Chapter 3

Application of Electron Paramagnetic Resonance Spectroscopy in Ophthalmology

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Additional information is available at the end of the chapter

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1. Introduction

Electron paramagnetic resonance (EPR) spectroscopy is the method of examination of free radicals and the others paramagnetic centers [1-22]. EPR measurements reveal applications in medicine [8-11, 23-34], biology [8-11], pharmacy [35-48], cosmetology [49, 50], biotechnology [51-54]. EPR method is useful to examination of tissues, cells, biopolimers, drugs, cosmetic substances, herbs, and biomaterials [8-11, 23-34]. Free radicals in such mentioned samples may be characterized by EPR spectra, and the influence of physical and chemical factors on them may be tested. The important information about light, oxygen, temperature, gamma irradiation on the biological or pharmacological objects may be obtain from analysis of their absorption signals [8-11, 23-54]. Free radical properties and concentration are determined by the use of electron paramagnetic resonance [1-8]. Applications of EPR method to determine the optimal conditions of photodynamic therapy [24-26], the best conditions of sterilization of drugs [35-48], herbs [22] and cosmetic substances [49, 50], was proposed.

1.1. Aim

The aim of this work is to present usefulness of EPR analysis in ophthalmology. EPR studies of free radicals and their interactions with tissues structures are described. Melanins, which contain o-semiquinone free radicals (S=1/2) and biradicals (S=1) exist in the eye. EPR studies of paramagnetic centers in melanin biopolymers are presented. The effect of light irradiation and temperature on free radicals in melanin biopolymer is shown. The effect of dia- and paramagnetic metal ions on free radicals in melanins is discussed. The effect of drugs on free radicals in melanins tested by EPR is presented. Different types of free radicals, their chemical and thermodynamic stability are compared. Free radicals and reactive oxygen species in ophthalmology are presented.

The effect of electron paramagnetic resonance as the absorption of electromagnetic waves by the sample located in magnetic field and the EPR spectrometer are described. The positive aspects of the EPR analysis in the technical meaning such are bring to light. EPR measurements are not destructive to the samples and only the low amount of the sample is needed. The types of EPR spectrometers and microwave frequencies are presented. EPR spectra of tissues may be multi-component and the frequency of microwaves influences the resolution of detection of EPR lines of different types of free radicals. The parameters of the EPR spectra: amplitudes, integral intensities, linewidths, g-factors, and both physical and practical meaning of them are shown. Amplitudes and integral intensities increase with increasing of free radical concentration in the sample [1-8]. Linewidth depends on molecular structure of the samples and magnetic interactions in the chemical units [1-6]. g-Values let us to determine the type of free radicals [1-3]. Free radical concentration determination and the references for these measurements are shown. The concentration is proportional to the area under the absorption lines [1-8]. The spin probes in EPR investigation to ophthalmology are presented. The professional spectroscopic programs are characterized.

Sample preparation to EPR measurements are presented. The measurements in the wide range of temperatures and microwave powers relative to their usefulness in ophthalmologic samples are discussed. The methods of differentiation of free radicals and biradicals in melanin biopolimers are presented.

2. Paramagnetic centers

2.1. Types of paramagnetic centers

Paramagnetic centers are the molecular units with unpaired electrons and they have characteristic behavior in magnetic field applied in EPR spectroscopy [1-8]. Paramagnetic centers are formed during photolysis, thermolysis, radiolysis, electrolysis, and the others chemical reactions [11]. Oxygen is very active in generation of paramagnetic centers [1, 11, 55]. The most known paramagnetic centers are free radicals, biradicals, paramagnetic metal ions, oxygen O_2 molecules in triplet ground state and paramagnetic conducting species [l, 5, 11]. Paramagnetic centers differ in spins and in stability. Free radicals have spin with the value of 1/2, biradicals and O_2 spins are equal of 1, paramagnetic ions mainly reveal spin of 1/2, delocalized electrons in conducting materials have spins of 1/2. Magnetic moments of paramagnetic centers result from their spins, and they are responsible for the orientations in magnetic field during their EPR detection.

Paramagnetic centers differ in lifetime [8, 11]. Stability of paramagnetic centers is connected with their chemical building and the external conditions in the environment. There is known stabile organic free radicals and labile reactive oxygen species. Samples in vacuum have usually free radicals for the longer times than the samples in air, where oxygen molecules O_2 in paramagnetic triplet states with spin S=1 exist [1, 11]. The reactions with oxygen is stronger in the higher temperatures [11]. The intensive reactions between paramagnetic centers become in the structures with the large amount of unpaired electrons.

2.2. Stable free radicals

Stabile free radicals with different localization of unpaired electrons exist in organic molecular units [10, 11, 55]. Thermodynamic stability of free radicals depend on their chemical structure [55]. The lifetime is longer for free radicals in aromatic units than in aliphatic units. The major types of free radicals exist in cells and tissues, because of differentiation on their building and composition. o-Semiquinone free radicals are the aromatic free radicals [55]. There is known the chain the aliphatic free radicals [55]. The exemplary popular free radicals in living organism are peroxyl radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$).

