Acta Poloniae Pharmaceutica – Drug Research, Vol. 72 No. 5 pp. 901–907, 2015
ISSN 0001-6837
Polish Pharmaceutical Society

EFFECT OF CADMIUM(II) ON FREE RADICALS IN DOPA-MELANIN TESTED BY EPR SPECTROSCOPY*

MAGDALENA ZDYBEL[1]**, BARBARA PILAWA[1] and EWA CHODUREK[2]

Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine, [1]Department of Biophysics, [2]Department of Biopharmacy, Jedności 8, 41-200 Sosnowiec, Poland

Abstract: Electron paramagnetic resonance (EPR) spectroscopy may be applied to examine interactions of melanin with metal ions and drugs. In this work EPR method was used to examination of changes in free radical system of DOPA-melanin - the model eumelanin after complexing with diamagnetic cadmium(II) ions. Cadmium(II) may affect free radicals in melanin and drugs binding by this polymer, so the knowledge of modification of properties and free radical concentration in melanin is important to pharmacy. The effect of cadmium(II) in different concentrations on free radicals in DOPA-melanin was determined. EPR spectra of DOPA-melanin, and DOPA-melanin complexes with cadmium(II) were measured by an X-band (9.3 GHz) EPR spectrometer produced by Radiopan (Poznań, Poland) and the Rapid Scan Unit from Jagmar (Kraków, Poland). The DOPA (3,4-dihydroxyphenylalanine) to metal ions molar ratios in the reaction mixtures were 2 : 1, 1 : 1, and 1 : 2. High concentrations of o-semiquinone (g ~2.0040) free radicals (~10^{21}-10^{22} spin/g) characterize DOPA-melanin and its complexes with cadmium(II). Formation of melanin complexes with cadmium(II) increase free radical concentration in DOPA-melanin. The highest free radical concentration was obtained for DOPA-melanin-cadmium(II) (1 : 1) complexes. Broad EPR lines with linewidths: 0.37-0.73 mT, were measured. Linewidths increase after binding of cadmium(II) to melanin. Changes of integral intensities and linewidths with increasing microwave power indicate the homogeneous broadening of EPR lines, independently on the metal ion concentration. Slow spin-lattice relaxation processes existed in all the tested samples, their EPR lines saturated at low microwave powers. Cadmium(II) causes fastening of spin-lattice relaxation processes in DOPA-melanin. The EPR results bring to light the effect of cadmium(II) on free radicals in melanin, and probably as the consequence on drug binding to eumelanin.

Keywords: DOPA-melanin, cadmium(II), DOPA-melanin-cadmium(II) complexes, free radicals, EPR spectroscopy

Free radical content in melanins is important for their ability of binding drugs to the chemical structure of these polymers (1-6). EPR studies show that in melanins exist o-semiquinone free radicals, and their concentrations depend on metal ions (1, 4-9) and drugs (4, 6, 8-11). Free radicals with unpaired electrons take part during formation of melanin-drug complexes (2, 4, 12). Free radical concentrations strongly depend on diamagnetic metal ions content in melanin polymers (4, 6, 13), so the knowledge about free radical systems in melanins is important to evaluate the binding ability of melanin to drugs. Our earlier EPR studies pointed out strong changes in free radical concentrations in *Cladosporium cladosporioides* melanin biopolymers (13-15). In this work, interactions of the model eumelanin – DOPA-melanin with cadmium(II) was examined by electron paramagnetic spectroscopy. Eumelanin generally exists in living organism (16-19), so it was the sample interesting for pharmacy. It was expected that interactions of eumelanin with cadmium(II) modify its ability to binding drugs.

The aim of this work is to examine the effect of cadmium(II) on properties and concentrations of free radicals in model eumelanin. The influence of different cadmium(II) concentrations on o-semiquinone free radicals in DOPA-melanin was tested. Magnetic interactions in DOPA-melanin complexes with cadmium(II) were compared with those in DOPA-melanin. Electron paramagnetic resonance spectroscopy was used as the method of direct studies of free radicals. EPR measurement do not change chemical structure of the tested samples, and the low amounts of the samples are needed (20-22).

