Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/jab

Original Research Article

Free radicals in ultraviolet irradiated melanins and melanin complexes with Cd(II) and Cu(II) – EPR examination

Magdalena Zdybel [a,*], *Ewa Chodurek* [b], *Barbara Pilawa* [a]

[a] *School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Katowice, Poland, Chair and Department of Biophysics, Jedności 8, 41-200 Sosnowiec, Poland*

[b] *School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Katowice, Poland, Chair and Department of Biopharmacy, Jedności 8, 41-200 Sosnowiec, Poland*

ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form
12 January 2015
Accepted 13 January 2015
Available online 27 January 2015

Keywords:
Ultraviolet irradiation
Eumelanin
Pheomelanin
Free radicals
EPR spectroscopy

ABSTRACT

Effect of UV irradiation on free radicals in different types of melanins and melanin complexes with diamagnetic Cd(II) and paramagnetic Cu(II) was examined by the use of electron paramagnetic resonance (EPR) spectroscopy. The aim of this studies was to compare o-semiquinone free radicals formation in two model eumelanins synthesized from 3,4-dihydroxyphenylalanine (DOPA) and tyrosine in the presence of tyrosinase, and in synthetic pheomelanin, under exposition on ultraviolet, because of the important role of free radicals and melanins in human organism. UV may change free radical concentrations in melanin. Changes in EPR spectra of DOPA-melanin-Cd(II) and DOPA-melanin-Cu(II) complexes after UV irradiation were determined. Diamagnetic Cd(II) strongly increased free radical concentrations in DOPA-melanin. UV irradiation during 30 and 60 min slightly increased and decreased free radical concentrations in DOPA-melanin-Cd(II) complexes, respectively. Paramagnetic Cu(II) quenched free radical lines of DOPA-melanin, and only the Cu(II) signals were detected for both UV-irradiated and nonirradiated samples. Free radical concentration in both eumelanins increased after UV irradiation, but it decreased in irradiated pheomelanin. EPR spectra of free radicals in the studied samples were homogeneously broadened. Slow spin-lattice relaxation processes exist in all the examined melanins and DOPA-melanin-Cd(II) complexes. Fast spin-lattice relaxation processes characterized Cu(II) in DOPA-melanin-Cu(II) complexes.

Introduction

Pigmentation of the skin, eyes and hair in humans is a result of the activity of melanocytes (Bennett, 2008; Brenner and Hearing, 2008; Simon et al., 2009; Hearing, 2011). These cells are found in the basal layer of the epidermis and produce two structurally different types of pigment: eumelanin and pheomelanin (Bandyopadhyay et al., 2001; Costin and Hearing, 2007; Rees, 2011).

* *Corresponding author*. Tel.: +48 32 364 11 62; fax: +48 32 364 11 66.
E-mail address: mzdybel@sum.edu.pl (M. Zdybel).

1214-021X/$ – see front matter
http://dx.doi.org/10.1016/j.jab.2015.01.002

Differences in skin color are caused by two factors: the amount and type of melanin (for example, the ratio of eumelanin to pheomelanin) and the form and distribution of melanosomes (Nissan et al., 2011). Light skin is rich in 5,6-dihydroxyindole-2-carboxylic acid (DHICA) – eumelanin and yellow-red pheomelanin. In this type of skin melanosomes are smaller, less pigmented and arranged in groups. Dark skin contains higher amount of 5,6-dihydroxyindole (DHI) – eumelanin and larger, more pigmented melanosomes, which are spread singly and homogenously (Meredith and Sarna, 2006).

Eumelanin, acting as an antioxidant and free radical scavenger, plays an important role in photoprotection (Gidanian et al., 2008; Jiang et al., 2010; Peles and Simon, 2011). However, pheomelanin is considered to be a potential photosensitizer and promoter of carcinogenesis induced by UV radiation (Takeuchi et al., 2004; Chodurek et al., 2012).

It is known that both eu- and pheomelanins are paramagnetic (Sarna, 1981; Sealy et al., 1982; Pasenkiewicz-Gierula and Sealy, 1986; Pilawa et al., 2002; Beberok et al., 2010). Electron paramagnetic resonance (EPR) studies indicated that o-semiquinone free radicals exist in melanins (Sarna, 1981; Sealy et al., 1982; Shima et al., 1997; Ito and Wakamatsu, 2003; Latocha et al., 2004b; Beberok et al., 2010). EPR spectra of eu- and pheomelanins differ in shape, and it is used to determine the type of natural melanin in the biological samples and biomaterials (Sarna, 1981; Sealy et al., 1982; Shima et al., 1997; Ito and Wakamatsu, 2003; Latocha et al., 2004a, 2004b; Matuszczyk et al., 2004; Buszman et al., 2006). EPR spectra of synthetic and different natural pheomelanins were analyzed by Sealy et al. (1982). Pheomelanin and its complexes with Zn (II) and Cd(II) were characterized by the use of electron paramagnetic resonance spectroscopy. The dependence of complex shape of pheomelanin on pH, Zn(II), and Cd(II) existence in polymer was presented (Sealy et al., 1982). Natural samples may contain both eu- and pheomelanins. For example we studied melanins in pigmented soil fungi *Cladosporium cladosporioides*, and the EPR spectra were superposition of signals from free radicals of these two polymers (Matuszczyk et al., 2004; Buszman et al., 2006). Content of free radicals in melanins is changed under the interactions of the physical and chemical factors (Sarna, 1981; Ito and Wakamatsu, 2003; Pilawa et al., 2004; Buszman et al., 2005a, 2005b; Tran et al., 2008; Najder-Kozdrowska et al., 2009; Zdybel et al., 2011).

Melanin biopolymers are capable to bind many chemical compounds, i.e., metal ions, chemicals, drugs (Sarna, 1981; Pasenkiewicz-Gierula and Sealy, 1986; Shima et al., 1997; Pilawa et al., 2002; Ito and Wakamatsu, 2003; Beberok et al., 2010; Najder-Kozdrowska et al., 2010; Wiernek et al., 2014). By binding metal ions and chemicals, melanins play a significant role in reducing levels of toxic compounds entering the environment. Melanins contain carboxyl, hydroxyl, amine groups, which are potential binding sites for metal ions (Sarna, 1981; Pasenkiewicz-Gierula and Sealy, 1986). Paramagnetic centers of melanin reveal also high activity in binding of metal ions. This feature is important for detoxification processes in human tissues or in environmental pollution (Sarna, 1981).

Complexation of melanin polymers with metal ions strongly affects their free radical systems (Sarna, 1981; Matuszczyk et al., 2004; Buszman et al., 2006; Najder-Kozdrowska et al., 2009; Zdybel et al., 2011). EPR studies pointed out that diamagnetic metal ions increase free radicals in melanins (Sarna, 1981; Matuszczyk et al., 2004; Buszman et al., 2006). Paramagnetic metal ions decrease EPR signals of free radicals in melanins (Sarna, 1981; Buszman et al., 2006; Najder-Kozdrowska et al., 2009; Zdybel et al., 2011). The influence of UV irradiation of melanin containing Cd(II) and Cu(II) was not tested by us earlier.

In our earlier paper (Chodurek et al., 2013) we examined free radicals in eumelanins: DOPA-melanin and melanin synthesized from tyrosine. The effect of 5,7-dimethoxycoumarin (DMC) and valproic acid (VPA) on eumelanins was spectroscopically proved. Slow spin-lattice relaxation processes were found in DOPA-melanin and in melanin synthesized from tyrosine. It was interesting to continue these researches (Chodurek et al., 2013) and to characterize the effect of electromagnetic irradiation on the paramagnetic centers in melanins.