2.3. Reactive oxygen species

The group of reactive oxygen species consists of both paramagnetic free radicals and diamagnetic non-radical compounds [11, 55]. The reactive oxygen species are not stabile, they have the short lifetimes often lower than one second, and they easy react with others molecules. The exemplary paramagnetic reactive oxygen species are hydroxyl radical ($^{\bullet}OH$), hydroperoxyl radical ($HO_2^{\bullet}$), superoxide radical anion ($O_2^{\bullet}$), and nitric dioxide ($NO_2^{\bullet}$) [11]. Reactive oxygen species appear in inflammatory states in human organism [10, 11], irradiated tissues, in thermally [35-43] and gamma [44-48] sterilized drugs.

2.4. Paramagnetic centers in eye

Paramagnetic centers in eye exist mainly in melanin biopolymer [56]. Melanin is a natural pigment that is found in most organisms. In humans, melanin is found in skin, eyes, hair, leucocytes, *Substantia nigra, Locus coerules* and inner ear [31, 32, 57-60]. Through a process called melanogenesis, melanin is produced by melanocytes [32, 58].

There are three basic types of melanin: eumelanin, which is a brown and black, pheomelanin, which is red or yellow, and neuromelanin, which is present in the brain [33, 59].

Chemically, melanins are amorphous biopolymers consisting of various monomer units i.a. 5, 6-dihydroxy indole-2-carboxylic acid and 5, 6-dihydroxy indole [59, 60]. Chemical structures of eu-and pheomelanins are compared in Figure 1 [59]. Eumelanin contains carbon (C), oxygen (O), and nitrogen (N) atoms. Besides C, O, and N, sulphur (S) exists in pheomelanin [59, 60].

Melanins are found in the eye in higher concentrations than anywhere else in the human body [61]. In the eye, melanin content in the iris, ciliary body, choroid and retinal pigment epithelium (RPE) [60]. Cornea and lens don't have the pigment [60, 62].

Content of melanin differs in various ocular tissues [60, 62]. In humans, scleral melanin levels are higher than retina and central choroid-RPE. Besides, it is also different distribution of melanin in human eyes. The peripheral tissue pigment levels are generally higher compared to the central regions [60]. The melanin content of human RPE decreases with age [56, 62].

Differences in iris color are caused by three factors: the concentration of pigment within stromal melanocytes, light scattering and absorptive properties of extracellular components, and the pigment granules in the iris pigment epithelium (IPE) (located on the back of the iris) [63].

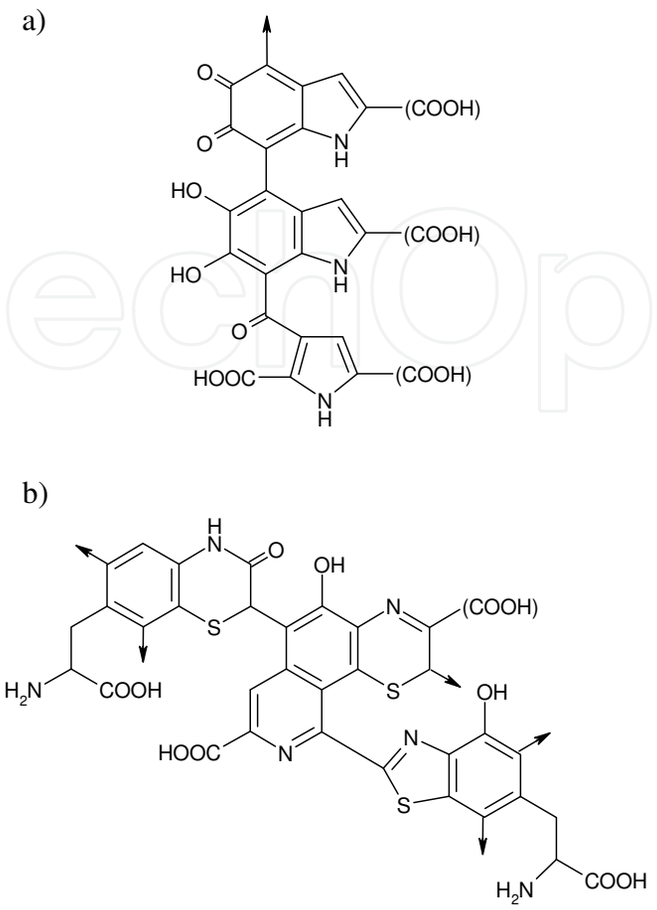

Figure 1. Chemical structure of eumelanin (a) and pheomelanin (b) [59].

Melanin from the IPE is essentially eumelanin, while melanin from IPE-scraped iris (consisting mainly of stroma plus anterior IPE) exhibiting content of both eumelanin and pheomelanin [63].

It is shown that the green iris appear to be more pheomelanic, whereas blue-green iris appear to be more eumelanic [63]. Blue eyes contain little of either pigment, while green-brown and brown irides feature a mixed pigment content [63].

Many drugs and metal ions bind to melanin [32, 52, 64-68]. The binding of drug to melanin is the result of physicochemical properties of the pigment [61]. Melanin protects the pigmented tissues through the absorption of many drugs and chemicals. On the other hand, this may lead to toxic accumulation of these substances in melanin, and consequently to the degradation of pigmented tissues [69, 70]. Differences in melanin content in ocular tissues might contribute to differences in drug binding and toxicity [60].

Melanin in the eyes helps protect them from ultraviolet and high-frequency visible light [34, 61, 70]. Besides, ocular melanin may also play a protective role against free radicals [61].