*Paper presented at IX MKNOL Conference, May, 2014

**Corresponding author, e-mail: mzdybel@sum.edu.pl

EXPERIMENTAL

Samples

DOPA-melanin and DOPA-melanin complexes with diamagnetic cadmium(II) ions of different concentrations were examined by EPR spectroscopy.

Synthetic DOPA-melanin – model eumelanin was obtained by autooxidative polymerization of 3,4-dihydroxyphenylalanine (L-DOPA, Sigma) in Tris-HCl buffer at pH 7.4 according to the Binns method (23).

Complexes of melanin samples with cadmium(II) ions were obtained as follows: 2.5, 5, and 10 mM $CdCl_2$ was added to 5 mM solution of L-DOPA in 50 mM Tris-HCl buffer at pH 7.4. The DOPA/cadmium(II) molar ratios in the reaction mixtures were 2 : 1, 1 : 1 and 1 : 2. Samples were incubated for 72 h at room temperature. Melanin sediments were centrifuged (2500 × g, 15 min) and then washed with deionized water.

EPR measurements

For free radicals in DOPA-melanin, and the complexes: DOPA-melanin-cadmium(II) (2 : 1), DOPA-melanin-cadmium(II) (1 : 1), and DOPA-melanin-cadmium(II) (1 : 2) EPR spectra were measured. The measurements were done by the use of an X-band (9.3 GHz) EPR spectrometer produced by Radiopan (Poznań, Poland). The modulation of magnetic field was 100 kHz. The Rapid Scan Unit from Jagmar (Kraków, Poland) was used for numerical detection of the lines. Microwave frequency was measured by MCM101 recorder of EPRAD (Poznań, Poland). The program LabVIEW 8.5 by National Instruments and the spectroscopic programs of Jagmar (Kraków, Poland) were applied in this work.

The following parameters of EPR spectra: g-values, linewidths (ΔB_{pp}) and integral intensities (I), were analyzed. Integral intensities depend on free radical concentration in the samples (20, 21). ΔB_{pp} changes with magnetic interactions in the samples and their values increase for the stronger dipolar interactions between free radicals (20-22). g-Value characterizes the type of free radicals. g-Values for free radicals were calculated from the resonance condition as (20): $g = h\nu/\mu_B B_r$, where: h - Planck constant; ν - microwave frequency; μ_B - Bohr magneton; B_r - induction of resonance magnetic field.

Free radical concentrations (N) in DOPA-melanin and DOPA-melanin-cadmium(II) complexes were determined by comparing the spectra of the analyzed sample with the spectra of references. The low microwave power of 2.2 mW was used to avoid microwave saturation effect. Free radical concentration is proportional to the integral intensity of EPR line, which is the area under the absorption curves (20-22). Integral intensities (I) were obtained by double integration of the first-derivative EPR spectra. The integral intensities of the EPR spectra of the tested DOPA-melanin and its complexes with cadmium(II) samples and for 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. The concentration of the free radicals (N) in the tested samples was calculated according to the formula (20):

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

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

The influence of microwave power (M) in the range of 2.2-70 mW on the EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes was studied. The changes of integral intensities and ΔB_{pp} of EPR spectra with increasing of microwave power was determined.

Table 1. Free radicals concentrations (N), g-values, and linewidths (ΔB_{pp}) of EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes. The data for EPR spectra recorded with microwave power of 2.2 mW.

