Electron paramagnetic resonance (EPR) study of DOPA–melanin complexes with kanamycin and copper(II) ions

Lidia Najder-Kozdrowska^{a,*}, Barbara Pilawa^b, Ewa Buszman^c, Dorota Wrześniok^c and Andrzej B. Więckowski^a

^a *Division of Spectroscopy of Functional Materials, Institute of Physics, Faculty of Physics and Astronomy, University of Zielona Góra, Zielona Góra, Poland*

^b *Department of Biophysics, School of Pharmacy, Medical University of Silesia, Sosnowiec, Poland*

^c *Department of Pharmaceutical Chemistry, School of Pharmacy, Medical University of Silesia, Sosnowiec, Poland*

Abstract. This work comprises the study of DOPA–melanin complexes with kanamycin and copper(II) ions made by electron paramagnetic resonance EPR spectroscopy. The high concentration of paramagnetic centers in melanin makes the use of EPR spectroscopy possible. The unpaired electron localized on the oxygen atom in indol-5,6-quinone groups is the paramagnetic centers in this polymer. The aim of this work was the analysis of EPR parameters of recorded spectra. For researches were prepared melanin complexes which differed in complexing order of drug and Cu(II) ions, and in concentration of CuCl₂ solutions used during synthesis. The introduction of drugs and metal ions into melanin causes changes in EPR parameters of recorded spectra. Kanamycin causes the increase and copper(II) ions the decrease of concentrations of paramagnetic centres in melanin. Shape of EPR signal of DOPA–melanin was analyzed by fitting method. Results indicate that EPR line of DOPA–melanin is superposition of Gaussian and Lorentzian function.

Keywords: EPR, DOPA–melanin, kanamycin, Cu(II)

1. Introduction

Electron paramagnetic resonance (EPR) is a very useful method for the study of melanin polymer [9,13,16,19]. Melanin contains a high concentration of paramagnetic centers. The unpaired electrons localized on the oxygen atoms in indol-5,6-quinone groups have the spin value $S = 1$ which obeys Curie law. Using the EPR method the existence of paramagnetic centres in thermally excited triplet state ($S = 1$) was proved [8]. Paramagnetic centers with spin $S = 1$ does not fulfil the Curie law. EPR method provides information on spin–spin and spin–lattice interactions in a sample. EPR is not invasive technique and do not influence on properties of melanin sample. The studies of melanin are making with natural and synthetic melanin [4,6–9,11,13,14,16,19]. Natural melanin exists in living organism. This polymer gives a colour for skin, ears and hair. Eumelanin gives a black and feomelanin a ginger

*Corresponding author: Dr. Lidia Najder-Kozdrowska, Division of Spectroscopy of Functional Materials, Institute of Physics, Faculty of Physics and Astronomy, University of Zielona Góra, Szafrana 4a, 65-516 Zielona Góra, Poland. Tel.: +48 68 328 29 05; E-mail: lkozdrowska@wp.pl.

colour. Pheomelanin contains sulphur and this element is responsible for this colour. All melanin have a high affinity to drugs and metal ions [4,7,9–11,19]. The mechanism of bonding drugs can have electrostatic character or can be caused by van der Waals forces. In melanin a role of bonding sites play carboxylic, orthophenolic, amine and hydroxyl groups. The mechanism of bonding drugs and metal ions with melanin has not been understood well so far. Metal ions change the concentrations of paramagnetic centres in melanin [4,9–11]. Paramagnetic ions cause decrease and diamagnetic ions cause increase of number of paramagnetic centres. After drug adding to melanin, the growth of the concentration of paramagnetic centers is observed. Melanin complexes with drug have a toxic influence on human organism (hyperpigmentation of the skin, inner ear lesions, hair blanching) [5,20]. Melanin reveals also insolubility and photo-protection. For our study we have used DOPA–melanin sample as the control, and its complexes with kanamycin and copper(II) ions. The aim of presented work was to analyse of the influence of kanamycin and Cu(II) on paramagnetic properties of melanin polymer and their impact on EPR parameters of melanin radicals. The fitting of experimental spectrum made the analysis of the lineshape by Gaussian and Lorentzian function.

