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NEW PARAMAGNETIC PROBES AND SINGLET OXYGEN FORMATION IN CELLS

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Paramagnetic oximetric probes have been examined in exemplary nasal polyps cells irradiated by laser without and with photosensitizer – chlorine e6. Laser irradiation causes excitation of oxygen O_2 from triplet to singlet state. Singlet oxygen damages pathological cells. The knowledge of singlet oxygen formation is important to determine the conditions of laser therapy. In this work new probes for oximetry in medicine have been proposed. As potential oximetric probes, coal samples thermally treated at temperatures (°C): 400, 500, 600, and 700, have been tested in cell cultures. In this study we used electron paramagnetic resonance (EPR) spectroscopy as the experimental technique. EPR spectrum of the oximetric probe should strongly depend on the amount of paramagnetic triplet oxygen molecules O_2 in cell cultures. It was proved that EPR spectrum of coal carbonized at 600°C is susceptible to paramagnetic O_2 molecules and it may be used in oximetry.

INTRODUCTION

Singlet oxygen molecules O_2 cause free radical reactions and toxic effects in living organisms [Bartosz, 2004; Jóźwiak, 2005; Jaroszyk, 2001; Antoszewski, Skalski & Skalska, 2004; Podbielska, Sieroń & Stręk, 2004; Rybak & Whitworth, 2005; Sha & Schacht, 1999; Terland, Alamas, Flamark, Andersson & Sorlie, 2006; Buszman, Wrześniok, Surażyński, Pałka & Molęda, 2006; Latocha, Pilawa, Chodurek, Buszman & Wilczok, 2006; Latocha, Pilawa, Zdybel & Wilczok, 2005; Latocha, Pilawa, Dudek, Biniszkiewicz, Kozdrowska, Sieroń & Wilczok, 2004; Eaton, Eaton & Salikhov, 1998]. Singlet oxygen is responsible for lipid peroxidation and it destroys cell membranes [Bartosz, 2004; Jóźwiak, 2005; Jaroszyk, 2001; Antoszewski, Skalski & Skalska, 2004; Eaton, Eaton & Salikhov, 1998]. Singlet oxygen plays an important role during photodynamic therapy (PDT) of tumor cells [Podbielska, Sieroń & Stręk, 2004]. PDT is accompanied by the following stages: 1) introducing of photosensitizer to tumor cells, 2) excitation of photosensitizer's molecules in cells by laser, 3) imparting of energy by photosensitizer to cell structures and oxygen molecules (formation of singlet O_2), 4) initiation of destructiwe free radicals processes in cells by singlet oxygen [Podbielska, Sieroń & Stręk, 2004]. Efficiency of photodynamic therapy increases with increasing of singlet oxygen molecules production in laser irradiated tumor cells. Excitation of triplet to singlet O_2 in cells depends on photosensitizer and time of laser irradiation. Knowledge about amount of reactive singlet O_2 molecules in cell cultures is necessary to determine optimal conditions of photodynamic processes.

Singlet oxygen molecules are diamagnetic and it is impossible to detect them directly by the use of electron paramagnetic resonance (EPR) spectroscopy [Wertz & Bolton, 1986; Stankowski & Hilczer, 2005; Kimse & Stach, 1994]. However diamagnetic O_2 concentration may be measured indirectly by EPR method and oximetric probes [Šentjurc, Čemažar & Serša, 2004; Pandian, Kutala, Parinandi, Zweier & Kuppusamy, 2003, Atsarkin, Demidov, Vasneva, Dzheparov, Ceroke, Odintsov & Clarkson, 2001; Santini, Cametti, Straface, Floridi, FFlamma, Paradise & Malorni, 1998; Manivannan, Yanagi, Ilangovan & Kuppusamy, 2001]. Paramagnetic samples ambient for cells and strongly interacting with paramagnetic O_2 molecules may be applied as oximetric probes in cells. Such probes are searched for and their interactions with O_2 are studied.