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The main paramagnetic centers in melanin are the o-semiquinone free radicals with spin of 1/2 and with unpaired electrons localized on oxygen atoms [64-68]. Additionally it was spectroscopically proved that biradicals with spin of 1 also exist in melanins [71, 72]. o-Semiquinone free radicals and biradicals were also found in melanin complexes with copper(II) ions and drugs, as kanamycin [71] and netilmicin [72]. So far in eye melanin only o-semiquinone free radicals were studied [56].

3. The effect of electron paramagnetic resonance – Basic theory

Electron paramagnetic resonance (EPR) is the effect which appears in the paramagnetic samples exposed to microwaves in magnetic field [1-3]. Magnetic field causes Zeeman splitting of energy levels. After splitting the energy levels of unpaired electrons in magnetic field relate to different orientation of their magnetic moments, i.e. parallel and non parallel to the magnetic induction vector B. Both the magnetic moments in magnetic field and the magnetic spin quantum number M_S have 2S+1 values [l, 5, 8]. The energy level of unpaired electron with the magnetic spin quantum number M_S in magnetic field is splitted into 2S+1 levels. The unpaired electron may be excited by microwaves and it comes to the higher energy levels, when the frequency and the energy of microwaves are equal as is given in the resonance condition formula [1]:

$$h\nu = E_2 - E_1 = g\mu_B B_r \tag{1}$$

where h – Planck constant, ν – microwave frequency, g – spectroscopic factor, μ_B – Bohr magneton, B_r – resonance induction of magnetic field, E_2 – energy of the excited level of unpaired electron, E_1 – energy of the ground level of unpaired electron.

The energy E of unpaired electron with the magnetic spin number M_S in magnetic field with induction **B** is given as [1, 4]:

$$E(M_S) = M_S g\mu_B B_r \tag{2}$$

The electron paramagnetic resonance (EPR) effect is also called electron spin resonance (ESR), because unpaired electrons in the paramagnetic samples have unpaired spins [1-8]. The described above effect is the basis of EPR (ESR) spectroscopy, which is the experimental method of examination of paramagnetic species. Paramagnetic samples are located in magnetic field and they absorb microwaves with energies fulfill the resonance conditions (1) [1-8]. EPR spectroscopy use to analysis the absorption and the first derivative lines. The first derivative curves are very important for the samples with complex paramagnetic center system, when the several types of paramagnetic species exist [1]. The resolution of the multicomponent EPR spectra to the component lines is easier for the first derivatives than for the absorption curves.

For paramagnetic samples with free radicals, when the spin is S=1/2 the Zeeman splitting of the individual energy level in magnetic field causes the appearance of two levels [1, 3, 5]. The Zeeman splitting for free radicals in magnetic field is shown in Figure 2 [3]. Free radicals mainly exist in eye, so this example is the most important for ophthalmology. In the Figure 2 the energy levels of unpaired electrons of free radicals outside and in magnetic field are presented, and the absorption and the first derivative lines are shown.

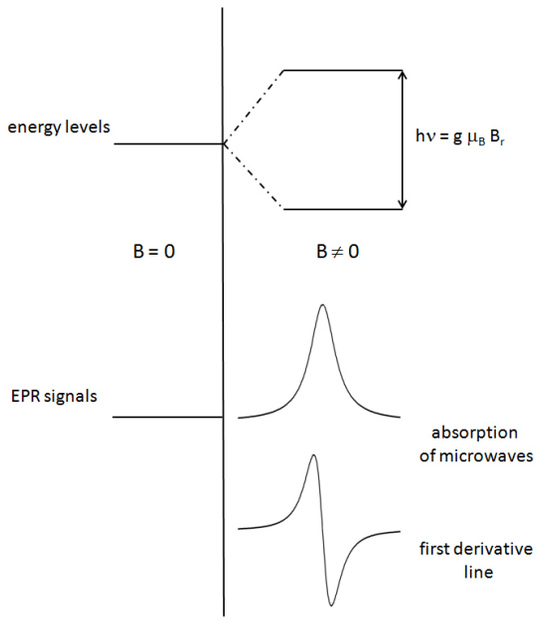

Figure 2. The Zeeman effect in magnetic field for free radicals with spin S=1/2. The absorption and the first absorption EPR curves are presented. B – induction of magnetic field, B_r – magnetic resonance induction, h – Planck constant, ν – microwave frequency, g – spectroscopic factor, μ_B – Bohr magneton, the resonance formula: $h\nu=g\mu_B B_r$. The scheme was prepared by the use of work [3].

4. EPR spectrometers useful in ophthalmology

4.1. The electronic blocks of the continuous microwave (CW) spectrometer

Electron paramagnetic spectrometer with continuous microwaves (CW-EPR) is the most useful apparatus for ophthalmology. For such type of the spectrometer samples are located in magnetic field and the microwaves are continuously send to the tested object [1, 3]. Unpaired electrons of the paramagnetic sample is continuously excited by microwaves and the spin-spin and spin-lattice relaxation processes occur [l, 3, 5, 8]. This spectrometer consists of microwave bridge, waveguides, resonance cavity, electromagnet, the modulation block, detector, and the amplifier. The source of microwaves and attenuator exist in microwave bridge. The attenuator is applied to change microwave power in the experiment. The popular source of microwaves is klystron. The microwave changes with attenuation according to the formula [4]:

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$$attenuation\left[dB\right] = 10lg\left(M_o / M\right) \tag{3}$$

where M_o – the total microwave power produced by klystron, M – microwave power used during the measurement of EPR spectra.