Sample	N × 10^{21} [spin/g]	g ± 0.0002	ΔB_{pp} [mT] ± 0.02
DOPA-melanin	4.1	2.0040	0.37
DOPA-melanin-Cd(II) (2 : 1)	8.0	2.0042	0.48
DOPA-melanin-Cd(II) (1 : 1)	14.9	2.0041	0.73
DOPA-melanin-Cd(II) (1 : 2)	10.4	2.0041	0.57

RESULTS

EPR examination of DOPA-melanin and its complexes with diamagnetic cadmium(II) ions indicated their paramagnetic character. For all the tested melanin samples strong EPR spectra were measured. Binding of cadmium(II) by melanin changed its EPR line. EPR spectra of DOPA-melanin-complexes depend on cadmium(II) concentration in the samples. EPR spectra of DOPA-melanin and

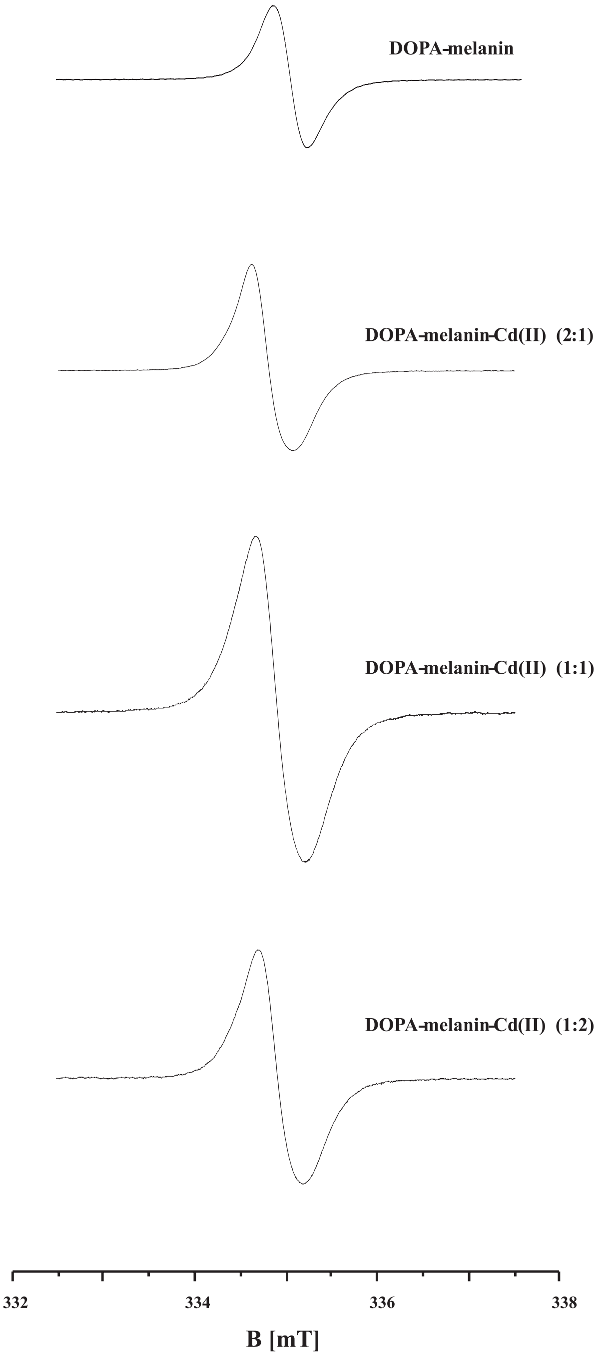

Figure 1. EPR spectra of DOPA-melanin, DOPA-melanin-cadmium(II) (2 : 1) complexes, DOPA-melanin-cadmium(II) (1 : 1) complexes, and DOPA-melanin-cadmium(II) (1 : 2) complexes. The EPR spectra were measured with microwave power of 2.2 mW. B - magnetic induction

DOPA-melanin-cadmium(II) complexes, for the DOPA/cadmium(II) molar ratios in the reaction mixtures amounts: 2 : 1, 1 : 1, and 1 : 2, are shown in Figure 1, respectively. EPR spectra of these samples are single asymmetric lines. The parameters of the measured EPR spectra recorded with microwave power of 2.2 mW and free radical concentrations in DOPA-melanin and the complexes DOPA-melanin with cadmium(II) are presented in Table 1.