2. Materials and methods

2.1. Samples

Synthetic DOPA–melanin was obtained according to the procedure given in paper [2]. The oxidative polymerisation of 3,4-dihydroxyphenylalanine (L-DOPA) in 1/15 M phosphate buffer at pH = 8 was performed for obtained synthetic DOPA–melanin. DOPA–melanin complexes with kanamycin and copper(II) ions are different in order of admixtures and in concentration of copper chloride CuCl_2 solution used during synthesis (0 , 1×10^{-5} , 5×10^{-5} , 1×10^{-4} , 5×10^{-4} , 1×10^{-3} M). The concentration of kanamycin was equal to 1×10^{-3} M in all cases. For researches were prepared 12 samples: DOPA–melanin, (DOPA–melanin)–kanamycin, five complexes [(DOPA–melanin)–kanamycin]–Cu(II) and five complexes [(DOPA–melanin)–Cu(II)]–kanamycin with different concentration of Cu(II) ions.

2.2. Analysis of drug and metal ion binding to melanin

The amounts of drug remaining in each filtrate after incubation with melanin with respect to the control samples were determined spectrophotometrically. All spectrophotometric measurements were performed by the use of JASCO model V-530, UV-VIS spectrophotometer. The amounts of metal ion bound to melanin with respect to the control samples were determined by the use of atomic absorption spectrophotometer type AAS 3 (Carl Zeiss, Jena). Control samples contained melanin and bidistilled water without ligand (drug or metal ion). The amounts of ligand bound to melanin, calculated as the difference between the initial amount of drug or metal ion administered to melanin and the amount of unbound ligand (in filtrate after incubation) [15], were expressed in moles of bound drug or metal ion per 1 mg melanin (Table 1).

2.3. EPR measurements

The EPR measurements were recorded using the spectrometer RADIOPAN Poznań at X-band (microwave frequency was 9.3 GHz). Modulation of magnetic field was 100 kHz. All spectra were recorded as the first derivative of absorption energy at room temperature. The measurements were done at the

Table 1
The amount of kanamycin and copper(II) ions bonded with melanin and in its complexes

Sample and concentrations of Cu(II) ions (M)		The amount of kanamycin and copper(II) ions bonded with melanin (μmol/mg)	
		Kanamycin	Cu(II) ions
(DOPA–melanin)–kanamycin	1×10^{-3}	0.4278	–
[(DOPA–melanin)–Cu]–kanamycin	1×10^{-5}	0.2096	0.0082
	5×10^{-5}	0.1886	0.0426
	1×10^{-4}	0.1623	0.0801
	5×10^{-4}	0.0678	0.3338
	1×10^{-3}	0.0520	0.5175
[(DOPA–melanin)–kanamycin]–Cu	1×10^{-5}	0.4051	0.0009
	5×10^{-5}	0.4031	0.0031
	1×10^{-4}	0.3997	0.0064
	5×10^{-4}	0.3955	0.0108
	1×10^{-3}	0.3907	0.0227

Notes: The concentration of drug in solutions was equal 1×10^{-3} M in each case. The concentrations of Cu(II) ions are given in the table.

value of microwave power 0.7 mW. The frequency of microwave radiation by MCM 101 recorder of EPRAD Firm (Poznań, Poland) was measured.

Spectroscopic splitting factor g was calculated from the condition of electron paramagnetic resonance [18]:

$$g = (h\nu)/\beta B_r, \quad (1)$$

where: h – Planck's constant, β – Bohr's magneton, ν – frequency of microwave light, B_r – magnetic resonance induction (mT).

