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Effect of UV irradiation on free radicals in synthetic melanin and melanin biopolymer from *Sepia officinalis* – EPR examination

Magdalena Zdybel, Barbara Pilawa

Abstract. Free radicals in synthetic melanin and melanin from *Sepia officinalis* were studied by electron paramagnetic resonance (EPR) spectroscopy. The effect of time of ultraviolet (UV) irradiation on free radicals in these melanins was tested. The samples were exposed to UV during 15, 30, and 60 minutes. EPR spectra were measured with microwaves from an X-band (9.3 GHz) in the range of microwave power of 2.2–70 mW. The performed EPR examinations indicate that high concentrations ($\sim 10^{21}$–10^{22} spin/g) of *o*-semiquinone free radicals with *g* factors of 2.0039–2.0045 exist in all the tested samples. For nonirradiated samples, free radical concentration was higher in natural melanin than in synthetic melanin. UV irradiation caused the increase of free radical concentrations in synthetic melanin samples and this effect depends on the time of irradiation. The largest free radical formation in the both melanins was obtained for 60 min of UV irradiation. Free radical concentrations after the UV irradiation of melanins during 30 min were lower than during irradiation by 15 min, and probably this effect was the result of recombination of the radiatively formed free radicals. EPR lines of the tested samples broadened with increasing microwave power, so these lines were homogeneously broadened. The two types of melanins differed in the time of spin-lattice relaxation processes. Slower spin-lattice relaxation processes exist in melanin from *Sepia officinalis* than in synthetic melanin. UV irradiation did not change the time of spin-lattice relaxation processes in the tested melanins. The performed studies confirmed the usefulness of EPR spectroscopy in cosmetology and medicine.

Key words: EPR spectroscopy • free radicals • melanin • *Sepia officinalis* • UV irradiation

M. Zdybel✉, B. Pilawa
Department of Biophysics,
School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec,
Medical University of Silesia in Katowice,
8 Jedności Str., 41-200 Sosnowiec, Poland,
Tel.: +48 32 364 1162, Fax: +48 32 364 1166,
E-mail: mzdybel@sum.edu.pl

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Introduction

Electron paramagnetic resonance (EPR) investigations confirmed the existence of free radicals in melanins [1–10], and melanin complexes with metal ions [1, 7, 11–15] and drugs [7, 12, 16–21]. Dia- and paramagnetic metal ions [11–15] and pharmacological substances [16–21] change the intensity of EPR lines of melanin. In this study, we concentrate on free radicals in melanins exposed to UV irradiation. The aim of our work was to compare the influence of UV irradiation on free radicals in synthetic melanin and natural melanin from *Sepia officinalis*. The effect of UV irradiation time on free radical formation in synthetic melanin and melanin biopolymer was evaluated.

The nonirradiated melanin from *Sepia officinalis* was examined by us earlier [22]. EPR spectra pointed out that mainly eumelanin exists in *Sepia officinalis*. The synthetic melanin was chosen to compare in the present studies. The EPR results for the melanins will be useful in the future examination of interactions of melanoma cells with drugs. Additionally, melanin polymers are used in cosmetics [22, 23]. UV irradiation may affect free radical contents

in melanin [1, 24, 25]. Our studies are important from the point of view of the potential usefulness of these two types of melanins in cosmetology. The formation of free radicals may modify interactions of cosmetics with skin.

In this work additionally we would like to test the formation of free radicals in the melanins as the potential component of cosmetics, which protects the skin under UV irradiation. It is known that the natural melanin from *Sepia officinalis* is usually used in cosmetics [22, 23]. We would like to test the usefulness of the other type of melanin as synthetic melanin obtained by the oxidation of tyrosine with hydrogen peroxide in cosmetology. Its examination, relative to the natural melanin from *Sepia officinalis*, was performed. Free radicals with unpaired electrons are the very active species, so the high amounts of free radicals should not be produced by UV in the melanins used as components of the cosmetics. We try to find by EPR examination, the melanins which may be used in cosmetics. These melanins should characterize the relatively lower free radical concentrations after UV irradiation.

Experimental

Samples

The original nonirradiated and UV irradiated synthetic melanin and melanin from *Sepia officinalis* were tested. The synthetic melanin was prepared by the oxidation of tyrosine with hydrogen peroxide. The two examined melanins synthetic melanin and melanin biopolymer from *Sepia officinalis* were obtained from Sigma-Aldrich. The purity of the samples was 97%. The melanins were tested in the solid state as powdered samples.