EPR spectra of the samples are broad with ΔB_{pp} in the range from 0.37 to 0.73 mT (Table 1). Dipolar interactions are responsible for line broadening of the recorded EPR spectra. EPR lines of DOPA-melanin complexes with cadmium(II) are broader (ΔB_{pp}: 0.48-0.73 mT) than EPR lines of DOPA-melanin (ΔB_{pp}: 0.37 mT) (Table 1). The highest linewidth (ΔB_{pp}: 0.73 mT) characterizes EPR spectrum of DOPA-melanin-cadmium(II) (1 : 1) complexes. The relatively lower linewidths were obtained for DOPA-melanin-cadmium(II) complexes (2 : 1) (ΔB_{pp}: 0.48 mT) and DOPA-melanin-cadmium(II) complexes (1 :2) (ΔB_{pp}: 0.57 mT).

g-Values in the range of 2.0040-2.0042 (Table 1) point out that o-semiquinone free radicals exist in DOPA-melanin and DOPA-melanin-cadmium(II) complexes.

The high values (~10^{21}-10^{22} spin/g) of o-semiquinone free radical concentrations for DOPA-melanin and DOPA-melanin-cadmium(II) complexes were measured (Table 1). Addition of cadmium(II) to melanin polymer causes an increase of free radical concentration. Free radical concentrations in DOPA-melanin and DOPA-melanin-cadmium(II) (2 : 1) complexes are equal ~10^{21} spin/g, while free radical concentrations in DOPA-melanin-cadmium(II) (1 : 1) and DOPA-melanin-cadmium(II) (1 : 2) complexes are ~10^{22} spin/g. Free radical concentration in DOPA-melanin complexes with cadmium(II) increases as follows: DOPA-melanin-cadmium(II) (2 : 1) < DOPA-melanin-cadmium(II) (1 : 2) < DOPA-melanin-cadmium(II) (1 : 1) (Table 1).

Magnetic interactions in melanin samples were tested by the continuous microwave saturation of their EPR spectra. The parameters of the spectra depend on microwave power. The effect of microwave power on linewidth and integral intensity of the EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes are presented in Figures 2 and 3, respectively.

The ΔB_{pp} values increase with increasing microwave power both for DOPA-melanin and DOPA-melanin-cadmium(II) complexes, independ-

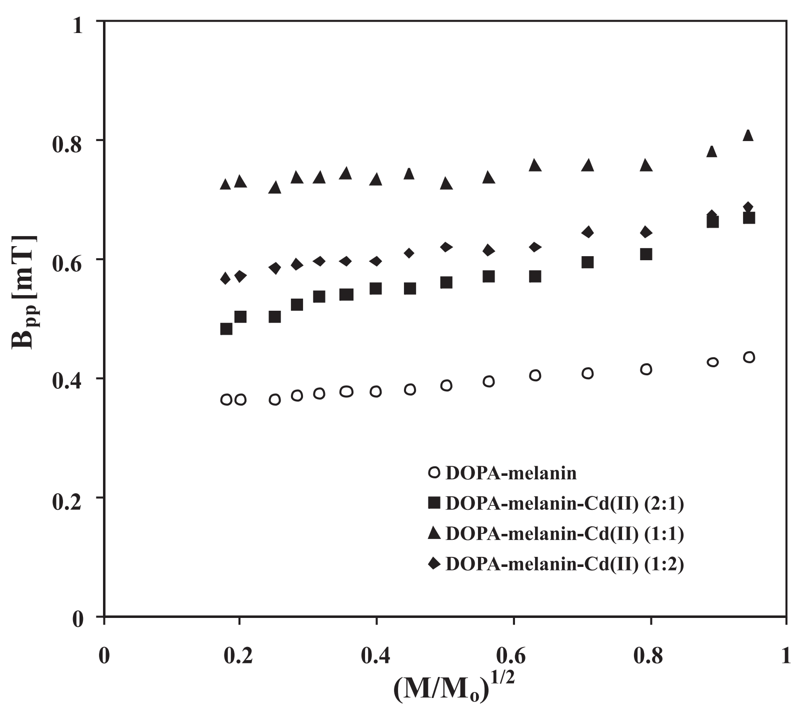

Figure 2. Influence of microwave power (M/M_o) on linewidth (ΔB_{pp}) of EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes. M, M_o - the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively

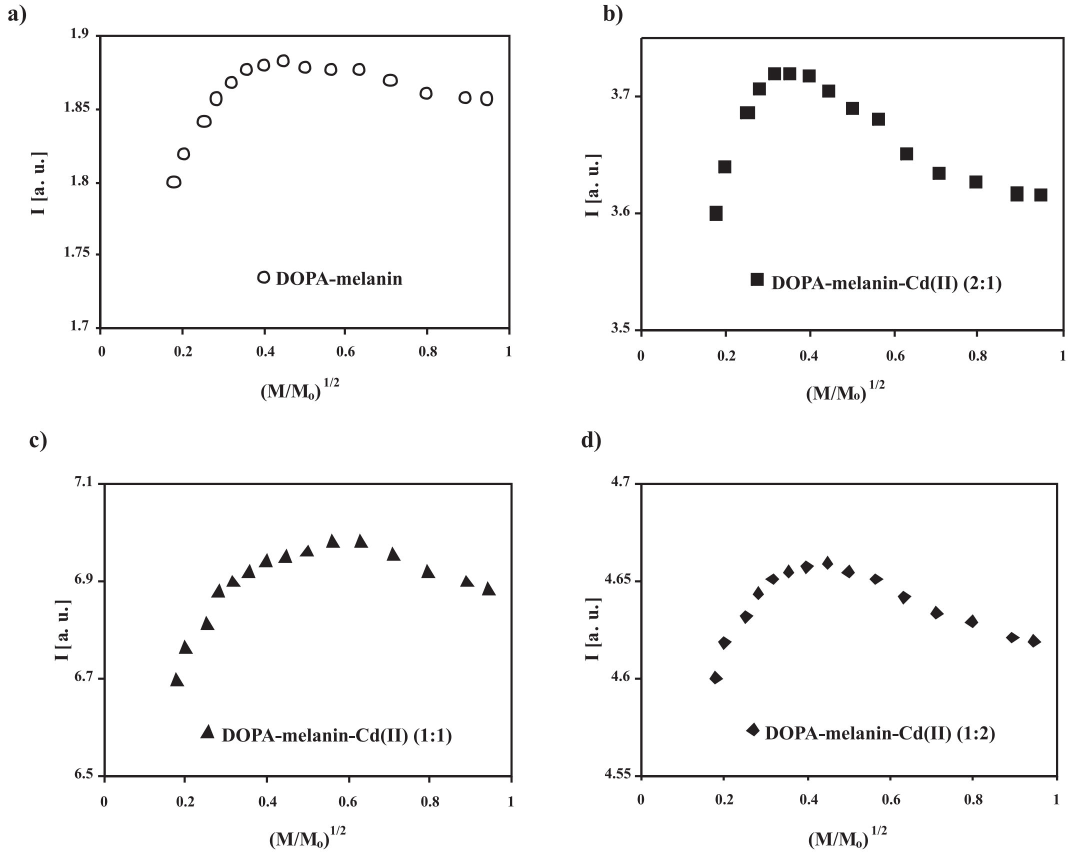

Figure 3. Influence of microwave power (M/M_o) on integral intensity (I) of EPR spectra of DOPA-melanin (a), DOPA-melanin-cadmium(II) (2 : 1) complexes (b), DOPA-melanin-cadmium(II) (1 : 1) complexes (c) and DOPA-melanin-cadmium(II) (1 : 2) complexes (d). M, M_o - the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively

ent on cadmium amount in melanin polymer (Fig. 2). Integral intensities increase with increasing microwave power, reach the maximum value, and after they decrease (Fig. 3). Such correlations between microwave power and both linewidths (Fig. 2) and integral intensities (Fig. 3) point out that the EPR lines of DOPA-melanin and DOPA-melanin-cadmium(II) complexes are homogeneously broadened.