The EPR spectra should be detect with low microwave power without the saturation effect to obtain the proper amounts of paramagnetic centers in the tested samples [1, 3]. The changes of microwave power and detection of EPR lines is used to characterize magnetic interactions in the samples.

Electromagnetic waves are send from microwave bridge via attenuator by waveguides to the resonance cavity [1-8]. The paramagnetic sample is located in the resonance cavity in the magnetic field produced by electromagnet. The absorption of the microwaves took place in the resonance cavity. Modulation of magnetic field is done by modulator and the signal is measured by detector. The receiver gain is used during the detection. Numerical detection of the EPR lines is done. Magnetic field is measured by NMR detector. Microwave frequency is usually measured, but sometimes the references are used. The measurement of the microwave frequency is necessary to accurately determine g-factor, which is important to study the type of paramagnetic centers in the samples.

The classic CW-EPR spectrometer of Bruker BioSpin GmbH is shown in Figure 3. The exemplary resonance cavity of Bruker BioSpin GmbH is presented in Figure 4. Electromagnet – the source of magnetic field is presented in Figure 5.

Figure 3. Continuous microwave EPR spectrometer EMXplus produced by Bruker BioSpin GmbH.

Figure 4. The resonance cavity of CW-EPR spectrometer of Bruker BioSpin GmbH.

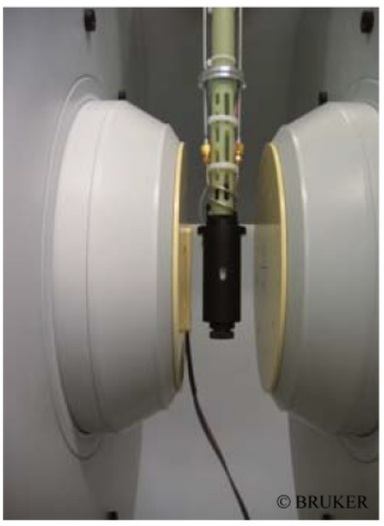

Figure 5. Electromagnet and the resonance cavity of CW-EPR spectrometer produced by Bruker BioSpin GmbH.

The pulsed EPR spectrometers are also used in medicine [1, 8]. These types of spectrometers are useful in examination of kinetics of processes. Microwaves are sent to the paramagnetic sample between poles of electromagnet and the decrease of the absorbed signal is detected. The pulsed EPR spectrometers are applied to test magnetic interactions in the samples [1]. Time of spin-lattice relaxation processes may be obtained by the pulsed method [1, 3, 5, 8]. The different spin-lattice relaxation times of unpaired electrons of major paramagnetic centers brings to light the component signals of them. It is used to determine the number of different types of paramagnetic centers in the samples. Determination of the number of component lines in the CW-EPR spectra is performed by fitting the resultant spectra by superposition of Gauss and Lorentz lines [1, 4].

4.2. Types of microwave bands in EPR spectrometers

Different microwave frequencies are used in the EPR spectrometers [1, 2]. The most popular is the frequency about 9.3 GHz (X-band). The typical microwave bands and corresponding frequencies of electromagnetic waves are presented in Table 1 [1, 2].

Microwave band	Microwave frequency [GHz]
L	1.5
S	3.0
C	6.0
X	**9.3**
K	23
Q	36
V	50
W	95

Table 1. Microwave bands used in EPR spectroscopy [1, 2].

5. EPR spectra

5.1. The parameters of EPR spectra and their practical meaning in studies of eye free radicals

The basic parameters of the first derivative EPR spectra give important information about type of paramagnetic centers in the sample, their amounts, and magnetic interactions which reflect chemical structure of the tested object [1-8]. The following parameters of EPR spectra: amplitudes (A), integral intensities (I), linewidths (ΔB_{pp}), and g-factors are usually analyzed. The parameters for the model eumelanin – DOPA-melanin (from SIGMA-ALDRICH) are shown in Figure 6. The first derivative EPR spectrum of DOPA-melanin is presented, because of the eumelanin mainly exists in eye.

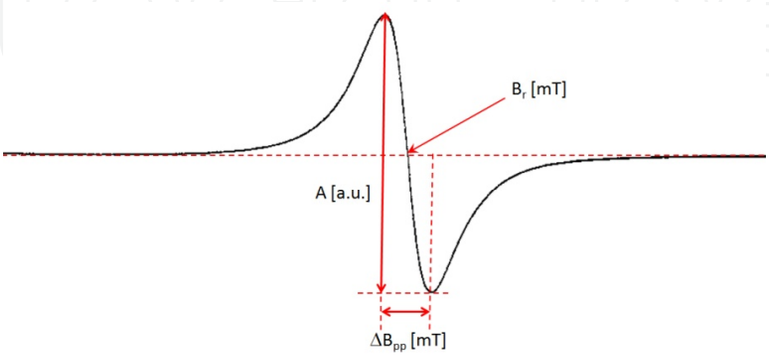

Figure 6. The first derivative EPR spectrum of the model eumelanin – DOPA-melanin and the basic parameters: amplitude (A), linewidth (ΔB_{pp}), and the resonance magnetic induction (B_r).