This work expands the knowledge about paramagnetic properties of melanins. The free radicals formed during UV irradiation may interact with paramagnetic centers of melanin (Bennett, 2008; Brenner and Hearing, 2008; Gidanian et al., 2008) and its complexes with metal ions. The aim of this work was to determine the effect of UV irradiation on free radicals in synthetic eu- and pheomelanin, and model eumelanin complexes with metal ions. The complexes of DOPA-melanin with diamagnetic Cd(II) and paramagnetic Cu(II) exposed to UV were examined. The changes in free radical concentrations and properties were measured.

Correlations between dipolar interactions and broadening of EPR lines were pointed out for the tested samples. Dipolar interactions of paramagnetic centers are interactions between magnetic moments of their unpaired electrons (Wertz and Bolton, 1986; Eaton et al., 1998). These interactions are stronger for the lower distances between unpaired electrons. The lower distances of free radicals result in the higher dipolar interactions and the broadening of EPR lines.

Velocity of spin–lattice interactions in the examined samples was discussed. Times of spin-lattice relaxation processes were not determined, because of the use of EPR spectroscopy with the continuous waves. The conclusions about relatively fast or slow spin-lattice relaxation processes in the samples were presented. To obtain such information the changes of amplitudes of the EPR lines with increase of microwave power were drawn. The increase of microwave power causes the increase of amplitude of EPR line up to the moment, when the line begins to saturate (Wertz and Bolton, 1986; Eaton et al., 1998). Then the amplitude of the EPR line decreases with the increase of microwave power. This effect is connected with spin-lattice relaxation (Wertz and Bolton, 1986; Eaton et al., 1998). For the fast spin-lattice relaxation in the sample the effect of decreasing of amplitudes with increasing of microwave power appears latter than for the slow spin-lattice relaxation. The decrease of amplitude of EPR line for the higher microwave power means that the higher number of unpaired electrons are located on the higher energy levels than in the ground energy levels, and there are now enough number of unpaired electrons to absorb microwaves. The eventual changes of dipolar interactions and velocity of

spin-lattice relaxation in the UV-irradiated samples reflect the changes in their paramagnetic centers systems.

Biomedicine is the interdisciplinary science, which develops modern diagnostic and therapeutic methods in medicine (Berger, 2011). The performed electron paramagnetic resonance examination of the model samples is interesting for free radicals and melanins in biomedicine. Free radicals play an active role in different processes in human organism (Eaton et al., 1998; Bartosz, 2006). Many diseases that afflict human (atherosclerosis, asthma, diabetes, inflammatory joint disease, degenerative eye disease, cancers) have their origin in free radical processes (Bartosz, 2006). Free radicals of melanin biopolymers may modify free radicals in organism during illness states, so the knowledge about free radicals in melanin is important for medicinal therapy. Melanins bind also drugs (Sarna, 1981; Pilawa et al., 2002; Beberok et al., 2010; Najder-Kozdrowska et al., 2010; Wiernek et al., 2014), it may change effectiveness of major therapies. Free radicals in cosmetics containing melanins may change during exposition to UV irradiation, so our investigation is expected to be helpful to determine storage conditions of such samples. In our work we proposed EPR spectroscopy as the useful tool in biomedicine.

Materials and methods

Preparation of synthetic eumelanins

Synthetic eumelanins were prepared by tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenylalanine (DOPA-melanin) and tyrosine (Tyr-melanin). Melanin precursors were dissolved in 50 mM sodium phosphate buffer (pH 6.8) to obtain the final concentration of 2 mM, then tyrosinase 100 U/ml (Sigma, 5370 U/mg) was added and the reaction mixtures were incubated for 48 h at 37 °C with vigorous stirring and protection from light. The obtained DOPA-melanin and Tyr-melanin pigments were collected by centrifugation (5000 × *g*, 15 min) and washed several times with deionized water. To remove possible traces of tyrosinase, eumelanin standards were treated with SDS and methanol, NaCl, then rewashed with deionized water and dried to a constant weight at 37 °C.

Preparation of synthetic pheomelanin

The mixture of L-DOPA (0.5 mM), L-cysteine (0.5 mM), 25 U tyrosinase in 50 mM phosphate buffer (pH 6.8) was incubated for 48 h at 37 °C. After centrifugation sediment pheomelanin was washed several times with deionized water. To remove possible traces of tyrosinase, pheomelanin standards were treated with SDS and methanol, NaCl, then rewashed with deionized water and dried to a constant weight at 37 °C.

Preparation of DOPA-melanin complexes with metal ions

To obtain DOPA-melanin-Cd(II) and DOPA-melanin-Cu(II) complexes, 5 mM $CdCl_2$ and 5 mM $CuSO_4$ were added to 5 mM solution of L-DOPA in Tris–HCl buffer (50 mM, pH 7.4). Samples were incubated at room temperature for 72 h. Melanin sediments were centrifuged (2500 × *g*, 15 min) and washed with deionized water.

Experimental conditions

The original nonirradiated samples and UV-irradiated samples were tested. The times of melanin exposition on UV were 30 and 60 min, respectively.

The melanins were irradiated by UVA 315–400 nm light. The irradiation was done by the use of Medisun 250 lamp (Schulze & Böhm GmbH) with 4 radiators each with the power of 20 W. The distance from the lamp to the samples was 30 cm.

The melanin samples for the EPR measurements were placed in the thin walled glass tubes with the external diameter of 3 mm. The mass of the samples in the tubes was determined.

EPR measurements

Free radicals in melanins and DOPA-melanin complexes with Cd(II) and Cu(II) were studied by electron paramagnetic resonance (EPR) spectroscopy at the X-band with microwave frequency of 9.3 GHz. Microwave frequency (ν) was measured by MCM101 recorder of EPRAD (Poznań, Poland). The EPR spectra were measured as the first derivative of absorption curves by Radiopan (Poznań, Poland) spectrometer with magnetic modulation of 100 kHz. The total microwave power (M_o) produced by klystron was 70 mW. The Rapid Scan Unit from Jagmar (Kraków, Poland) was used to perform the numerical acquisition of the EPR spectra. The EPR spectra were collected and analyzed by the use of spectroscopic programs SWAMP by Jagmar (Kraków, Poland) and LabVIEW 8.5 by National Instruments.

The following parameters of EPR spectra: *g*-values, amplitudes (*A*), integral intensities (*I*), and linewidths (ΔB_{pp}), were analyzed. Amplitudes (*A*) and integral intensities (*I*) increase with the increasing of free radical concentration in the samples (Wertz and Bolton, 1986; Eaton et al., 1998). Linewidth (ΔB_{pp}) increases for the stronger dipolar interactions of free radicals in the samples (Wertz and Bolton, 1986; Eaton et al., 1998). *g*-Values were calculated according to the formula (Wertz and Bolton, 1986): $g = h\nu/\mu_B B_r$, where h is the Planck constant, ν the microwave frequency, μ_B the Bohr magneton and B_r is the induction of resonance magnetic field.