The samples were irradiated by UVA (315–400 nm) with Medisun 250 lamp (Schulze & Böhm GmbH). The used lamp has four radiators, each of power of 20 W. Different times of UV irradiation 15, 30, and 60 min were used. UV irradiation was performed for the individual melanin sample with the use of definite time. UV irradiation was done from the lamp – sample distance of 20 cm. The doses corresponding to these times are given by Schulze & Böhm GmbH Firm as [J/cm^2]: 3.2, 6.4, and 12.7. The UVA irradiation of the samples was performed out of the EPR cavity. The stable free radicals were measured.

The samples were located in thin-walled glass--tubes with the external diameter of 1 mm. The mass of the samples was determined by the use of analytical balance of Sartorius (Germany). The masses of synthetic melanin and melanin from *Sepia officinalis* in the tubes were 0.0038 and 0.0059 g, respectively. EPR signals for the empty tubes were not observed.

EPR measurements

EPR spectra of melanins were measured with the EPR spectrometer of Radiopan firm (Poznań, Poland) with the magnetic modulation of 100 kHz and the system of numerical detection of Rapid Scan Unit produced by Jagmar firm (Kraków, Poland). The first-derivative EPR spectra were collected with a microwave power from 2.2 mW to 70 mW at room temperature. Microwave frequency (ν) was measured by MCM101 recorder of EPRAD firm (Poznań, Poland).

The following parameters of the EPR spectra *g* factors, amplitudes (A), integral intensities (I), and linewidths (ΔB_{pp}) were determined. *g*-Factors were calculated from the resonance condition as [26, 27]:

$$g = h\nu/\mu_B B_r$$

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

The integral intensities (I) of the EPR lines were calculated by the double integration of the first--derivative curves.

Ultramarine was the reference during examination of free radical concentration. Additionally the amplitudes of the EPR lines of the tested melanins and ultramarine were divided by amplitudes of the ruby crystal permanently located in the resonance cavity during the measurements. Free radical concentrations (N) in melanins were determined according to the formula [26, 27]:

$$N = N_u[(W_u A_u)/I_u][I/(WAm)]$$

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

The number of spin in the reference sample – ultramarine was 1.2×10^{19} spin.

Spectroscopic programs of Jagmar (Kraków, Poland) and LabVIEW 8.5 by National Instruments (U.S.A.), were used to measure the EPR spectra and to obtain their parameters: amplitude (A), integral intensity (I), linewidths (ΔB_{pp}), and the resonance magnetic induction (B_r).

Results and discussion

The measurements confirmed paramagnetic character of the tested melanin samples. For the samples, strong EPR lines were observed in all the used microwave powers (2.2–70 mW). EPR spectra of synthetic melanin and melanin from *Sepia officinalis* measured with low microwave power (2.2 mW) are shown in Fig. 1. The parameters of the EPR spectra of synthetic and natural melanin are presented in Tables 1 and 2, respectively. The exemplary EPR spectra of the tested melanins recorded at the higher microwave power of 22.1 mW are presented in Fig. 2.

The broad EPR lines were detected for both synthetic melanin (ΔB_{pp}: 0.53–0.70 mT) (Table 1) and natural melanin from *Sepia officinalis* (ΔB_{pp}:

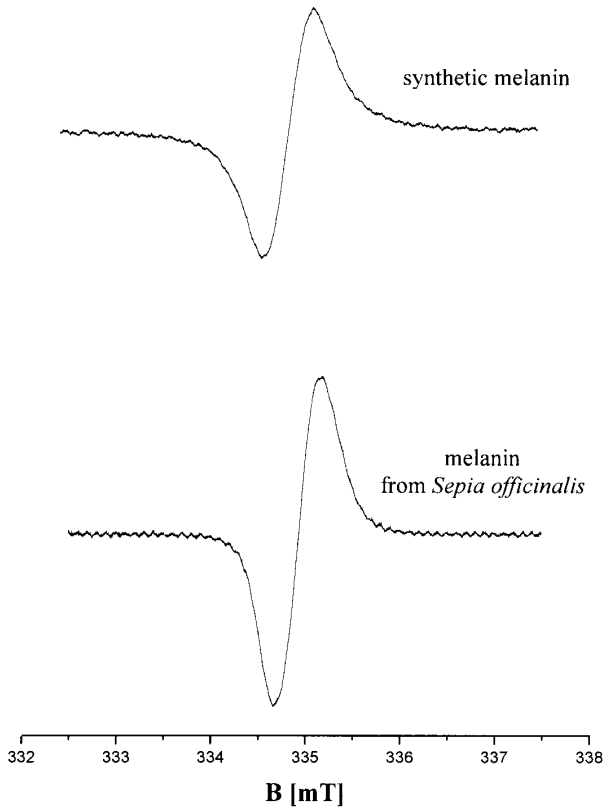

Fig. 1. EPR spectra of synthetic melanin and melanin from *Sepia officinalis* measured at room temperature with microwave power of 2.2 mW (attenuation of 15 dB). *B* – magnetic induction.

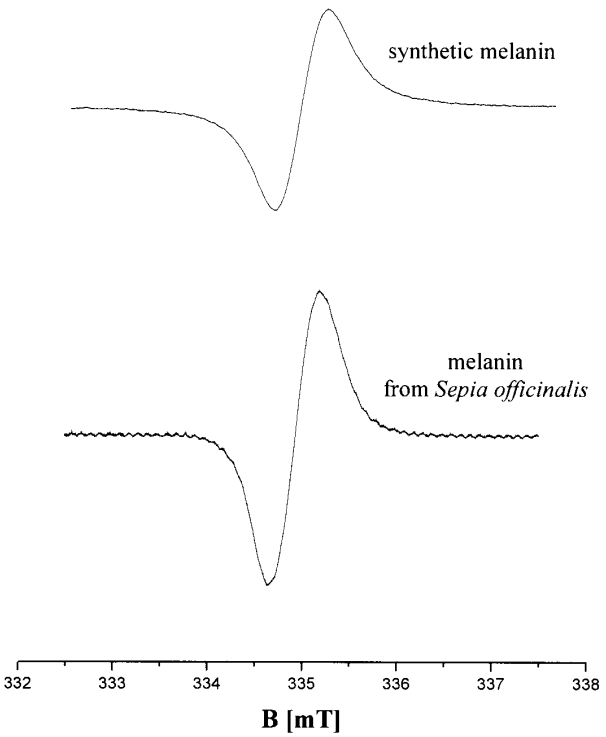

Fig. 2. EPR spectra of synthetic melanin and melanin from *Sepia officinalis* measured at room temperature with microwave power of 22.1 mW (attenuation of 5 dB). *B* – magnetic induction.

0.49–0.52 mT) (Table 2). Dipolar interactions are responsible for the broadening of EPR signals. This broadening is higher for the lower distances between free radicals in the sample [26, 27]. Dipolar interactions increase with free radical concentration in the samples, but they also depend on the chemical structure of the sample. It explains the higher linewidth (ΔB_{pp}) of the tested synthetic melanin with a significantly lower spin concentration (Tables 1 and 2). The observed correlation between linewidth and free radical concentrations in synthetic and natural melanins without proportionality (Tables 1, 2) is the important problem which should be examined in the future. Such effect may be observed in different samples with different chemical structures. The dipolar interactions increase with decreasing distances between unpaired electrons in the sample. The higher linewidth for the tested synthetic melanin

Table 1. Free radical concentrations (N), g factors, and linewidths (ΔB_{pp}) of EPR spectra of nonirradiated and UV irradiated synthetic melanin. N^{UV}/N^{nonUV} – free radical concentration in melanin after UV irradiation (N^{UV}) relative to free radical concentration in the non UV irradiated samples (N^{nonUV}). Times of UV irradiation were: 15, 30, and 60 min, respectively. EPR spectra were recorded with microwave power of 2.2 mW

Sample	$N \times 10^{21}$ ($\pm 0.2 \times 10^{21}$ spin/g)	N^{UV}/N^{nonUV}	g (± 0.0002)	ΔB_{pp} (± 0.02 mT)
Melanin	6.7	–	2.0039	0.53
Melanin (UV 15)	11.7	1.75	2.0042	0.70
Melanin (UV 30)	7.7	1.15	2.0040	0.55
Melanin (UV 60)	12.6	1.88	2.0041	0.65

Table 2. Free radical concentrations (N), g factors, and linewidths (ΔB_{pp}) of EPR spectra of nonirradiated and UV irradiated melanin from *Sepia officinalis*. N^{UV}/N^{nonUV} – free radical concentration in melanin after UV irradiation (N^{UV}) relative to free radical concentration in the non UV irradiated samples (N^{nonUV}). Times of UV irradiation were: 15, 30, and 60 min, respectively. EPR spectra were recorded with microwave power of 2.2 mW