The shape of EPR line of DOPA–melanin cannot be described only by Gaussian or Lorentzian function so it was fitted separately by Gaussian (G) and Lorentzian (L) function, and by their superposition ($G + G, L + L, G + L$):

$$Y_G = (-A_G)((B - B_{rG})/\Delta B_G^2) \exp(-0.5(((B - B_{rG})/\Delta B_G)^2)) - \text{Gaussian curve}, \quad (2)$$

$$Y_L = ((-2)A_L(B - B_{rL})/(\Delta B_L^2((1 + (((B - B_{rL})/\Delta B_L)^2))^2)) - \text{Lorentzian curve}, \quad (3)$$

where: A_G, A_L – the amplitude of absorption signal, B – magnetic induction, B_{rG}, B_{rL} – magnetic resonance induction, $\Delta B_G, \Delta B_L$ – linewidth.

The least squares method was used for determination of the quality of fitting by the value of χ^2 factor. The lowest value of χ^2 that means the best fitting. From this method we obtained the number and the shape of component lines.

The concentration of paramagnetic centres was calculated in accordance with the formula [12]:

$$N = n_u[(P_p/A_{rp}W_p m_p)/(P_u/A_{ru}W_u)],$$

where: n_u – the number of paramagnetic centers in ultramarine (was used as the reference of the concentration), P_p , P_u – the areas under the absorption curves for the sample and ultramarine, A_{rp} , A_{ru} – the amplitudes of the ruby signal for the sample and ultramarine (ruby crystal was used as the inner reference which is permanently placed in resonance cavity), W_p , W_u – the receiver gains for the sample and ultramarine, m_p – the mass of the sample. Areas under the absorption curves were obtained by double integration of the first derivative EPR spectra.

3. Results and discussions

3.1. The lineshape of DOPA–melanin

The EPR spectrum of DOPA–melanin is shown on Fig. 1. The obtained spectrum is lightly asymmetric. Its multicomponent character can cause this asymmetry. The analysis of lineshape provides information on interactions between paramagnetic centres in solid [1,17]. Mainly Gaussian and Lorentzian functions describe the shape of EPR spectrum. When spin–spin interactions exist and when there is a lack of exchange interactions, the EPR line is described by Gaussian function. When the exchange interaction exists, the EPR line is in Gaussian and Lorentzian type. Each of these functions, describe the EPR signal in appropriate range of magnetic field. The middle of the EPR signal has Lorentzian shape, and the wings are in Gaussian shape. The EPR signal of DOPA–melanin was recorded at low value of microwave power for avoidable of saturation phenomenon. The fitting results for Gaussian, Lorentzian and their superposition are presented on Figs 2–6. The fitting by Gaussian and Lorentzian function shows that shape of EPR signal of DOPA–melanin cannot be described by these function (Figs 2–3) what is according to work [3]. The shape of EPR signal of melanin must be superposition of Gaussian and Lorentzian function. The best solution of this problem for DOPA–melanin seems to be superposition of $(G + G)$ and $(G + L)$ functions (the smallest value of χ^2) (Figs 4–6). More probably is superpositions $(L + G)$. DOPA–melanin consist high concentrations of paramagnetic centres and exchange interaction can exist which are responsible for Lorentzian shape of part of EPR spectrum [1,17].

3.2. The parameters of EPR signals and the concentrations of melanin radicals

The obtained values of EPR linewidth of melanin radicals are in the range 0.36–0.43 mT (Table 2). Those values testify to moderate dipole–dipole interactions that are responsible for broadening of EPR lines [1,17]. The dipole–dipole interactions of unpaired electrons being in mutual distance r leads to broadening of the Zeemann energy levels about values $h/\beta^2 r^3$, where: h – Planck's constant, β – Bohr's magneton, r – the distance between electrons. The ordering of adding of drug and metal ions

Fig. 1. The EPR spectrum of DOPA–melanin. The spectra were recorded at microwave power 0.7 mW.

Fig. 2. The fitting of EPR spectrum of DOPA–melanin by Gaussian curve ($\chi^2 = 0.1293$).

Fig. 3. The fitting of EPR spectrum of DOPA–melanin by Lorentzian curve ($\chi^2 = 0.0677$).