It is known that coal samples are paramagnetic [Smirnova, Smirnov, Clarkson & Belford, 1994; Pilawa & Więckowski, 2007; Pilawa, Więckowski, Pietrzak & Wachowska, 2007; Pilawa, Więckowski, Wachowska & Kozłowski, 2003; Pilawa, Pietrzak, Wachowska & Babeł, 2005; Pilawa & Więckowski, 1997] and some of them strongly interact with

paramagnetic oxygen molecules [Pilawa & Więckowski, 2007; Pilawa, Pietrzak, Wachowska & Babeł, 2005; Pilawa & Więckowski, 1997]. In this work we described a new type of paramagnetic probe for oximetry and its behavior in selected types of cells. In oximetry we used carbonized coals which revealed stable paramagnetism. The process of carbonization of coals is accompanied by an increase in the carbon atom contents in the samples and by condensation of aromatic rings to larger units. Probability of interactions between paramagnetic centers and O_2 rises for multi-ring aromatic units. We compared EPR spectra of carbonized coals in biological systems differ in level of singlet oxygen. We tested paramagnetic probes during laser irradiation of nasal polyps. Irradiation of cells at different conditions was examined.

MATERIALS AND METHODS

Samples

Cells of nasal polyps irradiated by laser were tested. The cells cultures were observed by a diverse OLYMPUS IX 50 Firm microscope (Figure 1). Microscopic image of nasal polyps is shown in Figure 2. Samples were obtained through preparation of cell cutlure from nasal polyps after polypectomies [Rostkowska-Nadolska, Kuśmierz, Kapral, Latocha, Świątkowska & Fraczek, 2007]. Testing was performed on the polyps cells after the sixth passage after the primary culture was established. The nasal polyps cells were grown in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) and 1000 U/ml penicillin and 100 mg/ml streptomycin. Cell cultures were maintained at 37 ^{o}C in 95 % air and 5 % CO_2. The cells at the confluent state were used in the experiment.

Photodynamic therapy of nasal polyps was done by the use of laser with power 500 mW and wavelength 662 nm produced by Kriotechnika Medyczna (Wrocław). The cells were grown in glass plates without photosensitizer and with the photosensitizer – chlorine e6. These control cells and the cells irradiated by laser for 15 min were examined (Figure 3). We used 0.01 mg/ml concentrations of chlorin e6. Chlorin e6 was added and the cells were incubated for 1 h. The volume of 4 mm^3 of cells was studied by EPR method. Chemical structure of chlorine e6 is shown in Figure 4. [Graczyk A. (Ed.), 1999].

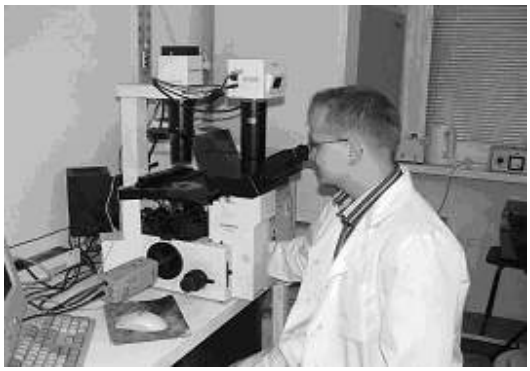

Fig. 1. Optical microscop Type OLYMPUS IX 50.

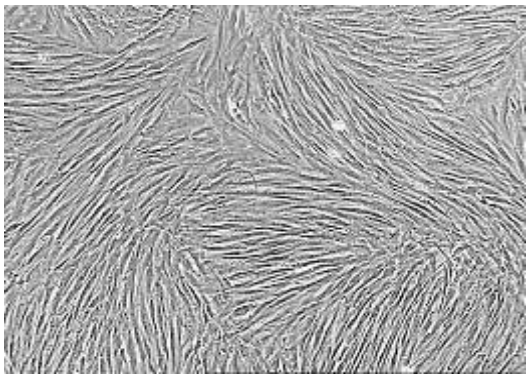

Fig. 2. Microscopic image of nasal polyps.

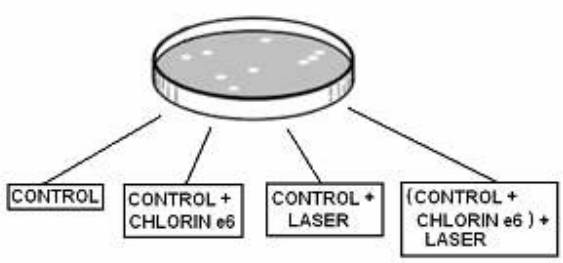

Fig. 3. Conditions of examination of nasal polyps cells.

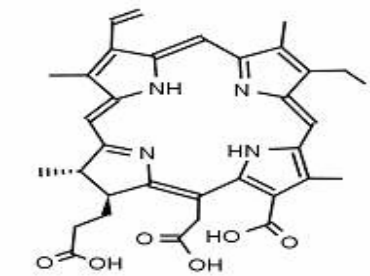

Fig. 4. Chemical structure of photosensitizer chlorine e6. [Graczyk A. (Ed.), 1999].