Sample	$N \times 10^{21}$ ($\pm 0.2 \times 10^{21}$ spin/g)	N^{UV}/N^{nonUV}	g (± 0.0002)	ΔB_{pp} (± 0.02 mT)
Melanin from *Sepia officinalis*	67.8	–	2.0045	0.49
Melanin from *Sepia officinalis* (UV 15)	66.3	0.98	2.0045	0.49
Melanin from *Sepia officinalis* (UV 30)	62.2	0.92	2.0044	0.49
Melanin from *Sepia officinalis* (UV 60)	89.3	1.32	2.0045	0.52

with the lower concentration of unpaired electrons compared to the natural melanin may be probably caused by different chemical structures of these two melanin polymers. The chemical structures of synthetic melanin and melanin from *Sepia officinalis* were examined by the use of pyrolysis connected with gas chromatography and mass spectrometry (Py-GC/MS), and atomic force microscopy (AFM) [28]. It was pointed out [28] that these two types of melanin polymers differ in chemical composition because different precursors are used. Different contents of the following derivatives benzene, pyrrole, pyridine, phenol, and indole were observed for these two melanins [28]. Structural differences were also obtained by Nofsinger *et al*. [29].

UV irradiation increases dipolar interactions in synthetic melanin (Table 1). This effect is correlated with increase of the spin concentration in the irradiated samples (Table 1). Line broadening was not observed for UV irradiated melanin from *Sepia officinalis* (Table 2). It may be explained by the lower increase of free radicals concentrations in this melanin exposed on UV (Table 2) compared to synthetic melanin (Table 1). The broad EPR lines were measured earlier for synthetic DOPA--melanin [1, 2, 5, 7, 11–13, 17, 19], melanin form *Cladosporium cladosporioides* [18], melanin from tumor cells [20, 21]. The linewidths changed after the UVA irradiation of DOPA-melanin-moxifloxacin complexes [19]. Linewidths of melanin polymers changed after drug binding to melanin [16–21] and metal ions [11–15].

For the tested melanins *g* factors of 2.0039–2.0045 (Tables 1 and 2) were obtained. These values are in the range obtained earlier for melanins with *o*-semiquinone free radicals [1, 3, 11–13, 17–19]. However *g* factor for broad EPR lines with unresolved hyperfine structure does not directly determine the type of free radicals in the samples. The high free radical concentrations in the samples were determined (Tables 1 and 2). The concentration (*N*) of *o*-semiquinone free radicals in nonirradiated synthetic melanin ($\sim 10^{21}$ spin/g) (Table 1) was lower than in nonirradiated natural melanin from *Sepia officinalis* ($\sim 10^{22}$ spin/g) (Table 2). It is expected that probably drug binding via free radicals will be more active in the natural melanin biopolymer than in the synthetic melanin.

UV irradiation caused the increase of free radical concentrations in all the examined synthetic melanin samples, irradiated during 15, 30, and 60 min (Table 1). The highest increase of free radical concentrations (*N*) in the UV irradiated synthetic melanin was observed for irradiation during 60 min. The free radical concentration (*N*) in the synthetic melanin irradiated by UV for 30 min was lower than in the UV irradiated sample for 15 min. The effect of decrease of free radical concentration in the synthetic melanin samples exposed to UV during 30 min (Table 1) is not clear; probably the recombination of free radicals or interactions with oxygen may be responsible for it.

Similar to the tested synthetic melanin (Table 1), the largest free radical formation in the natural melanin from *Sepia officinalis* was observed for 60 min of UV irradiation (Table 2). Free radical concentrations (*N*) in the natural melanin UV irradiated during 15 min and 30 min were lower than in the nonirradiated melanin (Table 2). The free radical concentration (*N*) after UV irradiation of melanin from *Sepia officinalis* for 30 min was also lower than in the sample irradiated during 15 min (Table 2). The strongest effect of free radical recombination in this sample is proposed. The strongest binding of drugs to melanin from *Sepia officinalis* exposed to UV irradiation for 60 min is expected.