Fig. 4. The fitting of EPR spectrum of DOPA–melanin by superposition of two Gaussian curves ($\chi^2 = 0.0119$).

Fig. 5. The fitting of EPR spectrum of DOPA–melanin by superposition of two Lorentzian curves ($\chi^2 = 0.0283$).

into melanin do not influence significant on linewidths of melanin radicals (Table 2). High value of spectroscopic splitting factor g (2.0042–2.0047) (Table 2) indicates to localization of unpaired elec-

Fig. 6. The fitting of EPR spectrum of DOPA–melanin by superposition of Gaussian and Lorentzian curves ($\chi^2 = 0.0115$).

Table 2

Linewidth of EPR signal ΔB_{pp} , spectroscopic splitting factor g , concentrations of paramagnetic centres N in studied melanin complexes in dependence on concentrations of Cu(II) ions in solution and on ordering of adding components

Sample	Concentrations of Cu(II) ions (M)	ΔB_{pp} (± 0.02 mT)	g (± 0.0002)	N (10^{18} spin/g)	Comparative concentration
DOPA–melanin	–	0.39	2.0045	13.2	1
(DOPA–melanin)–kanamycin	–	0.39	2.0044	15.3	1.16
[(DOPA–melanin)–Cu]–kanamycin	1×10^{-5}	0.39	2.0044	14.1	1.07
	5×10^{-5}	0.43	2.0045	10.2	0.77
	1×10^{-4}	0.42	2.0045	8.1	0.61
	5×10^{-4}	0.36	2.0044	3.0	0.23
	1×10^{-3}	0.36	2.0044	2.6	0.20
[(DOPA–melanin–kanamycin)]–Cu	1×10^{-5}	0.40	2.0045	14.3	1.08
	5×10^{-5}	0.37	2.0046	14.0	1.06
	1×10^{-4}	0.41	2.0047	10.4	0.79
	5×10^{-4}	0.39	2.0045	11.3	0.86
	1×10^{-3}	0.39	2.0045	10.4	0.79

Notes: Comparative concentrations of radicals versus DOPA–melanin sample. The concentration of kanamycin was equal 1×10^{-3} M. The EPR measurements were made in room temperature.

tron on oxygen atom in melanin polymer. The concentrations of paramagnetic centres in studied complexes are depended on ordering of adding drug and Cu(II) ions into melanin and on concentrations of metal ions in solutions using during synthesis (Table 2). Kanamycin causes increase and Cu(II) ions decrease of melanin paramagnetic centres. The concentrations of melanin radicals in [(DOPA–melanin)–kanamycin]–Cu(II) complexes are higher than in [(DOPA–melanin)–Cu(II)]–kanamycin complexes (Table 2). Kanamycin in [(DOPA–melanin)–kanamycin]–Cu(II) complexes blockades the bonding sites for Cu(II) ions which are responsible for decrease of concentrations of melanin radicals. The amount of bounded Cu(II) ions with melanin in [(DOPA–melanin)–kanamycin]–Cu(II) complexes is lower in comparison with [(DOPA–melanin)–Cu(II)]–kanamycin complexes (Table 1). The adding kanamycin into melanin caused increment of concentrations of melanin radicals about 16% (Table 2) in comparison with DOPA–melanin sample. Metal ions were added first to polymer and bonded with melanin through radical structures blockade bonding sites for drug. Radical structures in melanin are characterized by higher force of bonding than structures that have diamagnetism character. The highest decrease of concentrations of melanin radicals (about 83%) in comparison with (DOPA–melanin) is noticed for [(DOPA–