Natural medium-rank coal carbonized at temperatures (^{o}C): 400, 500, 600, and 700 were tested as oximetric probes. These individual coal probes (0.01 mg/ml) were located in cells cultures and their EPR spectra were measured. Coal probes were located in control culture, control culture with chlorine e6, control culture irradiated by laser, and in laser irradiated culture with chlorine e6.

Measurements

The spectra of coal probes were measured by an X-band (9.3 GHz) EPR spectrometer produced by RADIOPAN (Poznań) and Rapid Scan Unit produced by JAGMAR (Kraków) (Figure 5). The magnetic modulation was 100 kHz. To avoid signals saturation the first-derivative EPR spectra were recorded at a low microwave power of 0.7 mW.

Fig. 5. An X-band (9.3 GHz) EPR spectrometer of RADIOPAN Firm (Poznań) with Rapid Scan Unit of JAGMAR Firm (Kraków).

The following parameters of the EPR spectra were determined: amplitude (A), integral intensity (I), and linewidth (ΔB_{pp}). The concentration of the probe free radicals in the cells is proportional to the integral intensities. Integral intensities were normalized by dividing its value by mass of the probe studied. g-Values were calculated directly from the resonance condition according to the formula [Wertz & Bolton , 1986; Stankowski & Hilczer, 2005; Kirmse & Stach, 1994]

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

where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – resonance magnetic field. Microwave frequency (ν) was measured by an MCM 101 recorder produced by EPRAD Firm (Poznań).

The influence of the microwave power on the amplitudes and linewidths of the EPR spectra of the coal probes carbonized at 600^{o}C was determined. We compare the mentioned above correlations for the coal probe located in control cell culture, control cell culture with chlorine e6, cells irradiated without and with chlorine e6.

RESULTS AND DISCUSSION

Electron paramagnetic resonance spectra of exemplary coal sample obtained at 600^{o}C and located in control cell culture are shown in Figure 6. This spectrum with unresolved hyperfine structure is typical for coal samples. Similar EPR spectra were measured for the others probes obtained at 400, 500, and 700^{o}C. Only a very low EPR signal was observed for coal heated at 700^{o}C and located in cells, so we rejected this sample as oximetric probe.

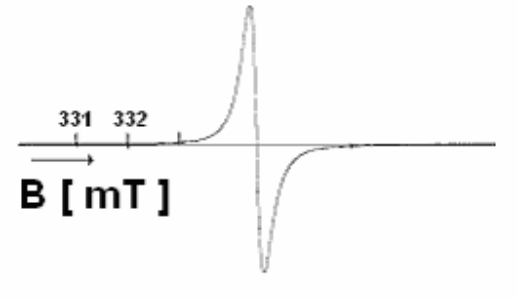

Fig. 6. EPR spectrum of coal probe carbonized at 600^{o}C for sample in control cell culture.

Parameters of the probes spectra located in control cells without and with chlorine e6, and cells irradiated without and with chlorine e6, are compared in Table 1. Amplitudes, integral intensities, linewidths and g-factors for the tested coal samples obtained by heating at 400, 500, 600 and 700^{o}C are presented. The same processes occur in all the studied probes used in the same conditions in cells culture. For good oximetric probe parameters of its EPR lines will change during PDT, because of changes of singlet oxygen concentration in environment. Probes with only weak changing EPR data during PDT should be rejected as oximetric probe.

Paramagnetic centers with low g-values exist in coal samples (Table 1). We expected that the unpaired electrons are located mainly on carbon atoms, and the low spin-orbit coupling constant is responsible for the detected g-values.

Broad EPR lines characterize the coals samples heated at 400^{o}C (ΔB_{pp}: 0.61-0.64 mT) and 500^{o}C (ΔB_{pp}: 0.56-0.59 mT) and located in the cell cultures (Table 1). Dipolar broadening is characteristic of the EPR lines of unpaired electrons located in coal units consisting of a few aromatic rings [Pilawa & Więckowski, 2007]. A comparison of the EPR data for the samples in air and in vacuum indicated that the paramagnetic centers of such

units weakly interact with paramagnetic oxygen atoms [Pilawa & Więckowski, 2007]. It was confirmed by our data for coal samples in the cell culture (Table 1). Paramagnetic samples heated at 400°C and 500°C were also rejected as oximetric probes (Table 1).