The relative values of the changes of free radical concentrations in the tested synthetic (Table 1) and natural melanin (Table 2) are presented as N^{UV}/N^{nonUV}. Generally the lowest changes of free radical concentrations after exposition to UV were observed for natural melanin from *Sepia officinalis* (Table 2), but the lowest free radical concentrations were obtained for nonirradiated and UV irradiated synthetic melanin (Table 1). It seems that these two types of melanins may be used in cosmetics. The advantage for the synthetic melanin is the lower free radical concentration than for melanin from *Sepia officinalis*. The advantage of the natural melanin from *Sepia officinalis* is the lower free radical formation during UV irradiation.

Parameters of the EPR lines changed with microwave power. The influence of microwave power on amplitudes (*A*) of synthetic melanin for nonirradiated sample and the samples irradiated for 15, 30, and 60 min, is compared in Fig. 3. The changes of amplitudes (*A*) of nonirradiated and UV irradiated melanin samples from *Sepia officinalis* are shown in Fig. 4. The influences of microwave power on linewidths (ΔB_{pp}) of EPR spectra of nonirradiated and UV irradiated synthetic melanin and melanin from *Sepia officinalis* are presented in Figs. 5 and 6, respectively.

The correlations between amplitudes (*A*) of EPR lines and microwave power (Figs. 3 and 4) indicated that slow spin-lattice relaxation processes existed in

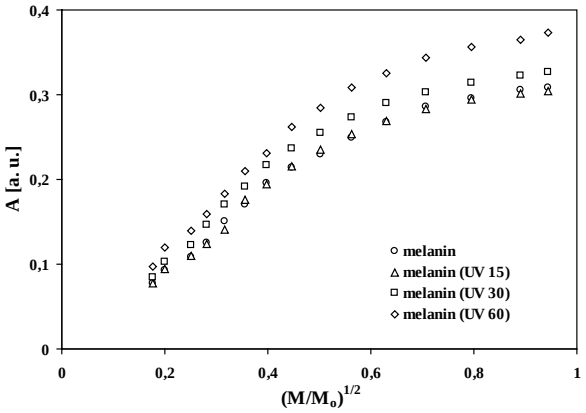

Fig. 3. The influence of microwave power (M/M_o) on amplitude (*A*) of the EPR line of nonirradiated and UV irradiated synthetic melanin, where M, M_o – microwave power used during the measurement of the EPR spectra and the total microwave power produced by klystron (70 mW), respectively. Times of UV irradiation were: 15, 30, and 60 min, respectively.

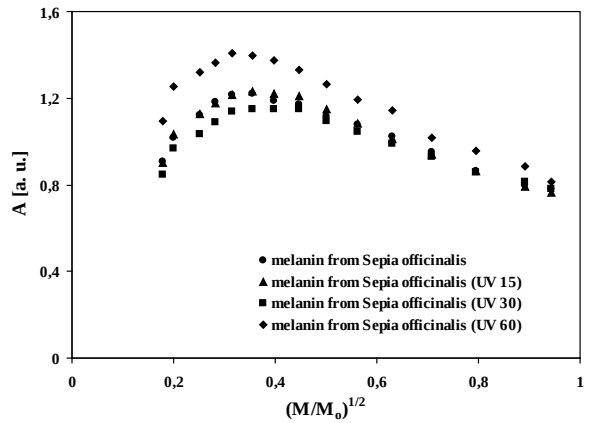

Fig. 4. The influence of microwave power (M/M_o) on amplitude (A) of the EPR line of nonirradiated and UV irradiated melanin from *Sepia officinalis*, where M, M_o – microwave power used during the measurement of the EPR spectra and the total microwave power produced by klystron (70 mW), respectively. Times of UV irradiation were: 15, 30, and 60 min, respectively.

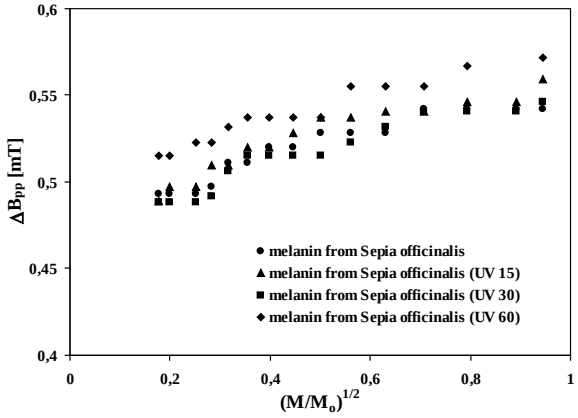

Fig. 6. The influence of microwave power (M/M_o) on linewidth (ΔB_{pp}) of the EPR line of nonirradiated and UV irradiated melanin from *Sepia officinalis*, where M, M_o – microwave power used during the measurement of the EPR spectra and the total microwave power produced by klystron (70 mW), respectively. Times of UV irradiation were: 15, 30, and 60 min, respectively.