melanin)–Cu(II)]–kanamycin complex for concentrations of metal ions equal 1×10^{-3} M (Table 2). The increase of paramagnetic centers in DOPA–melanin was observed also for other aminoglycoside antibiotics [10]. Melanin polymer binds the antibiotic introduced to organism that leads to prolongation of its act on tissues. The generation of radicals in melanin by dint of aminoglycoside antibiotic is probably responsible for toxic effects that are incidental effect of activity of drugs on human organism. Radicals in (DOPA–melanin)–aminoglycoside antibiotics complexes initiate adverse biochemical reactions in human organism. By the using of EPR spectroscopy and by biomedical analysis was shown that aminoglycoside antibiotics inducing the strong toxic effects are responsible for generations of high amount radicals in melanin [10,11]. The exemplary EPR spectra of complexes of DOPA–melanin with kanamycin and copper(II) ions are shown on Fig. 7. The wide EPR signals are characteristic for the EPR signals of Cu(II) ions (over 7 mT). The value of g -factor for copper(II) ions is around 2.1. The value of amplitude of Cu(II) ions change with concentrations of metal ions in solutions using during synthesis of

Fig. 7. Exemplary spectra of DOPA–melanin complexes with Cu(II) ions and kanamycin differing on order of adding Cu(II) ions and drug. The concentration of Cu(II) ions was 1×10^{-3} M. The spectra were recorded at microwave power 0.7 mW.

Table 3
The amplitude A of EPR signal of Cu(II) ions in melanin complexes

Sample	Concentrations of Cu(II) ions (M)	Amplitude A (arb. u.)
[(DOPA–melanin–Cu)]–kanamycin	1×10^{-5}	–
	5×10^{-5}	2.0
	1×10^{-4}	2.4
	5×10^{-4}	6.6
	1×10^{-3}	8.6
[(DOPA–melanin)–kanamycin]–Cu	1×10^{-5}	–
	5×10^{-5}	–
	1×10^{-4}	0.2
	5×10^{-4}	2.1
	1×10^{-3}	3.2

Notes: The concentration of kanamycin was equal 1×10^{-3} M. The EPR measurements were made in room temperature.

samples, and with the order of adding drug and Cu(II) ions into melanin. The values of EPR amplitude of Cu(II) ions are shown in Table 3. The EPR signal of Cu(II) ions were not detected for the lowest concentrations of them in all prepared complexes (Table 3). The amplitude of EPR signal of copper(II) ions bonded with melanin polymer in [(DOPA–melanin)–Cu(II)]–kanamycin and [(DOPA–melanin)–kanamycin]–Cu(II) complexes grows with increase of concentration of Cu(II) (5×10^{-5} – 1×10^{-3} M) (Table 3). This effect is caused by the increment of amount of bonded Cu(II) ions with the increment of concentration of these ions used during preparation of samples (Table 1).

4. Conclusions

The analysis of lineshape of EPR signal of DOPA–melanin shows that its shape is the superposition of Gaussian and Lorentzian lines. Kanamycin and Cu(II) ions have influence on concentrations of radicals in melanin polymer. Kanamycin makes for increase and Cu(II) ions for decrease of them. The decline of paramagnetic centers in melanin was observed with increase of metal ions in solution. The bonding sites of melanin polymer participate in bonding of Cu(II) into melanin. The EPR study confirms the phenomenon of blocking of bonding sites in the structure of melanin polymer by kanamycin or copper(II) ions in dependence of ordering of admixture. The concentrations of paramagnetic centers in [(DOPA–melanin)–kanamycin]–Cu(II) are higher than in [(DOPA–melanin)–Cu(II)]–kanamycin complexes.

References

- [1] P.W. Anderson and P.R. Weiss, Exchange narrowing in paramagnetic resonance, *Rev. Mod. Phys.* **25**(1) (1953), 269–276.
- [2] F. Bins, R.F. Chapman, N.C. Robson, G.A. Swan and A. Waggoner, Studies related to the chemistry of melanins. Part VIII. The pyrrolecarboxylic acid formed by oxidations or hydrolysis of eumelanin derived from 3,4-dihydroxyphenethylamine or 3,4-dihydroxyphenylalanine, *J. Chem. Soc.* (1970), 1128–1134.
- [3] M.S. Blois, A.B. Zahlan and J.E. Maling, Electron spin resonance studies on melanin, *Biophys. J.* **4**(6) (1964), 471–490.
- [4] E. Buszman, B. Pilawa, T. Witoszyńska, M. Latocha and T. Wilczok, Effect of Zn^{2+} and Cu^{2+} on free radical properties of melanin from *Cladosporium cladosporioides*, *Appl. Magn. Reson.* **24** (2003), 401–407.
- [5] A. Forge and J. Schacht, Aminoglycoside antibiotics, *Audiol. Neurotol.* **5** (2002), 3–22.