Amplitudes (A) and integral intensities (I) increase with the increasing of free radical concentration in the samples [1, 8]. The comparison of amplitudes (A) of EPR spectra of different samples from eye give information about the relative contents of free radicals in them. But the free radical concentration in the individual biological sample is determined by integral intensity (I), and it is proportional to the value of this parameter (I). Integral intensity (I) of the EPR spectra is the area under the absorption line, so for the first derivative EPR curve double integrations should be done. Linewidth (ΔB_{pp}) increases for the stronger dipolar interactions of free radicals in the samples [1, 8]. Dipolar interactions increases for decrease distances between free radicals [1, 8].

g-Values are calculated from the resonance condition according to the formula [1]:

$$g = h\nu / \mu_B B_r \tag{4}$$

where h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – induction of resonance magnetic field.

Determination of g-value is possible for known microwave frequency (ν) and resonance induction (B_r). Microwave frequency is detected by the recorder, and resonance induction is obtained from the EPR spectrum (Figure 6). g-Values characterize type of free radicals existing in the samples [1, 5]. The individual free radicals have EPR lines in the correspond to their chemical structures magnetic field. The resonance magnetic induction effect on the g-factor of free radicals (formula 4). g-Values are used in EPR spectroscopy to identification of the species which causes paramagnetic character of the sample.

5.2. EPR spectra of complex biological samples

Free radical system in biological samples, for example for species obtained from eye, is usually complex. In cells or tissues several groups of free radicals may exist [8]. The EPR spectra are then multi-component as the superposition of the lines of all the groups of free radicals. The information about the each group of free radicals is obtain by numerical fitting of the shape of the resultant EPR spectrum of the sample by sum of theoretical lines. The shape of the component lines is gaussian or lorentzian [1-4]. The percentage fraction of the individual component lines in the total EPR spectrum means the percentage fraction of their concentration in the sample [1]. Such numerical fitting were done for example for model neuromelanins [29, 30].

The analysis of shape of the EPR spectra and determination of their parameters are performed by spectroscopic programs. The known modern program to spectral analysis is WINEPR of Bruker, ORIGIN 6.0 of Microcal Software, LabVIEW 8.5 by National Instruments (Austin, Texas) or programs of JAGMAR (Kraków, Poland) and EPRAD (Poznań, Poland).

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6. Application of EPR spectroscopy in ophthalmology

6.1. Sample preparation to the examination

EPR spectra can be measure for solid state, liquid and gaseous samples [1, 4, 8]. Solid state samples, for example melanin from eye, are examined in glass or quartz tubes with the diameters higher than for liquid samples. The mass of the solid samples located in the tubes should be determined to obtain the content of free radicals in one gram of the probe. The melanin in the glass tubes for spectroscopic studies is shown in Figure 7. The external diameter of the thin walled tube is 3 mm. The materials used in tubes should not reveal EPR signals for the measurement parameters used in the experiment. The EPR spectrum of DOPA-melanin is presented in Figure 8.

Figure 7. The model eumelanin – DOPA-melanin in the glass tube with diameter of 3 mm for EPR measurements.

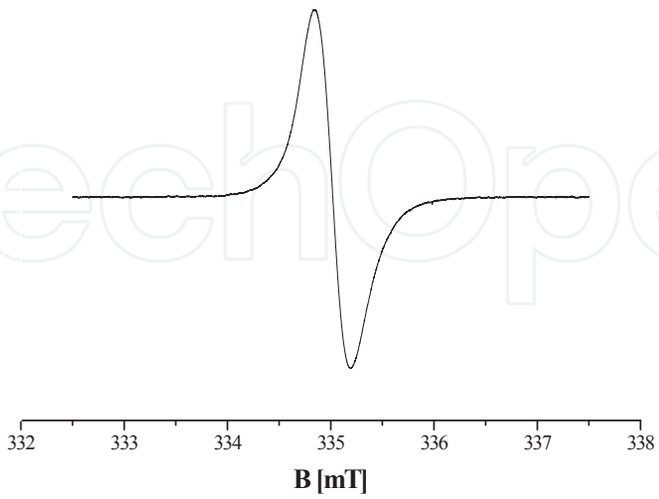

Figure 8. The EPR spectrum of model eumelanin – DOPA-melanin recorded at room temperature with low microwave power of 2.2 mW. B is the induction of magnetic field.

Liquid samples should be measure in the thin glass or quartz tubes with diameter below 1 mm. The special flat cells are also used. Water in the sample quenches EPR signals, so the large dimensions of wet samples are practically not used.

Paramagnetic gases in the environment usually decrease EPR lines of the paramagnetic samples. Such effects were observed for example for melanins [73, 74]. The samples susceptible to oxygen may be evacuated before the EPR measurements.

6.2. Determination of free radical concentration in the samples in ophthalmology

6.2.1. The measurement of the concentration

Free radicals concentrations in the samples are determined by the use of integral intensities of their EPR spectra and the spectra of the reference with the known amounts of paramagnetic centers [1, 3, 8]. Free radical concentrations (N) in the samples are determined as the value which is proportional to the area under the absorption curves and the integral intensity (I) [1, 3, 8]. Integral intensities (I) of the absorption line is obtained by integration of this curve. Double integration of the first-derivative EPR spectra give us the value of integral intensity (I).

To obtain free radical concentration the EPR lines of the tested samples and the references are measured. In our EPR studies of melanin polymers two references were used: ultramarine and the ruby crystal. Amplitudes of the EPR lines of the ruby crystal (A) located with the analyzed sample and ultramarine (A_u) in the resonance cavity were determined. The integral intensities (I) of the EPR spectra of the tested melanin samples and the reference-ultramarine (I_u) were compared.