the examined synthetic melanin and melanin from *Sepia officinalis*. The slow spin-lattice relaxation processes exist in both synthetic melanin and melanin from *Sepia officinalis*, but the relatively slower spin-lattice interactions characterized the natural melanin biopolymer. Amplitude (A) of EPR lines of melanin from *Sepia officinalis* reached maximum and decreased for higher microwave powers (Fig. 4). The effect of microwave saturation in the example of this natural melanin is clearly visible. The saturation at low microwave power is typical for slow spin-lattice relaxation processes [26, 27]. The decrease of amplitudes (A) at higher microwave powers was not observed for the tested synthetic melanin, but the state near the maximum is visible (Fig. 3). The difference between correlations presented in Figs. 3 and 4 is probably the result of different chemical structure of the melanin samples. UV irradiation did not change the spin-lattice relaxation processes in both melanins (Figs. 3 and 4). The character of changes of the amplitudes (A) was similar for nonirradiated and UV irradiated melanin samples. Slow spin-lattice relaxation processes were observed earlier, for example, for DOPA-melanin, which is the model eumelanin [1, 2, 5, 7, 11–13, 17, 19].

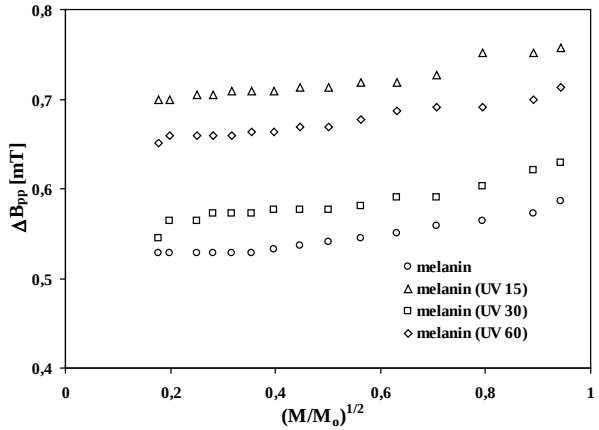

Fig. 5. The influence of microwave power (M/M_o) on linewidth (ΔB_{pp}) of the EPR line of nonirradiated and UV irradiated synthetic melanin, where M, M_o – microwave power used during the measurement of the EPR spectra and the total microwave power produced by klystron (70 mW), respectively. Times of UV irradiation were: 15, 30, and 60 min, respectively.

The linewidths (ΔB_{pp}) of EPR spectra of all the tested melanin samples increased with an increase microwave power (Figs. 5 and 6). The EPR lines of the synthetic melanin and natural melanin from *Sepia officinalis* were homogeneously broadened (Figs. 3–6), according to the theoretical base [26, 27]. Homogeneously broadened EPR lines for nonirradiated melanins were observed earlier [7, 11–13, 17–22].

This work confirmed the usefulness of an X-band electron paramagnetic resonance spectroscopy in examination of free radicals in melanins. The results are important for cosmetology and medicine. Changes of free radicals in different types of melanin polymers after UV irradiation were evaluated. Modifications of binding of drugs after UV irradiation of melanins are suggested.

Conclusions

EPR examination of synthetic melanin and melanin from *Sepia officinalis* pointed out that:

1) *o*-Semiquinone free radicals with characteristic g factors in the range of 2.0039–2.0045 exist in both tested synthetic and natural melanins.
2) The higher free radicals concentrations were measured for nonirradiated natural melanin ($\sim 10^{22}$ spin/g) than for synthetic melanin ($\sim 10^{21}$ spin/g).
3) Free radicals are formed in both tested melanins after exposition of the samples on UV during 60 min.
4) Free radical concentration after UV irradiation of melanin polymers depend on time of exposition on the electromagnetic waves in the following order: samples irradiated for 60 min > samples irradiated for 15 min > samples irradiated for 30 min.

5) EPR lines of both nonirradiated and UV irradiated synthetic and natural melanin from *Sepia officinalis* are homogeneously broadened.
6) Relatively slower spin-lattice relaxation processes exist in nonirradiated melanin from *Sepia officinalis* than in synthetic melanin. The effect of UV irradiation on spin-lattice relaxation processes in the examined melanins was not observed.

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