Free radical concentrations (*N*) in the melanins were determined as the value which is proportional to the integral intensity (*I*) of EPR line (Wertz and Bolton, 1986; Eaton et al., 1998; Stankowski and Hilczer, 2005). Integral intensities (*I*) as the areas under the absorption curves were calculated by double integration of the first-derivative EPR spectra. The integral intensities (*I*) of the EPR spectra of the tested melanin samples and the reference – ultramarine (I_u) were compared. The second reference – a ruby crystal (Al_2O_3: Cr^3) was permanently placed in a resonance cavity. For each sample and for ultramarine the EPR line of a ruby crystal was measured. 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 concentration of the free radicals (*N*) in the melanins was calculated as (Wertz and Bolton, 1986): $N = N_u[(W_u A_u)/I_u][I/(WAm)]$, where N_u is the number of paramagnetic centers in the ultramarine reference, W and W_u the receiver gains for sample and the ultramarine, *A*

and A_u the amplitudes of ruby signal for the sample and the ultramarine, I and I_u the integral intensities for the sample and ultramarine, and m is the mass of the sample.

Mass of the samples was measured by the use of analytical balance of Sartorius (Germany). The mass was calculated as the difference between the mass of the tube with the sample and the mass of the empty tube.

The influence of microwave power (M) on the EPR spectra of free radicals and Cu(II) in the melanin samples was examined. Microwave powers (M) in the range of 2.2–70 mW were used. The changes of amplitudes (A) and linewidths (ΔB_{pp}) of EPR spectra with increasing of microwave power were determined. The effect of microwave power (M) on amplitudes (A) and linewidths (ΔB_{pp}) of the EPR spectra depend on broadened EPR lines (homogeneous or inhomogeneous). For homogeneous broadened EPR lines the amplitude (A) increases with increasing of microwave power (M) and for the higher microwave powers it value decreases (Wertz and Bolton, 1986). The increase of linewidth (ΔB_{pp}) with increasing of microwave power (M) is characteristic for the homogeneously broadened EPR lines (Wertz and Bolton, 1986). For inhomogeneous broadening of spectral lines the amplitude (A) increases with increasing of microwave power (M) and for the higher microwave powers its value does not change (Wertz and Bolton, 1986). Linewidth (ΔB_{pp}) of the inhomogeneously broadened EPR lines is unchanged with increasing of microwave power (M) (Wertz and Bolton, 1986). In this work the effect of microwave power on parameters of the EPR lines for the nonirradiated and UV-irradiated melanins was compared. The slow and fast spin-lattice relaxation processes in the samples differ in microwave saturation of EPR lines (Wertz and Bolton, 1986). 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 (Wertz and Bolton, 1986; Stankowski and Hilczer, 2005).

Results and discussion

All the tested melanin polymers were paramagnetic and the EPR spectra for both nonirradiated and UV-irradiated samples were observed. EPR lines of free radicals were measured for melanins and DOPA-melanin complexes with diamagnetic Cd(II), while EPR signals of Cu(II) were obtained for DOPA-melanin-Cu(II) complexes. Hyperfine structure was not visible in all the analyzed EPR spectra. Dipolar interactions broadened the spectra and the single lines were measured for both free radicals and paramagnetic Cu(II) ions. EPR spectra of melanin complexes with Cd(II) ions revealed the same character as for melanin free of Cd(II). Cu(II) ions quenched EPR lines of free radicals of DOPA-melanin, and only EPR lines of Cu(II) were detected.

The high of EPR signals of DOPA-melanin-Cd(II) complexes changed after their UV irradiation. The exemplary EPR lines of DOPA-melanin-Cd(II) samples before and after UV irradiation during 60 min are compared in Fig. 1, respectively. The EPR lines of DOPA-melanin samples with Cd(II) were very broad, what is characteristic for strong dipolar interactions of unpaired electrons of free radicals with low distances in

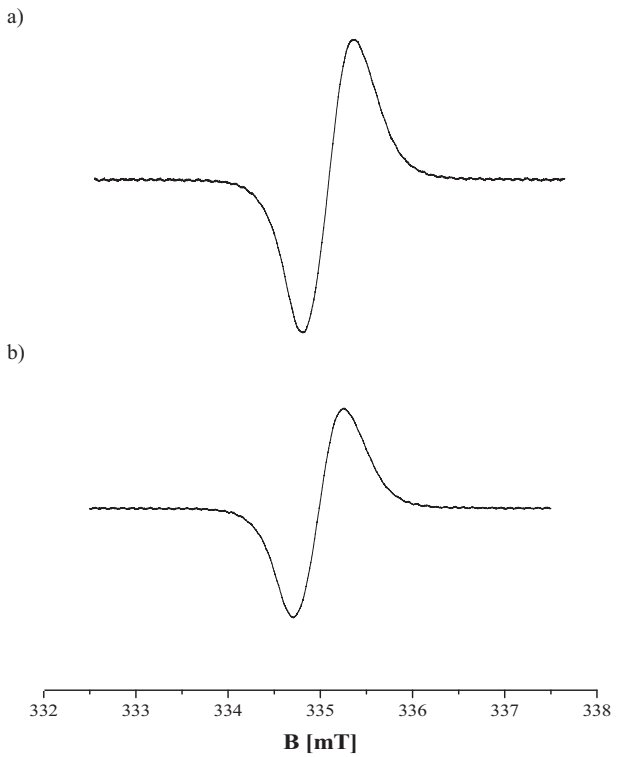

Fig. 1 – EPR spectra of DOPA-melanin-Cd(II) complexes for nonirradiated (a) and UV-irradiated (b) during 60 min samples. The EPR spectra were measured with microwave power of 2.2 mW. The qualitative data are shown.

melanin. The low distances between free radicals in DOPA-melanin-Cd(II) complexes resulted from the high ($\sim 10^{22}$ spin/g) o-semiquinone free radical concentrations in this model eumelanin. The linewidths and free radical concentrations in the non- and UV-irradiated DOPA-melanin-Cd(II) are compared in Fig. 2, respectively. Similar linewidths of DOPA-melanin-Cd(II) complexes before and after UV irradiation during 30 and 60 min were observed (Fig. 2a). Diamagnetic Cd (II) increased strongly free radical content in DOPA-melanin, but only the slight changes of free radical concentrations after UV irradiation were obtained (Fig. 2b).

EPR investigations proved that o-semiquinone free radicals exist in melanins, both eu- and pheomelanins (Sarna, 1981; Meredith and Sarna, 2006). High free radical concentration in melanin polymers is responsible for broadening of their EPR lines. Hyperfine structure is usually not visible in EPR spectra of melanins in solid state. The EPR lines of o-semiquinone free radicals strongly depend on the existence of paramagnetic Cu(II) and diamagnetic Cd(II) ions (Sarna et al., 1976; Sarna, 1981). Sarna et al. (1976) proposed physicochemical mechanism of the effect. Magnetic interactions of unpaired magnetic moments of Cu(II) and o-semiquinone free radicals decrease EPR lines of free radicals of melanins (Sarna et al., 1976). Diamagnetic Cd(II) ions effect on the quinone–hydroquinone equilibrium and the highest semiquinone forms appear, what increase o-semiquinone free radical concentration and increase of the EPR lines of free radicals (Sarna et al., 1976;

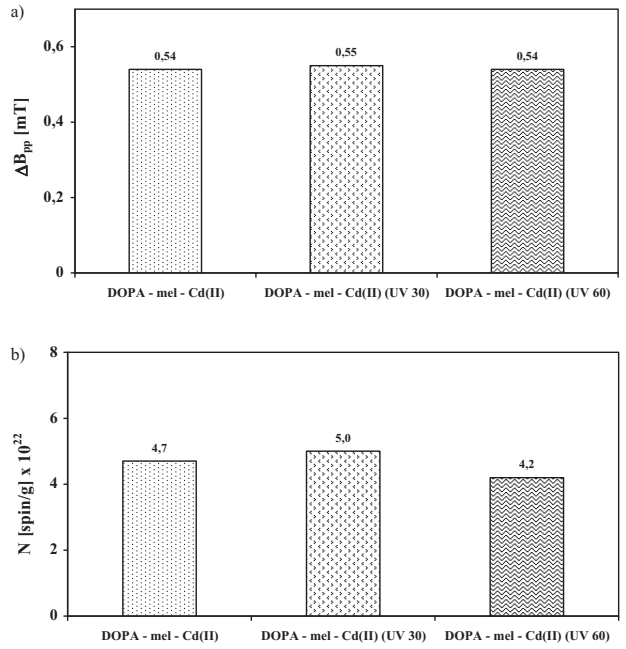

Fig. 2 – Linewidths (ΔB_{pp}) (a) of EPR spectra and free radical concentrations (*N*) (b) in DOPA-melanin-Cd(II) complexes for nonirradiated and UV-irradiated samples. Times of UV-irradiated were 30 and 60 min, respectively.