Table 1. Parameters of EPR lines of coal probes located in nasal polyps culture: amplitude (A), integral intensity (I), linewidth (ΔB_{pp}), and g-factor. Data for coal probes obtained during carbonization at different temperatures: 400°C, 500°C, 600°C, and 700°C. The individual coal probes were located in nasal polyps cultures: control, control with chlorine e6, control and irradiated by laser, control with chlorine e6 and irradiated by laser. Time of laser irradiation was 15 minutes.

PROBE	SAMPLE	A (±0.1) [a. u.]	I (±0.1) [a. u.]	ΔB_{pp} (±0.02) [mT]	g (±0.0002)
400 °C	Control	1.0	0.6	0.63	2.0031
	Control + e6	0.9	0.3	0.61	2.0031
	Control + laser	1.3	0.5	0.64	2.0031
	(Control + e6) + laser	1.2	0.4	0.64	2.0031
500 °C	Control	2.7	0.8	0.56	2.0028
	Control + e6	3.3	1.1	0.58	2.0027
	Control + laser	4.4	1.4	0.57	2.0028
	(Control + e6) + laser	3.0	1.0	0.59	2.0029
600 °C	Control	45.6	6.1	0.37	2.0031
	Control + e6	35.3	5.1	0.38	2.0031
	Control + laser	44.7	5.8	0.36	2.0029
	(Control + e6) + laser	30.1	4.0	0.36	2.0032
700 °C	Control	0.04	0.01	0.36	2.0025
	Control + e6	0.05	0.01	0.45	2.0026
	Control + laser	0.08	0.02	0.45	2.0026
	(Control + e6) + laser	0.06	0.01	0.47	2.0026

The linewidths of the EPR lines decrease for samples heated at higher temperatures: 600°C (ΔB_{pp}: 0.36-0.38 mT) and 700°C (ΔB_{pp}: 0.36-047 mT) (Table 1). Super-exchange interactions of unpaired electrons may be responsible for the narrowing of these EPR lines, which are located in multi-ring aromatic structures [Pilawa & Więckowski, 2007]. It is in agreement with our expectations, because the number of the aromatic rings in coal units increases with increasing carbonization temperature. Additional changes in EPR lines with microwave power confirmed the existence of large aromatic structures in the sample heated at 600°C (Figure 7). In figures 7 and 8 the influence of microwave power on amplitudes and linewidth of its EPR lines are shown, respectively. The EPR lines of the coal carbonized at 600°C and located in the cell culture do not saturate in the microwave power range used (up to 70 mW) (Fig. 7). It was stated earlier that EPR lines of coals with large multi-ring aromatic units saturate at high microwave powers [Pilawa & Więckowski, 2007]. Such correlations are also characteristic of the samples with fast spin-lattice relaxation processes [Wertz & Bolton , 1986; Stankowski & Hilczer, 2005]. EPR lines of this samples broaden for higher microwave powers (Fig. 8).

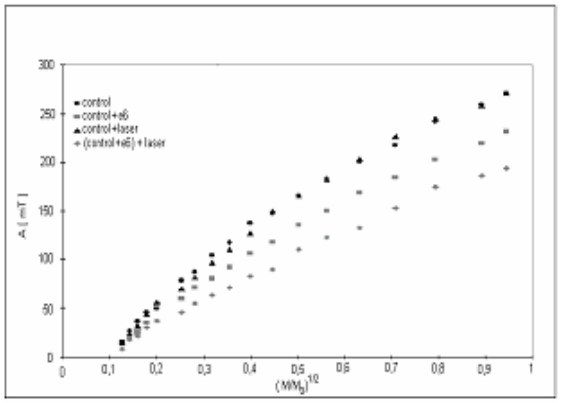

Fig. 7. Influence of microwave power on amplitudes (A) of EPR lines of coal probes obtained by carbonization at 600°C and located in cell cultures: control, control with chlorine e6, control and irradiated by laser, control with chlorine e6 and irradiated by laser, respectively. M – microwave power used during the measurement, and M_o - total microwave power produced by klystron (70 mW).