The concentration of the free radicals (N) in the melanins was calculated as [3]:

$$N = N_u\left[\left(W_u A_u\right)/I_u\right]\left[I/\left(WAm\right)\right] \tag{5}$$

where N_u – the number of paramagnetic center in the ultramarine reference; W, W_u – the receiver gains for sample and the ultramarine; A, A_u – the amplitudes of ruby signal for the sample and the ultramarine; I, I_u – the integral intensities for the sample and ultramarine, m – the mass of the sample.

6.2.2. The paramagnetic references and their properties

The paramagnetic references should contain high amounts of stabile paramagnetic centers [1-8]. In our studies of melanin biopolymer from eye [56], others melanins [64-68, 71-78], cells [24-26, 68], drugs [35-48], ultramarine was used as the reference. In Figure 9 ultramarine in the glass tube is shown. The broad EPR line of ultramarine with paramagnetic centers with unpaired electrons located on sulfur atoms is presented in Figure 10.

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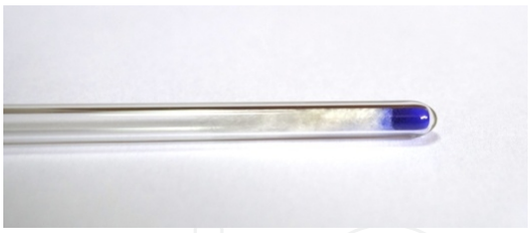

Figure 9. Ultramarine in the glass tube to EPR measurements.

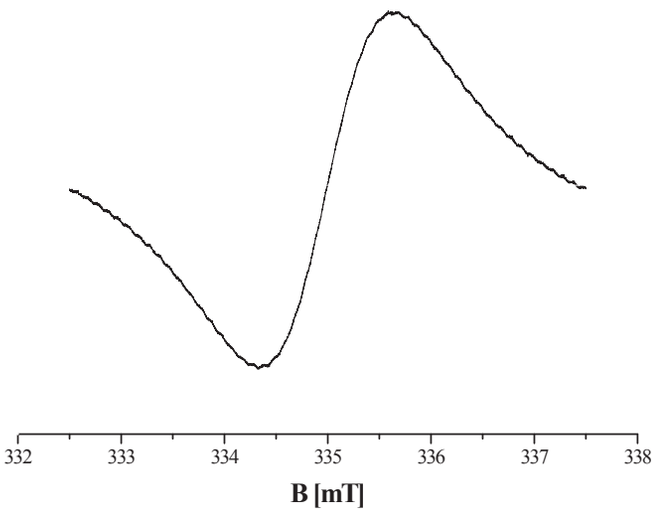

Figure 10. EPR spectrum of ultramarine – the reference for free radical concentration recorded with low microwave power of 2.2 mW. B is the induction of magnetic field.

The second reference – a ruby crystal (Al_2O_3: Cr^3) was permanently placed in a resonance cavity. For tested sample and for ultramarine the EPR line of a ruby crystal are measured. The ruby crystal is shown in Figure 11.

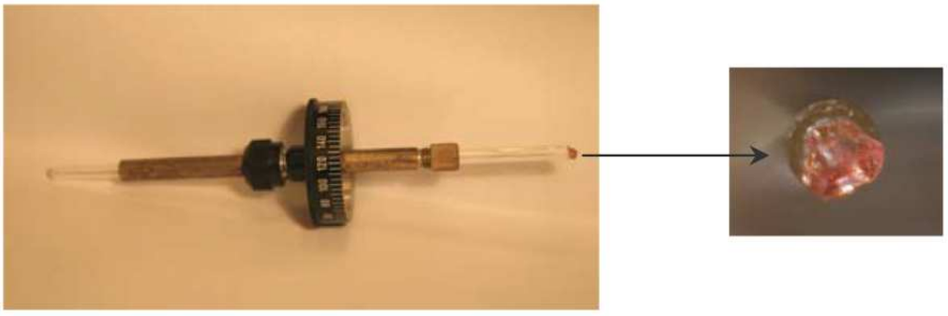

Figure 11. A ruby crystal.

The other reference for free radical concentration is DPPH (2, 2-diphenyl-1-picrylhydrazyl) [4]. Unpaired electron in DPPH is localized on nitrogen atom. DPPH is not so stabile such as ultramarine, because it is susceptible for oxygen. During storage of DPPH in air its free radical concentration decreases, so it should be evacuated.

6.3. Magnetic interactions in ophthalmological samples

Information about magnetic interactions between unpaired electrons in paramagnetic samples gives linewidth and changes of the spectra with microwave power [1-3]. The influence of microwave power (M) on the EPR spectra of the melanin samples from eye [56] and the others melanins [64-68] were examined. The changes of amplitudes (A) and linewidths (ΔB_{pp}) of EPR spectra with increasing of microwave power were analysed to determine type of broadening of EPR lines. The influence of microwave power (M) on amplitudes (A) and linewidths (ΔB_{pp}) of the EPR spectra depend on free radicals distribution (homogeneous or inhomogeneous) in the samples. For homogeneous broadened EPR lines the amplitude (A) increases with increasing of microwave power (M) and for the higher microwave powers it value decreases [1]. The increase of linewidth (ΔB_{pp}) with increasing of microwave power (M) is characteristic for the homogeneously broadened EPR lines [1]. For inhomogeneous broadening of spectral lines the amplitude (A) increases with increasing of microwave power (M) and for the higher microwave powers it value does not change [1]. Linewidth (ΔB_{pp}) of the inhomogeneously broadened EPR lines is unchanged with increasing of microwave power (M) [1].