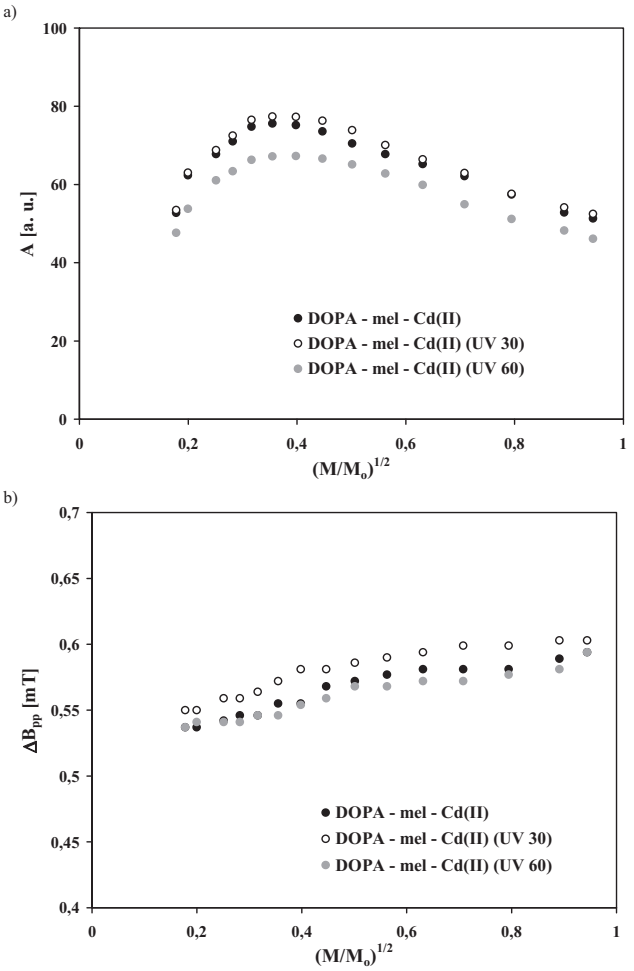

Fig. 3 – Influence of microwave power (M/M_o) on amplitude (*A*) (a) and linewidth (ΔB_{pp}) (b) of EPR spectra of nonirradiated and UV-irradiated DOPA-melanin-Cd(II) complexes. *M* and M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively.

Sarna, 1981). The complex mechanism of binding of metal ions to melanin polymer is studied so far. Unfortunately the examination of EPR lines without hyperfine structure did not give information about chemical reactions during binding Cu (II) and Cd(II) ions to melanins.

The changes of amplitudes (*A*) and linewidths (ΔB_{pp}) of EPR lines of DOPA-melanin complexes with Cd(II) for nonirradiated and UV-irradiated samples, are compared in Fig. 3, respectively. UV irradiation did not change the character of these changes. Amplitudes (*A*) increased with increasing of microwave power and after maximum they decreased (Fig. 3a). Linewidths (ΔB_{pp}) broadened with increasing of microwave power (Fig. 3b). The correlations between amplitudes, linewidths, and microwave power were characteristic for homogeneously broadened EPR lines (Wertz and Bolton, 1986). The microwave saturation of EPR lines of DOPA-melanin-Cd(II) complexes (Fig. 3a) brought to light slow spin-lattice relaxation processes (Wertz and Bolton, 1986). Slow spin-lattice relaxation processes existed in non- and UV-irradiated samples.

Only Cu(II) signals were detected for both UV-irradiated and nonirradiated samples. Microwave saturation of EPR lines of the non- and UV-irradiated DOPA-melanin complexes with Cu (II) was similar (Fig. 4). Amplitude (*A*) of the EPR lines of Cu(II) ions increases with increasing of microwave power in whole range (up to 70 mW). Fast spin-lattice relaxation processes existed in Cu(II) system in DOPA-melanin complexes with Cu (II).

Free radicals were found in all the tested nonirradiated and UV-irradiated melanins. The EPR spectra were obtained for DOPA-melanin, Tyr-melanin, and pheomelanin. The EPR

Table 1 – Free radical concentrations (*N*), linewidths (ΔB_{pp}), and *g*-values of EPR spectra of the studied synthetic eu- and pheomelanins.

Sample	*N* [$\times 10^{19}$ spin/g]	ΔB_{pp} [mT] [±0.02]	*g* [±0.0002]
DOPA-melanin	4.2	0.32	2.0043
DOPA-melanin (UV 30)	4.9	0.29	2.0044
DOPA-melanin (UV 60)	6.2	0.33	2.0040
Tyr-melanin	0.8	0.35	2.0044
Tyr-melanin (UV 30)	1.4	0.41	2.0043
Tyr-melanin (UV 60)	2.0	0.52	2.0043
Pheomelanin	1.7	0.63	2.0046
Pheomelanin (UV 30)	1.2	0.56	2.0053
Pheomelanin (UV 60)	1.2	0.61	2.0036

The EPR spectra were measured with microwave power of 2.2 mW.

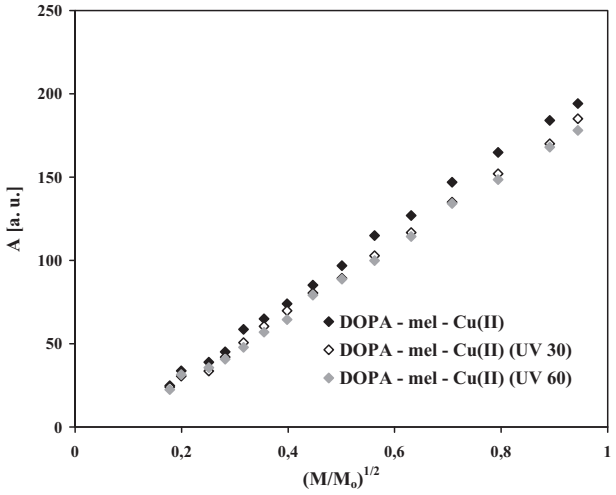

Fig. 4 – Influence of microwave power (M/M_o) on amplitude (A) of EPR lines of paramagnetic Cu(II) ions of nonirradiated and UV-irradiated DOPA-melanin-Cu(II) complexes. M and M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively.

