper layers of the membrane. The complex kinetics of the decay of the signal of all three used spin-probes indicates that AUVO contains more

than one antioxidative compound active in both media. Such complex kinetics may be deconvoluted into components in order to evaluate the number of active components in the system, as was shown on plant plasma membranes.[153]

3.5. Evaluation of the antioxidative capacity against lipid peroxidation

The ability of antioxidants to remove lipid peroxidation can be assessed by using a specific combination of spin-probing and radical-generating systems. The microenvironment of a spin-probe has a significant impact on its EPR spectrum; thus, specific probes could be used to evaluate membrane fluidity and some other important physiological parameters.[127] Although interesting in itself, EPR spin-probing measurements of fluidity could be used in antioxidant research based on the fact that the fluidity of membranes is dependent on lipid peroxidation.[160] The basic principle of this indirect approach is to provoke lipid peroxidation by exposing membranes to free radicals generated by the Fenton reaction or some other system. The fluidity of the membrane is measured prior and after the addition of a potential antioxidant. If the antioxidant is effective against lipid peroxidation, its introduction into the system should remove lipid radicals and compensate the decrease of membrane fluidity related to peroxidation.

This approach was applied in several studies using liposomes or erythrocytes exposed to the Fenton system as a model of a cellular membrane exposed to oxidative stress, before and after the addition of plant extracts[39,122] or chocolate.[41] Doxyl stearates in a membrane environment have restricted motion which results in a broadening of their spectra, a feature that could be used to measure the order parameter *S* (Fig. 11), which is reciprocally proportional to membrane fluidity.[127] Peroxidation leads to a decrease in fluidity, so an antioxidant active inside a membrane should be able to enable normal fluidity of the membrane to be regained. For example, the *S* value of an erythrocyte membrane labelled with 7-DS was around 0.752 in normal cells and ≈0.776 for erythrocytes the membrane of

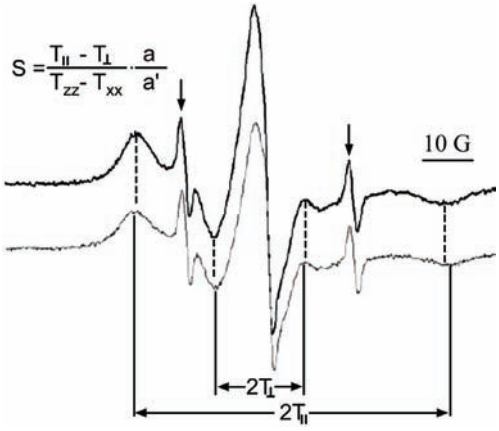

Fig. 11. EPR spectra of erythrocyte membrane labelled with 7-DS. Dark trace – untreated cells; pale trace – cells exposed to the Fenton system. *S*: order parameter. $2T_{II}$: outer hyperfine splitting. $2T_{\perp}$: inner hyperfine splitting; *a*: isotropic hyperfine coupling constant in crystal ($a = 1/3(T_{xx} + T_{yy} + T_{zz})$); *a*': isotropic hyperfine coupling constant in membrane ($a' = 1/3(T_{II} + 2T_{\perp})$). T_{xx}, T_{yy} and T_{zz}: hyperfine constants (for 7-DS, they were taken to be $T_{xx} = T_{yy} = 6.1$ G, $T_{zz} = 32.4$ G[161]). The two narrow lines originate from 7-DS in the solution (arrows).[39]

which was subjected to lipid peroxidation *via* the Fenton reaction. The subsequent application of the extracts of chestnut catkin reverted the order parameter to 0.754, showing that some lipophilic compound(s) in the extract possess the capacity to remove lipid peroxides in biomembranes.

4. CONCLUSIONS

EPR spectroscopy has played a vital role in redox research and its applications are still growing. Herein two approaches that have not hitherto received full attention were addressed. In the first section, it was demonstrated that endogenously present stabile radicals could be used for measuring the oxidative status. Although such an approach is less versatile than the application of EPR spin-traps and spin-probes, its advantage lies in the measurement of the oxidative status of biological systems without any interference with metabolic processes. Secondly, the manners in which different EPR spin-trapping and spin-probing techniques can be used to establish the efficacy of various antioxidants to remove physiologically relevant free radicals and sequestrate metal ions, and thus protect cells from oxidative damage, were presented. The intention was to encourage fellow colleagues interested in redox research to complement the methods used in their studies with some of the EPR techniques outlined in this review and to enhance knowledge further in this exciting area.

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И З В О Д

УЛОГА ЕПР СПЕКТРОСКОПИЈЕ У ИСПИТИВАЊУ ОКСИДАТИВНОГ СТАТУСА БИОЛОШКИХ СИСТЕМА И АНТИОКСИДАТИВНИХ КАРАКТЕРИСТИКА РАЗЛИЧИТИХ ЈЕДИЊЕЊА

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У ери слободних радикала и антиоксиданата, електронска парамагнетна резонанција (EPR) је вероватно најбоља техника за редокс истраживања, посебно када су у питању биохемијски и биолошки системи. У овом прегледном раду, нису покривене све могућности примене EPR-а, него је пажња ограничена на две области које су нове и нису довољно описане у литератури. У првом делу описане су различите примене EPR-а у одређивању оксидативног статуса, употребом ендогених стабилних парамагнетних врста, као што су аскорбил радикал, семихинон, меланин и оксидовани биљни пигменти. Други део се односи на примену ЕПР-а у области испитивања антиоксиданата. Осим прегледа доступне литературе, приказани су детаљно примери (већином из досадашњег рада аутора) како би се илустровали различити начини за коришћење пуних капацитета ЕПР-а у овим областима. Разлог за овакав приступ је жеља да се подстакну биолози заинтересовани за редокс метаболизам, као и

хемичари и фармаколози који се баве хемијом слободних радикала или антиоксидативним особинама нових лекова и природних производа, да укључе ову технику у своја истраживања.

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