Spin-lattice interactions in the samples may be characterized by observation of changes of amplitudes (A) of EPR lines with increasing of microwave power [1-3]. The slow and fast spin-lattice relaxation processes in the samples differ in microwave saturation of EPR lines [1-3]. The higher power of microwave saturation of EPR lines reveal the samples with the fast spin-lattice relaxation processes than the samples with the slow spin-lattice relaxation processes [1-4].

6.4. EPR investigation of melanin biopolymer in eye

6.4.1. Free radicals and biradicals in melanin biopolymer

The important paramagnetic structures existing in eye are melanin biopolymers [56, 60-63]. EPR examination of melanins from low temperature of liquid nitrogen to room temperature proved that two types of paramagnetic centers are located in them [71, 72]. The characteristic for both free radicals with spin S=1/2 and biradicals with spin S=1 correlations between integral intensities (I) of EPR lines and the measuring temperature (T) were observed. IT value for free radicals was constant independent on temperature, and the IT values for biradicals depended on the measuring temperature.

Free radicals and biradicals play an important role during binding drugs to melanins [71, 72]. The amounts of these paramagnetic centers changes after formation of complex melanin-drug. Such effects were observed for example for kanamycin [71] and netilmicin [72]. Paramagnetic copper(II) ions influence on free radicals and biradicals in melanin complexes with kanamycin

and netlimicin was observed. It is expected that drugs applied in ophthalmology change free radicals and biradicals concentrations in melanin biopolymers in eye. So electron paramagnetic resonance spectroscopy seems to be very useful in examination of interactions of drugs with melanin in eye.

6.4.2. EPR results for melanin in eye

Electron paramagnetic resonance spectroscopy was used to examine free radicals in RPE melanosomes from different aged donors [56]. o-Semiquinone free radicals were identified in RPE melanosomes with the characteristic g-values and single broad EPR lines. Concentrations of free radicals in RPE melanosomes depend on the age of donors. The higher free radicals concentrations were obtained for donors aged > 45 years, than for donors aged < 22 years [56]. Free radicals concentrations in RPE melanosomes (~10^{17} spin/g) [56] were lower than the concentrations in model eumelanin – DOPA-melanin [56], A-375 melanoma cells [68], *Cladosporium cladosporioides* mycelium [52], and *Cladosporium herbarum* mycelium [53]. Free radical concentration in melanin changed after irradiation of eumelanin by visible light [34].

EPR spectra of RPE melanosomes were similar to those observed for DOPA-melanin (Figure 8), which is the synthetic model eumelanin. The unresolved hyperfine structure characteristic for pheomelanins was not observed for melanin of RPE. The exemplary complex shape of EPR spectra with signals of pheomelanin is shown in Figure 12 [52].

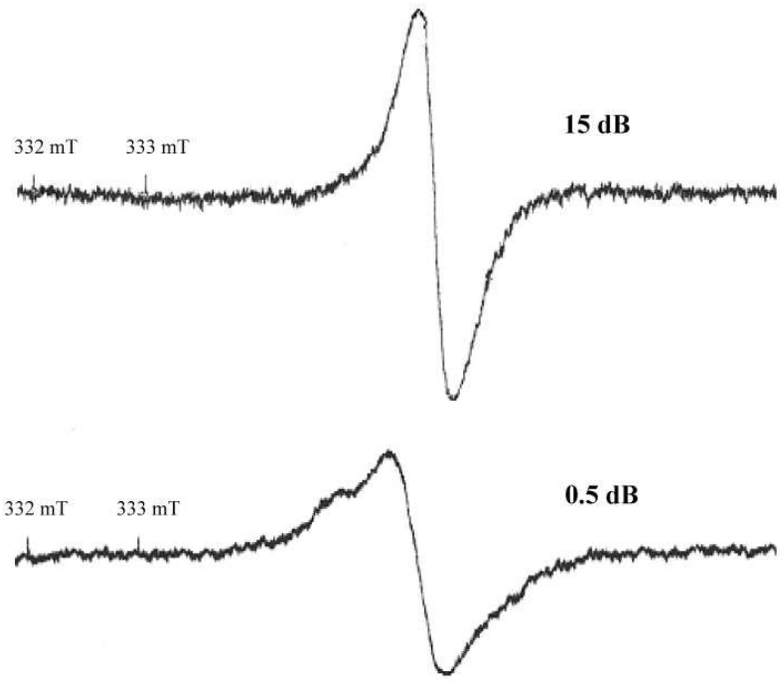

Figure 12. EPR spectra with signals of pheomelanin in *Cladosporium cladosporioides* recorded with different microwaves. The melanin samples were studied in paper [52].

Melanin in *Cladosporium cladosporioides* mycelium is the mixture of eu-and pheomelanin [52, 75-77]. The signal of pheomelanin is clearly visible in the EPR spectrum recorded with 0.5 dB attenuation, while it was not observed in the EPR spectrum measured with attenuation of 15 dB (Figure 12). It is proposed that the lower attenuation and higher microwave powers should be used to search pheomelanin in the biological samples.