The EPR lines of all the examined melanin samples are saturated at low microwave powers (Fig. 3), so slow spin-lattice relaxation processes exist in DOPA-melanin and DOPA-melanin complexes with cadmium(II). Cadmium(II) strongly affect spin-lattice relaxation processes in DOPA-melanin and change the values of microwave power of saturation. Integral intensities of EPR lines of DOPA-melanin-cadmium(II) complexes begin decrease with microwave power for its higher values (Fig. 3). The spin-relaxation processes in DOPA-melanin-cadmium(II) complexes (Fig. 3b-d) are relatively faster than in DOPA-melanin (Fig. 3a).

DISCUSSION AND CONCLUSIONS

The performed EPR examination of melanin samples are very important for drug binding to melanin polymer in the presence of cadmium(II). It is known that o-semiquinone free radicals in DOPA-melanin take a part in binding of drugs to its structure (1-11). Our studies point out an application of EPR spectroscopy to determine the type of free radicals in melanin complexes and their concentration in the samples, and to characterize magnetic interactions.

Diamagnetic cadmium(II) increases the free radical concentrations in DOPA-melanin (Table 1).

The high content of o-semiquinone free radical concentrations in DOPA-melanin-cadmium(II) complexes points out that probably the strong possibilities of binding of drugs *via* these paramagnetic centers exist. It is expected that participation of o-semiquinone free radicals in binding of drugs will increase similarly to an increase of free radical concentrations in melanin: DOPA-melanin-cadmium(II) (2 : 1) < DOPA-melanin-cadmium(II) (1 : 2) < DOPA-melanin-cadmium(II) (1 : 1) (Table 1). The lower free radical concentration in DOPA-melanin-cadmium(II) (1 : 2) (Table 1) is caused by recombination of free radicals during synthesis.

The increase of free radical concentration in melanin samples after complexing with cadmium(II) was observed by us earlier (15). Free radical concentration in DOPA-melanin complexes with cadmium(II) was ~10^{19} spin/g (15) and this value was lower than for DOPA-melanin complexes with cadmium(II) tested in this work (~10^{21}-10^{22} spin/g; Table 1). Differences between values of free radical concentrations result from different methods of obtaining the melanin samples. Complexes of cadmium(II) with DOPA-melanin in this work were formed during synthesis of this polymer ($CdCl_2$ was added to solution of L-DOPA in Tris-HCl buffer), whereas in previous work, cadmium(II) was added to DOPA-melanin after process of its synthesis.

The increase of free radical concentration in melanin after complexing with cadmium(II) may be responsible for major chemical reactions, because of chemical activity of free radicals containing unpaired electrons. Free radicals may interact with oxygen molecules in the melanin environment or with free radicals in others structures. Such reactions should be tested in the future.