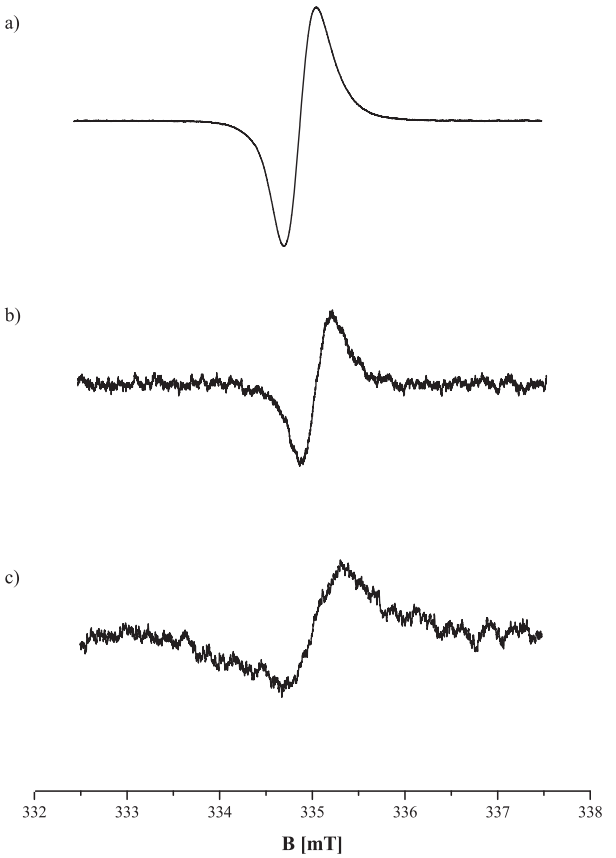

Fig. 5 – EPR spectra of nonirradiated DOPA-melanin (a), Tyr-melanin (b), and pheomelanin (c). The EPR spectra were measured with microwave power of 2.2 mW. The qualitative data are shown.

spectra of the two model eumelanins and pheomelanin for the original samples, before UV irradiation, were presented in Fig. 5. Similar shapes were observed for the UV-irradiated melanins. EPR spectra of eumelanins (Fig. 5a and b) were the single broad and asymmetrical lines. EPR spectrum of the pheomelanin reveals the complex character with the unresolved hyperfine structure (Fig. 5c). The differences in shapes of eumelanins (Fig. 5a and b) and pheomelanin (Fig. 5c) may be used to determine the type of melanin biopolymer in natural samples (Sarna, 1981; Matuszczyk et al., 2004; Buszman et al., 2006). In the human skin eumelanin and pheomelanin exist (Bennett, 2008; Brenner and Hearing, 2008; Simon et al., 2009; Hearing, 2011), so the EPR spectra of both model eu- and pheomelanins were tested by us.

The strong EPR spectra of the analyzed eumelanins and pheomelanin (Fig. 5) indicated that the high amount of free radicals exist in the samples. Free radical concentrations (N) and the parameters of EPR spectra: linewidths (ΔB_{pp}) and g-values, for the nonirradiated melanins and UV-irradiated melanins are presented in Table 1. Free radical concentrations in the examined melanins were about 10^{19} spin/g. The highest free radical concentration exists in the original DOPA-melanin (4.2×10^{19} spin/g), and the lowest free radical concentration (0.8×10^{19} spin/g) was obtained for nonirradiated Tyr-melanin (Table 1). Free radical concentration in nonirradiated pheomelanin (1.7×10^{19} spin/g) was lower than in DOPA-melanin and higher than in Tyr-melanin (Table 1).

Under UV irradiation of both tested eumelanins free radical concentrations increased in the samples (Table 1). We think that probably o-semiquinone free radicals were produced in photolysis. Because hyperfine structure is not visible in the measured EPR spectra of UV-irradiated melanins the other spectral analysis of types of free radicals is not possible.

The g-values (Table 1) were characteristic for o-semiquinone free radicals as usually exist in melanins. The energy of electromagnetic UV waves may rupture chemical bonds in melanin, and probably new o-semiquinone free radicals appear. The exposition of DOPA-melanin and Tyr-melanin caused the strong increase of free radical concentrations in the samples (Table 1). This increase was considerably higher for eumelanins irradiated during 60 min than 30 min (Table 1). It pointed out that free radical contents increased in irradiated samples at the presence of UV electromagnetic waves. It may be important during phototherapy, when free radicals may produce different reactions in skin and in the neighboring tissues. Free radicals may initiate peroxidation processes or they may react with cells in organism and the reactive oxygen species may appear. Increase of free radicals contents in skin and tissues may be responsible for their damage via free radical reaction. The formed products are the new molecules and units and the proper functions of cells may be stopped. It seems to be proper that this toxic free radical effect plays an important role during irradiation of skin during phototherapy. Free radicals are formed in eumelanin biopolymers and they may excite the reaction via their unpaired electrons. It is

known that melanins bind drugs and active substances of cosmetics (Sarna, 1981; Ito and Wakamatsu, 2003; Buszman et al., 2005a, 2005b; Beberok et al., 2010). Free radicals in melanins take a part in this process (Pilawa et al., 2002; Buszman et al., 2005a, 2005b; Beberok et al., 2010). It is possible that during UV irradiation in melanin biopolymers in skin the strong reactions with drugs and cosmetics will occur. We also observed changes of free radical concentration in melanotic tumor cells under red laser irradiation during photodynamic therapy (Latocha et al., 2005, 2006). The process of free radical formation in eumelanin in skin during photodynamic therapy with UV laser will influence on the result of this therapy. Probably the formed free radicals will lower the effectiveness of photodynamic therapy, because the resulting free radicals will be recombined with those of melanin. The interactions of UV generated free radicals in melanins in skin should be studied in the next works.

In this model studies free radicals in melanin polymers exposed to UV irradiation were examined. Tests of free radical processes in the natural samples, cells and tissues, are very difficult, because of the major reactions. Free radicals react not only with free radicals, but also with diamagnetic molecules without unpaired electrons. The chain reactions are possible. The product of reactions with free radicals may be paramagnetic containing unpaired electrons, but they may be also diamagnetic with paired electrons. The large number of possibilities exist, so the probability of modification of chemical structures in tissues is high. Modification of chemical structures in tissues interferes their functions. Our earlier EPR studies of free radicals in cells and in irradiated cells (Latocha et al., 2005; Chodurek et al., 2013) show the complex changes in their free radical system, so such complex changes are expected in melanotic cells after UV irradiation.

Free radical concentrations decreased after UV irradiation of pheomelanin (Table 1). The same free radical concentrations were obtained for pheomelanin irradiated by UV during 30 and 60 min. The decrease of free radical concentrations in pheomelanin may be the result of recombination of free radicals in this polymer or the result of major chemical reactions.

In the tested eu- and pheomelanins mainly exist o-semiquinone free radicals with the characteristic *g*-values of 2.0036–2.0053 (Table 1). *g*-Values for pheomelanin samples were higher than for eumelanin samples (Table 1). *g*-Values depend on the spin–orbit coupling and on the energy levels in molecular environment of the unpaired electrons in free radicals (Wertz and Bolton, 1986). The higher spin–orbit coupling may be responsible for the higher *g*-values of pheomelanin. This effect is connected with different chemical structures of pheo- and eumelanins. o-Semiquinone free radicals were detected in melanins earlier (Sarna, 1981; Shima et al., 1997; Ito and Wakamatsu, 2003; Latocha et al., 2004b; Matuszczyk et al., 2004; Beberok et al., 2010).

The parameters of the EPR spectra of the melanins changed after irradiation by UV (Table 1). Amplitude of the EPR lines changes similarly to the changes of free radical concentrations described above (Table 1). The broad EPR lines were measured for all the tested melanins. The linewidths of the nonirradiated samples were in the range of 0.32–0.63 mT (Table 1). The strong dipolar interactions are responsible for line broadening (Wertz and Bolton, 1986). The strongest dipolar interactions exist in pheomelanin with EPR linewidth of 0.63 mT, and the lower dipolar interactions characterize DOPA-melanin with EPR linewidth of 0.32 mT (Table 1). After UV irradiation only the weak changes of dipolar interactions and linewidths appeared in DOPA-melanin (Table 1). These changes were stronger for eumelanin synthesized from tyrosine. The strong increase of dipolar interactions and the high broadening of EPR line were observed after UV irradiation of this sample (Table 1).