6.4.3. EPR method proposed to examine free radicals in drugs in ophthalmology

EPR may be applied to examine of free radicals formed in ophthalmological drugs in process of their sterilization. Drugs used in ophthalmology should not contain microorganisms, and the sterilization is performed to remove or killed them [35-48]. Sterilization methods are gamma irradiation or thermal treatment of drugs [35-48]. Radiative and thermal sterilization should not produce free radicals in drugs, because changes of their interactions on eye as the result of modification of their chemical structure. Free radicals in sterilized optalmological drugs may be responsible for toxic effects during therapy.

Electron paramagnetic resonance spectroscopy was used to optimize sterilization procedure and conditions of antibiotics and the other drugs [35-48]. The methods of sterilization and temperatures for which the low amounts of free radicals are produced in drugs were searched. Similar examination of drugs may be proposed in ophthalmology.

6.4.4. EPR studies of antioxidant properties of drugs in ophthalmology

Electron paramagnetic resonance spectroscopy may be used in ophthalmology to examine antioxidant properties of drugs. The interactions of drugs with free radicals are tested with DPPH as the reference [79-81]. Chemical structure of DPPH is shown in Figure 13 [4]. DPPH is the model source of free radicals in this study. The EPR spectrum of DPPH is presented in Figure 14.

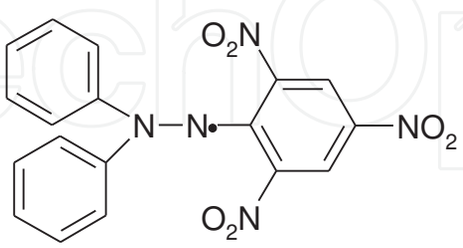

Figure 13. Chemical structure of DPPH [4].

The antioxidant properties of drugs reflects the decrease of amplitudes of the EPR line of DPPH after adding the tested samples to the solution [79-81]. The changes of integral intensities are also observed.

http://dx.doi.org/10.5772/58313

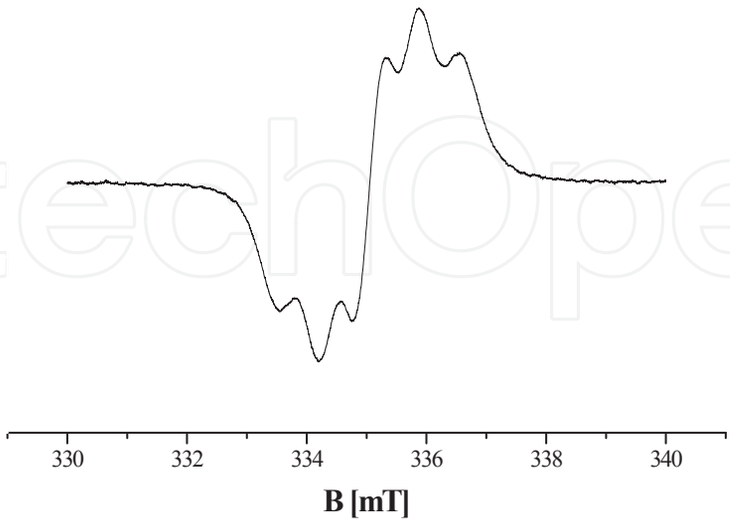

Figure 14. EPR spectrum of DPPH recorded with low microwave power of 2.2 mW. B is the induction of magnetic field.

7. Conclusions – Advantages of EPR measurements in ophthalmology

Electron paramagnetic resonance spectroscopy is the useful method to examine free radicals in eye, drugs and their interactions with free radicals (Table 2). Microbiological tests may be accompanied by EPR analysis to obtain the best conditions of sterilization process. Antioxidant properties of drugs may be determined by EPR measurements.

EPR IN OPHTHALMOLOGY	
Application	**Characteristics**
determination of free radical properties and concentrations in eye structures	type and chemical structure of free radicals, localization of unpaired electrons in free radicals, light and temperature effect on free radicals, oxygen molecules effect on free radicals, para- and diamagnetic metal ions effect on free radicals, changes in free radicals of eye melanin biopolymers after drug binding, spin-spin and spin-lattice interactions depend on chemical structures in eye
examination of biradicals in melanin biopolymers in eye	chemical structure and amounts of biradicals, effect of drugs and physical conditions on biradicals
studies of free radical interactions in eye	kinetics and products of free radicals

EPR IN OPHTHALMOLOGY	
Application	**Characteristics**
determination of antioxidants influence on free radicals in eye	decrease of the amount of free radicals in eye structures after interactions with antioxidant drugs and substances
use of spin-labels to examine biochemical units in eye	numerical analysis of shape of EPR lines of spin-labels to obtain information about chemical units in eyes
determination of antioxidant and free radical properties of ophthalmological drugs	analysis of quenching of free radicals by individual drugs, and study free radicals contents in pharmacological substances
optimization of thermal and radiative sterilization processes of ophthalmological drugs	searching temperatures or doses of irradiation, which do not produce high amounts of free radicals in drugs during heating or gamma irradiation
optimization of storage conditions of ophthalmological drugs	searching effects of light, temperature, and oxygen on free radical formation in drugs; the best conditions of drugs storage do not produce high amounts of free radicals in the samples

Table 2. Application of electron paramagnetic resonance (EPR) spectroscopy in ophthalmology [1-4, 11, 24-48, 52-56, 64-68, 70-81].

The most important advantages of EPR analysis in ophthalmology are the low amount of the samples necessary to test, the non destructive type of this analysis, the major information about free radicals. EPR spectra bring to light type and concentration of free radicals in eye, melanin biopolymer, and drugs.

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