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Carbon Blacks as EPR Sensors for Localized Measurements of Tissue Oxygenation

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New electron paramagnetic resonance (EPR) oximetry probes were identified in the class of carbon black materials. These compounds exhibit very high oxygen sensitivity and favorable EPR characteristics for biological applications. At low pO_2, the linewidth is particularly sensitive to changes in oxygen tension (sensitivity of 750 mG/mmHg). The application of the probes for oximetry was demonstrated in vivo: the pO_2 was measured in muscle in which the blood flow was temporarily restricted as well as in tumor-bearing mice during a carbogen breathing challenge. The responsiveness to pO_2 was stable in muscle for at least 3 months. No toxicity was observed using these materials in cellular experiments and in histological studies performed 2, 7, and 28 days after implantation. In view of their EPR characteristics (high sensitivity) as well as the well-characterized production procedure that make them available on a large scale, these probes can be considered as very promising tools for future developments in EPR oximetry. Magn Reson Med 51:1272–1278, 2004. © 2004 Wiley-Liss, Inc.

Key words: EPR oximetry; carbon; oxygenation; vivo; free radicals

Tissue oxygenation is an important parameter related to various physiological and pathophysiological processes in biological systems (1). Oxygen also plays a role in therapeutics, where the efficiency of radiotherapy depends on the oxygen tension in tumors (2). The assessment of tissue oxygenation is, therefore, of great physiological and clinical interest, and numerous methods have been developed to measure this parameter. The two classical methods involve the insertion of probes inside the tissues, with the measurement of partial pressure of oxygen (pO_2) carried out by polarographic electrodes (Eppendorf, Hamburg, Germany) (3) or fiber optics based on the fluorescence quenching of a ruthenium dye (OxyLite, Oxford Optronix, Oxford, UK) (4). However, each time a measurement is required the probes must be inserted into the location of interest. Several noninvasive methods based on MR principles have been developed: ^{19}F NMR spectroscopy (5,6), blood oxygen level-dependent (BOLD) imaging (7,8), EPR oxygen mapping (9), and Overhauser methods (10–12). In the EPR-based and Overhauser-based methods, it is possible to use soluble paramagnetic materials such as nitroxides or trityl radicals (10–13) or particulate materials. Although the spatial distribution of oxygen can be determined using the soluble materials, they have the limitation that they are metabolically converted to diamagnetic species or cleared by excretion (12). Solid probes are usually metabolically inert, and are characterized by a higher sensitivity for oximetry. Once introduced inside the tissue, the particles enable repeated measurement of pO_2 from the same site for days and weeks after implantation. Within the last few years, several new paramagnetic particulate materials have been found to exhibit a pO_2-dependent EPR linewidth: lithium phthalocyanine crystals (14), lithium naphthalocyanine crystals (15), particles of natural coals such as fusinite (16) or gloxy (17), analytical charcoals (18), and synthetic carbohydrate chars (19). In vivo EPR oximetry has already produced very useful results that have contributed significantly to solving important biological problems (20–27). It would be of major interest to transpose this technique into clinical practice. For this purpose, there is a critical need for paramagnetic sensors that are fully biocompatible. Although various strategies exist to improve the biocompatibility of the existing sensors (28–31), it would be easier to use a material that is already being used in human subjects. This was first achieved in the pioneering work of H.M. Swartz' group using India inks (32,33). This group demonstrated that it was feasible to measure pO_2 in tissues using inks, and carried out the first human EPR oximetry study on a volunteer with an extensive tattoo on his upper arm. Although these studies raised great hope for the immediate application of India ink as an oxygen sensor for clinical use, further development was hampered by the suboptimal EPR characteristics of these first probes, especially the low spin density and consequently the low signal-to-noise ratio (SNR) obtained in vivo. Together with H.M. Swartz' group, we have recently tried to select materials with more optimal EPR properties. Several dozen drawing-ink and tattoo inks were screened for the presence of paramagnetic materials, oxygen sensitivity, and performance in vivo (B. Gallez et al., in prep.). Interesting results were obtained, and it is likely that some of the materials will be further investigated for possible use in human EPR oximetry. However, it also appeared that EPR properties can vary from one batch to another within the same trademark of commercially available inks. Therefore, in order to achieve a long-term availability of the optimal EPR sensor(s) and distribute it through the EPR community, it would be very desirable to isolate the pure active component from ink that is sensitive to oxygen. The aim of the present study was to identify carbon blacks as possible EPR oxygen sensors. Carbon blacks are produced by the incomplete combustion or the thermal decomposition of aromatic oils or other hydrocarbons. Several classes of carbon blacks can be obtained from different production methods: gas