The influences of microwave power on amplitudes (*A*) and linewidths (ΔB_{pp}) of the EPR spectra of the tested nonirradiated and UV-irradiated synthetic melanins are shown in Figs. 6–8, respectively. Amplitudes (*A*) of the EPR spectra of DOPA-melanin and pheomelanin increase with increasing of microwave power and after reaching the maximum their values

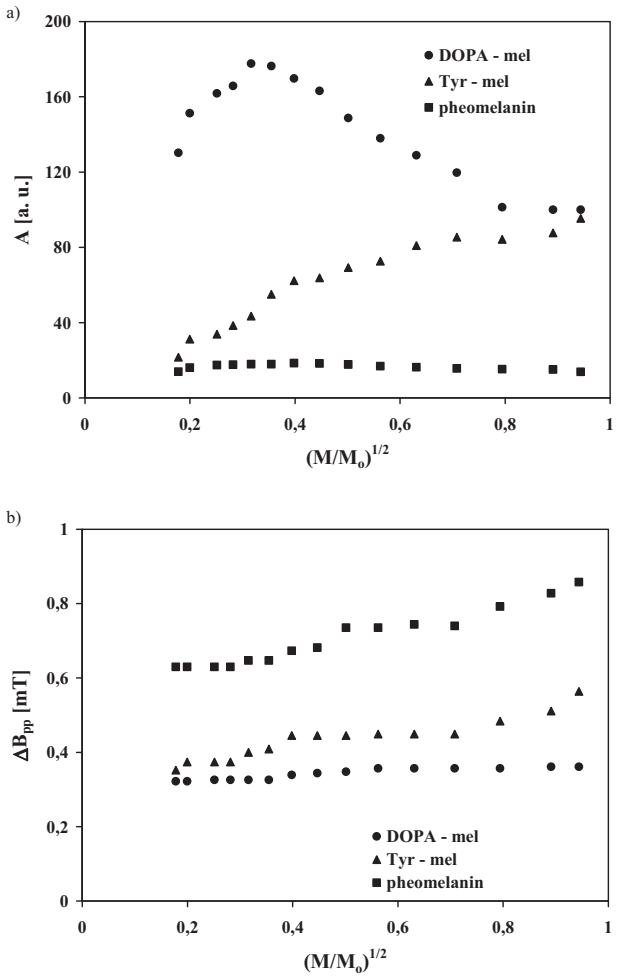

Fig. 6 – Influence of microwave power (M/M_o) on amplitude (*A*) (a) and linewidth (ΔB_{pp}) (b) of EPR spectra of nonirradiated DOPA-melanin, Tyr-melanin, and pheomelanin. *M* and M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively.

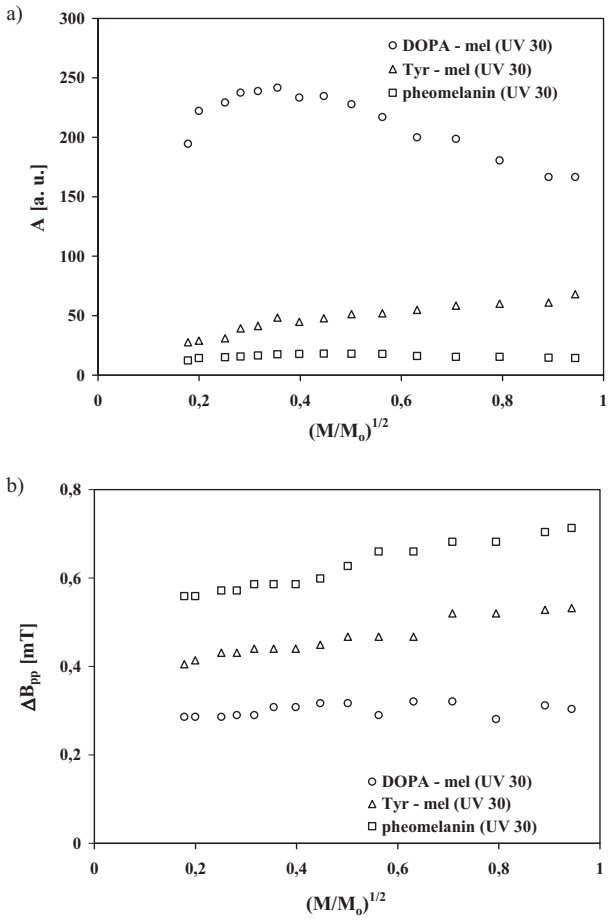

Fig. 7 – Influence of microwave power (M/M_o) on amplitude (A) (a) and linewidth (ΔB_{pp}) (b) of EPR spectra of DOPA-melanin, Tyr-melanin, and pheomelanin for the samples irradiated by UV during 30 min. M and M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively.

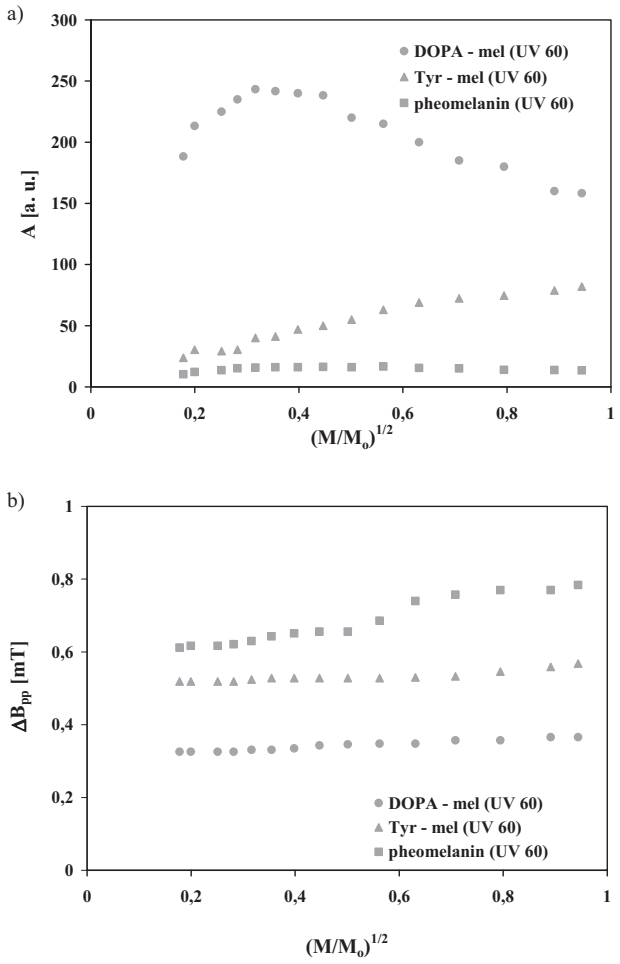

Fig. 8 – Influence of microwave power (M/M_o) on amplitude (A) (a) and linewidth (ΔB_{pp}) (b) of EPR spectra of DOPA-melanin, Tyr-melanin, and pheomelanin for the samples irradiated by UV during 60 min. M and M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively.

decrease (Figs. 6a, 7a and 8a). Amplitude (A) of the EPR spectra of Tyr-melanin increases with increasing of microwave power, reaches maximum, but its decrease was not observed (Figs. 6a, 7a and 8a). The reaching of maximum by the amplitudes (A) in the used microwave power range up to 70 mW indicates the slow spin-lattice relaxation processes. The slow spin-lattice relaxation processes exist in all the tested melanin samples. The increase of linewidth (ΔB_{pp}) of the EPR spectra with increasing of microwave power was observed (Figs. 6b, 7b and 8b). The changes of amplitudes (A) and linewidths (ΔB_{pp}) of the EPR spectra of the synthetic eu- and pheomelanins with microwave power (Figs. 6–8) are characteristic for homogeneously broadened lines. EPR lines of both nonirradiated and UV-irradiated melanins were homogeneously broadened.