In this work the effect of microwave power (2.2-70 mW) on the parameters of EPR lines of DOPA-melanin and its complexes with cadmium(II) (Figs. 2, 3) was compared. According to the theory of electron paramagnetic resonance, the effect of microwave power (M) on integral intensities and ΔB_{pp} of the EPR spectra depend on free radicals distribution (homogeneous or non-homogeneous) in chemical structure of the samples (20, 21). For homogeneous broadened EPR lines the integral intensity increases with increasing of microwave power and for the higher microwave powers its value decreases as the result of microwave saturation. The increase of ΔB_{pp} with increasing of microwave power is characteristic for the homogeneously broadened EPR lines. For non-homogeneous broadening of EPR lines, the integral intensity increases with increasing of microwave power (M), but for the higher microwave powers its value does not change. ΔB_{pp} of the non-homogeneously broadened EPR lines is constant and they do not depend on microwave power. Correlations presented in Figures 2 and 3 prove that the EPR lines of DOPA-melanin and DOPA-melanin complexes with cadmium(II) are homogeneously broadened. Homogeneous broadening of EPR lines of melanin was observed by us earlier (6, 7, 9, 10, 12, 13). Homogenous broadened EPR lines characterize DOPA-melanin-complexes with zinc(II) (6, 13), copper(II) (5, 6, 8, 9, 12, 13), and iron(III) (7). Homogeneously broadened EPR lines were measured for melanin complexes with netilmicin (6, 8), kanamycin (9, 12), and moxifloxacin, ciprofloxacin, lomefloxacin, norfloxacin and sparfloxacin (10, 11). Cadmium(II) changes magnetic interactions in DOPA-melanin. The broader EPR lines of DOPA-melanin-cadmium(II) complexes relative to DOPA-melanin (Table 1) indicate stronger dipolar interactions in DOPA-melanin after addition of cadmium(II). This effect is the highest for DOPA-melanin-cadmium(II) (1 : 1) complexes. Dipolar interactions increase with decreasing the distances between unpaired electrons of free radicals (20), so the short distances between o-semiquinone free radicals exist in DOPA-melanin-cadmium(II) (1 : 1) complexes. Dipolar interactions and distances between free radicals are relatively lower in DOPA-melanin-cadmium(II) (1 : 2) and DOPA-melanin-cadmium(II) (2 : 1) complexes with relatively narrower EPR lines (Table 1).

The changes of integral intensities (I) of EPR lines with microwave power (Fig. 3) are characteristic for slow spin-lattice relaxation processes. The slow and fast spin-lattice relaxation processes in the samples differ in microwave saturation of EPR lines (20). The higher power of microwave saturation of EPR lines reveals the samples with faster spin-lattice relaxation processes than the samples with the slow spin-lattice relaxation processes (20-22). Cadmium(II) in melanin causes fastening of spin-lattice relaxation processes, because the shift of microwave saturation of EPR lines to the higher values of microwave power is observed (Fig. 3b-d).

The several important conclusions for pharmacy may be drawn from our EPR studies of DOPA-melanin and DOPA-melanin-cadmium(II) complexes. Cadmium(II) increases free radical concentration in DOPA-melanin, so the binding ability of drugs to melanin *via* free radicals rises. This ability is the highest for DOPA-melanin-cadmium(II) (1 : 1) complexes, because of the highest o-semiquinone free radical concentration observed in these complexes. The relatively lower binding of drugs is expected for DOPA-melanin-cadmium(II) (1 : 2) complexes and DOPA-melanin-cadmium(II) (2 : 1) complexes with the lower free radical concentration compared to DOPA-melanin-cadmium(II) (1 : 1) complexes. The influence of cadmium(II) on the ability of melanin to binding drugs is only suggested by us, and it should be tested in the next works. Our suggestion results from our earlier EPR studies of melanin complexes with drugs: netilmicin, kanamycin, moxifloxacin, ciprofloxacin, lomefloxacin, norfloxacin and sparfloxacin (6, 8-12), where free radical contents in melanin changed after introducing of drug molecules to this polymer.

EPR examination broadens the knowledge about free radicals in DOPA-melanin complexes with cadmium(II) of different concentrations of these diamagnetic ions. All the tested complexes are strongly paramagnetic and the formation of o-semiquinone free radicals dominates in DOPA-melanin-cadmium(II) (2 : 1) and DOPA-melanin-cadmium(II) (1 : 1) complexes. Recombination of free radicals appears during complexation of DOPA-melanin with cadmium(II) for DOPA-melanin-cadmium(II) (1 : 2) complexes. Strong dipolar interactions and slow spin-lattice relaxation processes exist in all the tested melanin samples. Cadmium(II) changes both dipolar interactions and spin-lattice relaxation processes in DOPA-melanin. Dipolar interactions increase in DOPA-melanin complexes with cadmium(II) and faster spin-lattice processes appear, relatively to DOPA-melanin. EPR spectroscopy with microwave from the X-band (9.3 GHz) is the useful method to examine free radicals in melanin complexes with cadmium(II) ions. The fine example of application of EPR method in pharmacy was presented in this work.