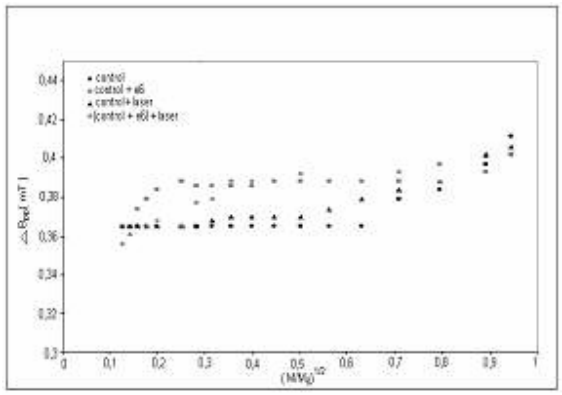

Fig. 8. Influence of microwave power on linewidths (ΔB_{pp}) of EPR lines of coal probes obtained by carbonization at 600°C and located in cell cultures: control, control with chlorine e6, control and irradiated by laser, control with chlorine e6 and irradiated by laser. M – microwave power used during the measurement, and M_o – total microwave power produced by klystron (70 mW).

Among other works our EPR studies of different coal samples [Pilawa & Więckowski, 2007] indicate that the paramagnetic centers located in large aromatic structures actively interact with the paramagnetic oxygen molecules O_2. This effect was also observed for coal heated at 600°C and located in cell cultures (Table 1, Figure 9). After irradiation by laser amplitude and integral intensity of its EPR lines decrease. The higher decrease in these EPR parameters was obtained for coal

located in cell culture with the photosensitizer – chlorine e6. The intensity of the EPR line of the coal probe located in the control cells culture was 6.1 [a.u], and it decreased to 4.0 [a.u] for the coal probe located in the cells irradiated with chlorine e6.

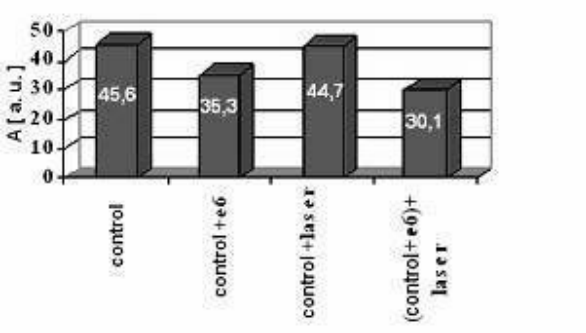

Fig. 9. Comparison of integral intensities (I) (±0.1) of EPR lines of coal probe carbonized at 600°C located in control culture (without and with chlorine e6), and in laser irradiated cells culture (without and with chlorine e6).

The quenching of the EPR lines of the coal sample carbonized at 600°C and located in cells cultures after laser irradiation is a result of recombination of free radicals generated by laser in the cells and the paramagnetic centers of the coal probe. During laser irradiation is excited oxygen O_2 from triplet to singlet state and as a consequence free radicals are formed in the cells. Free radicals of biological structures interact with the paramagnetic centers of the coal probe located in the cell culture, diamagnetic products of such reactions appear and the EPR lines of the coal probe decrease. We can say that the laser irradiation of the nasal polyps in the presence of chlorine e6 brings positive results of photodynamic therapy, because large amount of both singlet oxygen and free radicals are formed in the cells.

Only a very low EPR signal was observed for the coal sample heated at 700°C (Table 1) and located in the cells, so we rejected this sample as an oximetric probe.

The EPR studies performed indicate that selected coal samples may be useful in medicine and oximetry. It was shown that the paramagnetic coal samples containing large aromatic-units can act as good oximetric probes. The coal oximetric probes proposed by us are relatively easy to obtain in carbonization process.

CONCLUSIONS

Electron paramagnetic resonance examination of the coal probes located in nasal polyps cultures indicates that:

1) Coal samples carbonized at 400, 500, and 700°C were rejected as oximetric probes. Paramagnetic centers of the coal samples carbonized at 400°C and 500°C only weakly interact with paramagnetic O_2. Only a very low EPR signals were measured for coal carbonized at 700°C.

2) The coal sample carbonized at 600°C is proposed as an oximetric probe, because of strong interactions with paramagnetic oxygen molecules O_2 and the highest integral intensity of its EPR line for sample located in control cell culture.

3) Microwave saturation of EPR lines indicates that fast spin-lattice relaxation processes occur in coal probe carbonized at 600°C. Such a fast relaxation is characteristic of unpaired electrons in large multi-ring aromatic units in coal structure.

4) Laser irradiation of polyps both without photosensitizer and with chlorine e6 causes a decrease in the intensity of the EPR signal of coal probes carbonized at 600°C as a results of recombination of paramagnetic centers of this coal sample and free radicals formed by singlet oxygen generated during photodynamic processes.

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