EPR lines of DOPA-melanin and pheomelanin saturated at relatively low microwave powers lower than 70 mW (Figs. 6a, 7a and 8a), so the slow spin-lattice relaxation processes exist in this samples. The EPR lines of Tyr-melanin begun to saturate (Figs. 6a, 7a and 8a), so the spin-lattice processes in this eumelanin are faster than in DOPA-melanin and pheomelanin. UV irradiation did not change spin-lattice relaxation processes in DOPA-melanin (Fig. 9a) and Tyr-melanin (Fig. 9b), their EPR lines saturate similarly. Spin-lattice relaxation processes were faster in UV-irradiated pheomelanin (Fig. 9c). EPR lines of UV-irradiated pheomelanins saturated at higher microwave powers.

The performed comparative studies of EPR spectra of nonirradiated and UV-irradiated melanins and melanin complexes with diamagnetic Cd(II) and paramagnetic Cu(II) indicated that electron paramagnetic resonance spectroscopy is useful to examine changes in free radicals system in these polymers under ultraviolet irradiation. EPR may be helpful to determine the effect of metal ions complexation on free radicals in melanin.

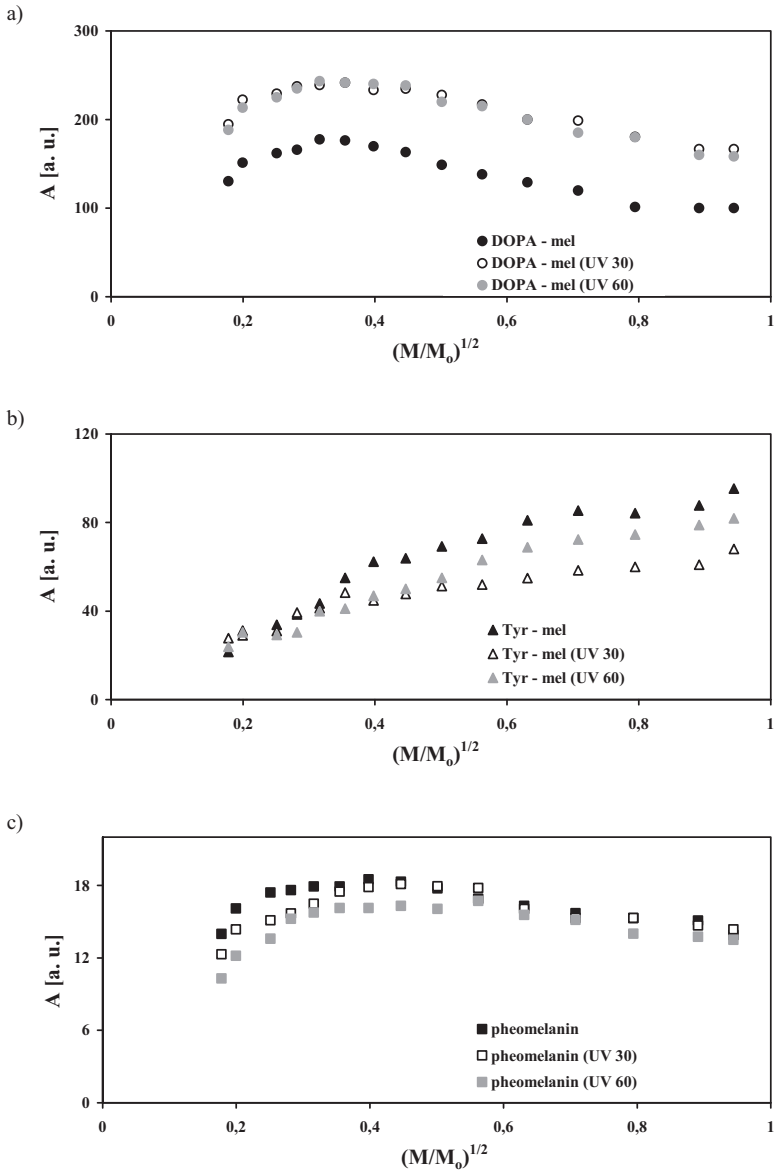

Fig. 9 – Comparison of the influence of microwave power (M/M_o) on amplitude (A) of EPR spectra of nonirradiated and UV-irradiated samples: DOPA-melanin (a), Tyr-melanin (b), and pheomelanin (c). M and M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively.

Conclusions

Usefulness of electron paramagnetic resonance (EPR) spectroscopy to examination of the effect of UV irradiation on free radicals in melanins and melanin complexes with Cd(II) and Cu(II) was stated. The high o-semiquinone free radical concentrations ($\sim 10^{19}$ spin/g) were measured in non- and UV-irradiated model eumelanins and pheomelanin. The influence of UV irradiation on free radicals depend on type of melanin, and Cu(II) or Cd(II) ions content in the polymer. For nonirradiated and UV-irradiated samples diamagnetic Cd(II) strongly increased free radical concentrations in model eumelanin – DOPA-melanin, while paramagnetic Cu(II) quenched free radical EPR lines. Free radical concentrations in eumelanins: DOPA-melanin and Tyr-melanin increased after UV irradiation, and the increase was higher for the longer time of irradiation. Free radical concentrations decreased in UV-irradiated pheomelanin, and the influence of irradiation time on this effect was not observed.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was financially supported by Medical University of Silesia, Katowice, Poland (grant no. KNW-1-005/K/4/0).

REFERENCES

Bandyopadhyay, D., Timchenko, N., Suwa, T., Hornsby, P.J., Campisi, J., Medrano, E.E., 2001. The human melanocyte: a model system to study the complexity of cellular aging and transformation in non-fibroblastic cells. Exp. Gerontol. 36, 1265–1275.

Bartosz, G., 2006. The Second Face of Oxygen. Free Radicals in Nature. PWN, Warsaw (in Polish).

Beberok, A., Buszman, E., Zdybel, M., Pilawa, B., Wrześniok, D., 2010. EPR examination of free radical properties of DOPA-melanin complexes with ciprofloxacin, lomefloxacin, norfloxacin and sparfloxacin. Chem. Phys. Lett. 497, 115–122.

Bennett, D.C., 2008. Ultraviolet wavebands and melanoma initiation. Pigment Cell Melanoma Res. 21, 520–524.

Berger, J., 2011. The age of biomedicine: current trends in traditional subjects. J. Appl. Biomed. 9, 57–61.

Brenner, M., Hearing, V.J., 2008. The protective role of melanin against UV damage in human skin. Photochem. Photobiol. 84, 539–549.

Buszman, E., Pilawa, B., Zdybel, M., Wrześniok, D., Grzegorczyk, A., Wilczok, T., 2005a. EPR examination of Zn^{2+} and Cu^{2+} effect on free radicals in DOPA–melanin–netilmicin complexes. Chem. Phys. Lett. 403, 22–28.