Acknowledgment

These studies were financially supported by Medical University of Silesia in Katowice (grant no. KNW-1-005/K/4/0).

REFERENCES

1. Pasenkiewicz-Gierula M., Sealy R. C.: Biochim. Biophys. Acta 884, 510 (1986).
2. Sealy R. C., Hyde J. S., Felix C. C., Menon I. A., Prota G.: Science 217, 545 (1982).
3. Sarna T.: Current Topics in Biophysics (in Polish) 6, 201 (1981).
4. Buszman E.: Habilitation thesis. Medical University of Silesia in Katowice 1994.
5. Najder-Kozdrowska L., Pilawa B., Więckowski A. B., Buszman E., Wrześniok D.: Appl. Magn. Reson. 36, 81 (2009).
6. Zdybel M., Pilawa B., Buszman E., Wrześniok D.: Chem. Phys. Lett. 556, 278 (2013).
7. Zdybel M., Chodurek E., Pilawa B.: Appl. Magn. Reson. 40, 113 (2011).
8. Zdybel M., Pilawa B., Buszman E., Wrześniok D., Krzyminiewski R., Kruczyński Z.: Appl. Magn. Reson. 43, 341 (2012).
9. Najder-Kozdrowska L., Pilawa B., Buszman E., Więckowski A.B., Świątkowska L. et al.: Acta Phys. Pol. A 118, 613 (2010).
10. Beberok A., Buszman E., Zdybel M., Pilawa B., Wrześniok D.: Chem. Phys. Lett. 497, 115 (2010).
11. Beberok A., Zdybel M., Pilawa B., Buszman E., Wrześniok D.: Chem. Phys. Lett. 592, 41 (2014).
12. Najder-Kozdrowska L., Pilawa B., Więckowski A. B., Buszman E., Wrześniok D.: Acta Phys. Pol. A 124, 112 (2013).
13. Buszman E., Pilawa B., Zdybel M., Wilczyński S., Gondzik A. et al.: Sci. Total Environ. 363, 195 (2006).
14. Zdybel M., Pilawa B., Buszman E., Witoszyńska T.: Nukleonika 58, 401 (2013).
15. Matuszczyk M., Buszman E., Pilawa B., Witoszyńska T., Wilczok T.: Chem. Phys. Lett. 394, 366 (2004).
16. Krzywda A., Petelenz E., Michalczyk D., Płonka P. M.: Cell. Mol. Biol. Lett. 13, 130 (2008).
17. Plonka P. M., Michalczyk D., Popik M., Handjiski B., Paus R.: J. Dermatol. Sci. 49, 227 (2008).
18. Roulin A., Ducrest A.L.: Eur. J. Pharmacol. 660, 226 (2011).
19. Ito S., Wakamatsu K.: Photochem. Photobiol. 84, 582 (2008).
20. Wertz J.E., Bolton J.R.: Electron spin resonance: elementary theory and practical applications. Chapman and Hall, London 1986.
21. Eaton G.R., Eaton S.S., Salikhov K.M.: Foundations of modern EPR. World Scientific, Singapore 1998.
22. Stankowski J., Hilczer W.: Introduction to magnetic resonance spectroscopy (in Polish). PWN, Warszawa 2005.
23. Binns F., Chapman R.F., Robson N.C., Swan G.A., Waggott A.: J. Chem. Soc. 8, 1128 (1970).

Received: 6. 08. 2014