- [6] W. Froncisz, T. Sarna and J.S. Hyde, Cu^{2+} probe of metal-ion binding sites in melanin using electron paramagnetic resonance, *Arch. Biochem. Biophys.* **202**(1) (1980), 289–303.
- [7] U. Mars and B.S. Larson, Pheomelanin as binding sites for drugs and chemicals, *Pigment Cell Res.* **12** (1999), 266–274.
- [8] L. Najder-Kozdrowska, B. Pilawa, E. Buszman, A.B. Więckowski, L. Świątkowska, D. Wrześniok and W. Wojtowicz, Triplet states in DOPA–melanin and in its complexes with kanamycin and copper(II) ions, *Acta Phys. Pol. A* **118**(4) (2010), 613–618.
- [9] L. Najder-Kozdrowska, B. Pilawa, A.B. Więckowski, E. Buszman and D. Wrześniok, Influence of copper(II) ions on radicals in DOPA–melanin, *App. Magn. Reson.* **36**(1) (2009), 81–88.
- [10] B. Pilawa, E. Buszman, D. Wrześniok, M. Latocha and T. Wilczok, Application of EPR spectroscopy to examination of gentamicin and kanamycin binding to DOPA–melanin, *Appl. Magn. Reson.* **23** (2003), 181–192.
- [11] B. Pilawa, M. Latocha, E. Buszman and T. Wilczok, Effect of oxygen on spin–spin and spin–lattice relaxation in DOPA–melanin complexes with chloroquine and metal ions, *Appl. Magn. Reson.* **25** (2003), 105–111.
- [12] B. Pilawa, A.B. Więckowski and S. Duber, EPR study of the bituminous coal, *Erdol. Kohle Erdgas Petrochemie Hydrocarbon Technol.* **43** (1990), 240–245.
- [13] P.M. Płonka, Electron paramagnetic resonance as a unique tool for skin and hair research, *Exp. Dermatol.* **18**(5) (2009), 472–484.
- [14] G. Prota, Some new aspects of eumelanin chemistry, *Prog. Clin. Biol. Res.* **256** (1998), 101–124.
- [15] M. Rizk and F. Younis, Spectrophotometric assay of certain aminoglycoside antibiotics using chloranil, *Annal. Lett.* **17** (1984), 1803–1809.
- [16] T. Sarna, J.M. Burke, W. Korytowski, M. Różanowska, C.M. Skumatz, A. Zareba and M. Zareba, Loss of melanin from human RPE with aging: possible role of melanin photooxidation, *Exp. Eye Res.* **76**(1) (2003), 89–98.
- [17] J.H. Van Vleck, The dipolar broadening of magnetic resonance lines in crystals, *Phys. Rev.* **74**(9) (1948), 1168–1183.
- [18] J.A. Weil, J.R. Bolton and J.E. Wertz, *Electron Paramagnetic Resonance*, Wiley, New York, 1994.
- [19] P.R. Williamson, K. Wakamatsu and S. Ito, Melanin biosynthesis in *Cryptococcus neoformans*, *J. Bacteriol.* **180**(6) (1998), 1570–1572.
- [20] D. Wrześniok, E. Buszman, J. Trzcionka and R. Różańska, Wiązanie streptomycyny i dihydrostreptomycyny do melaniny w aspekcie ototoksycznego działania aminoglikozydów, *Ann. Acad. Med.* **40** (1999), 63–76.