Buszman, E., Pilawa, B., Zdybel, M., Wrześniok, D., Grzegorczyk, A., Wilczok, T., 2005b. Paramagnetic centers in DOPA-melanin-dihydrostreptomycin complexes. Acta Physica Pol. A 108, 353–356.

Buszman, E., Pilawa, B., Zdybel, M., Wilczyńki, S., Gondzik, A., Witoszyńska, T., Wilczok, T., 2006. EPR examination of Zn^{2+} and Cu^{2+} binding by pigmented soil fungi *Cladosporium cladosporioides*. Sci. Total Environ. 363, 195–205.

Chodurek, E., Orchel, A., Orchel, J., Kurkiewicz, S., Gawlik, N., Dzierżewicz, Z., Stępień, K., 2012. Evaluation of melanogenesis in A-375 cells in the presence of DMSO and analysis of pyrolytic profile of isolated melanin. ScientificWorldJournal 854096, http://dx.doi.org/10.1100/2012/854096.

Chodurek, E., Zdybel, M., Pilawa, B., 2013. Application of EPR spectroscopy to examination of free radicals in melanins from A-375 and G-361 human *melanoma malignum* cells. J. Appl. Biomed. 11, 173–185.

Costin, G.E., Hearing, V.J., 2007. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J. 21, 976–994.

Eaton, G.R., Eaton, S.S., Salikhov, K.M., 1998. Foundations of Modern EPR. World Scientific, Singapore.

Gidanian, S., Mentelle, M., Meyskens Jr., F.L., Farmer, P.J., 2008. Melanosomal damage in normal human melanocytes induced by UVB and metal uptake – a basis for the pro-oxidant state of melanoma. Photochem. Photobiol. 84, 556–564.

Hearing, V.J., 2011. Determination of melanin synthetic pathways. J. Invest. Dermatol. 131, E8–E11.

Ito, S., Wakamatsu, K., 2003. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. 16, 523–531.

Jiang, S., Liu, X.M., Dai, X., Zhou, Q., Lei, T.C., Beermann, F., Wakamatsu, K., Xu, S.Z., 2010. Regulation of DHICA-mediated antioxidation by dopachrome tautomerase: implication for skin photoprotection against UVA radiation. Free Radic. Biol. Med. 48, 1144–1151.

Latocha, M., Pilawa, B., Buszman, E., Wilczok, T., 2004a. Identification of melanin biopolymers in cultured melanoma cells by the use of EPR spectroscopy. Pol. J. Med. Phys. Eng. 10, 119–126.

Latocha, M., Pilawa, B., Chodurek, E., Buszman, E., Wilczok, T., 2004b. Paramagnetic centers in tumor cells. Appl. Magn. Reson. 26, 339–344.

Latocha, M., Pilawa, B., Zdybel, M., Wilczok, T., 2005. Effect of laser radiation on free radicals in human cancer G361 cells. Acta Physica Pol. A 108, 409–412.

Latocha, M., Pilawa, B., Kuśmierz, D., Zielińska, A., Nawrocka, D., 2006. Changes in free radicals system of Imr-90 and C-32 cells during photodynamic therapy. Pol. J. Environ. Stud. 15, 154–156.

Matuszczyk, M., Buszman, E., Pilawa, B., Witoszyńska, T., Wilczok, T., 2004. Cd^{2+} effect on free radicals in *Cladosporium cladosporioides* – melanin tested by EPR spectroscopy. Chem. Phys. Lett. 394, 366–371.

Meredith, P., Sarna, T., 2006. The physical and chemical properties of eumelanin. Pigment Cell Res. 19, 572–594.

Najder-Kozdrowska, L., Pilawa, B., Więckowski, A.B., Buszman, E., Wrześniok, D., 2009. Influence of copper(II) ions on radical in DOPA-melanin. Appl. Magn. Reson. 36, 81–88.

Najder-Kozdrowska, L., Pilawa, B., Buszman, E., Więckowski, A. B., Świątkowska, L., Wrześniok, D., Wojtowicz, W., 2010. Triplet states in DOPA-melanin and in its complexes with kanamycin and copper Cu(II) ions. Acta Physica Pol. A 118, 613–618.

Nissan, X., Lemaitre, G., Peschanski, M., Baldeschi, C., 2011. Coloring skin with pluripotent stem cells. Cell Cycle 10, 3985–3986.

Pasenkiewicz-Gierula, M., Sealy, R.C., 1986. Analysis of the ESR spectrum of synthetic dopa melanin. Biochim. Biophys. Acta 884, 510–516.

Peles, D.N., Simon, J.D., 2011. UV-absorption spectra of melanosomes containing varying 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid content. J. Phys. Chem. B 115, 12624–12631.

Pilawa, B., Buszman, E., Wrześniok, D., Latocha, M., Wilczok, T., 2002. Application of EPR spectroscopy to examination of gentamicin and kanamycin binding to DOPA-melanin. Appl. Magn. Reson. 23, 181–192.

Pilawa, B., Latocha, M., Krzyminiewski, R., Kruczyński, Z., Buszman, E., Wilczok, T., 2004. Effect of temperature on melanin EPR spectra. Phys. Med. 20, 96–98.

Rees, J.L., 2011. The genetics of human pigmentary disorders. J. Invest. Dermatol. 131, E12–E13.

Sarna, T., 1981. Study of the structure and properties of the melanin active centers. Zag. Biofiz. Współcz. 6, 201–219 (in Polish).

Sarna, T., Hyde, J.S., Swartz, H.M., 1976. Ion-exchange in melanin: an electron spin body resonance study with lanthanide probes. Science 192, 1132–1134.

Sealy, R.C., Hyde, J.S., Felix, C.C., Menon, I.A., Prota, G., Swartz, H. M., Persad, S., Haberman, H.F., 1982. Novel free radicals in synthetic and natural pheomelanins: distinction between dopa melanins and cysteinyldopa melanins by ESR spectroscopy. Proc. Natl. Acad. Sci. U.S.A. 79, 2885–2889.

Shima, T., Sarna, T., Swartz, H.M., Stroppolo, A., Gerbasi, R., Zecca, L., 1997. Binding of iron to neuromelanin of human substantia nigra and synthetic melanin: an electron paramagnetic resonance spectroscopy study. Free Radic. Biol. Med. 23, 110–119.

Simon, J.D., Peles, D., Wakamatsu, K., Ito, S., 2009. Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res. 22, 563–579.

Stankowski, J., Hilczer, W., 2005. Introduction to Magnetic Resonance Spectroscopy. PWN, Warsaw (in Polish).
Takeuchi, S., Zhang, W., Wakamatsu, K., Ito, S., Hearing, V.J., Kraemer, K.H., Brash, D.E., 2004. Melanin acts as a potent UVB photosensitizer to cause an atypical mode of cell death in murine skin. Proc. Natl. Acad. Sci. U.S.A. 101, 15076–15081.
Tran, T.T., Schulman, J., Fisher, D.E., 2008. UV and pigmentation: molecular mechanisms and social controversies. Pigment Cell Melanoma Res. 21, 509–516.
Wertz, J.E., Bolton, J.R., 1986. Electron Spin Resonance: Elementary Theory and Practical Applications. Chapman and Hall, London.
Wiernek, B.K., Pilawa, B., Zdybel, M., Buszman, E., Wrześniok, D., 2014. Interaction of free radicals of DOPA-melanin-streptomycin complexes with paramagnetic oxygen O_2. J. Appl. Biomed. 12, 161–169.
Zdybel, M., Chodurek, E., Pilawa, B., 2011. EPR studies of DOPA-melanin complexes with Fe(III). Appl. Magn. Reson. 40, 113–123.