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Table 1
EPR Properties of the Carbon Blacks

No EPR signal	EPR signal Linewidth not oxygen sensitive	EPR signal Linewidth oxygen sensitive
From Degussa-Hüls	From Degussa-Hüls	From Degussa-Hüls
Printex 30	Printex XE2	Printex U
	Printex 35	Printex 140U
From Cabot	Printex 55	Spezialschwarz 4
BP880/No.068/P337905	Spezialschwarz 6	
Eltex 460	Spezialschwarz 350	
United 120	Spezialschwarz 550	
Elttex 570	Spezialschwarz FW2	
Elften TP	Spezialschwarz 160	
BP 800		
Ketjen Black EC 600	From Cabot	
Black pearl P 1000/GP 299B	CAB-O-JET 200	
Black Pearl L/GP3505	PANI/18168/60	
Black Pearls 1400/GP 3278	Black Pearl 2000	
Black Pearls 3700/CS 3862	R400/No. 039	
Black Pearls 1300/GP 3544	Denka Black	
Black Pearls 1000/GP 2996	Vulcan P	
Ketjen EC 600 JD	Mogul h 170	
Graphite EP 1010	N330	
Graphite EP 1040	Y50A/SN2A	
CAB-O-JET solid		
ENSACO 350	From Columbian Chemicals	
	Raven 760	
	Raven1225	
	Raven7000	
	Cond.Sc.Utra Powder	

blacks, furnace blacks, and lamp blacks. The three production processes result in a wide range of different carbon blacks with well-characterized particle size, structure, surface area, rheology, and electrical conductivity. We screened the three classes of carbon blacks for the presence of oxygen-sensitive paramagnetic centers. The steps used for the screening were: 1) to check for the presence of paramagnetic centers in the material; 2) to measure the sensitivity of the EPR linewidth to variations in oxygen environment and calibrate the oxygen-sensitive materials; 3) to test the sensitivity and the stability of responsiveness in animals; 4) to evaluate the usefulness of the probes for monitoring variations of pO_2 in a murine tumor model; and 5) to test the cell and tissue toxicity of the oxygen-sensitive materials.

MATERIALS AND METHODS

Carbon Blacks

Forty-three carbon blacks were analyzed: 12 were purchased from Degussa-Hüls (Frankfurt, Germany), four from Columbian Chemicals (Marietta, GA), and 27 from Cabott (Billerica, MA). The list of the compounds tested is given in Table 1.

X-Band EPR Spectroscopy

The calibration measurements (EPR linewidths as a function of the pO_2) were performed at 9.3 GHz with a Bruker (Fremont, CA) EMX EPR spectrometer equipped with a variable temperature controller BVT-3000. Suspensions of particles were placed in a gas-permeable Teflon tube (0.625 mm inner diameter; 0.05 mm wall). This tube was folded twice at both ends and placed in a quartz tube open at both ends. Gas with known concentrations of nitrogen and air (obtained using a gas mixer; Aalborg Instruments, Orangeburg, NY), equilibrated at 37°C, was flushed over the samples and the spectra were recorded every 5 min until equilibration was achieved. The oxygen content in the gas was analyzed using a Servomex oxygen analyzer OA540. Typical spectrometer parameters were modulation amplitude less than one-third of the peak-to-peak linewidth, and incident microwave power 1 mW.

Table 2
Properties of the Oxygen Responding Carbon Blacks

Carbon black	EPR linewidth (mT)			BET surface area* m^2/g	Particle size* nm
	0% oxygen	1% oxygen	21% oxygen		
Printex U	0.116	0.639	3.360	100	25
Printex 140	0.108	0.597	3.350	90	29
Specialschwarz 4	0.081	0.100	0.203	180	25

*Data provided by Degussa Huls.

In Vivo EPR Measurements

In vivo EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. Typical spectrometer parameters were as follows: modulation amplitude less than one-third of the peak-to-peak linewidth; scan range 1–2 mT. Male NMRI mice were used for this study. The mice (five mice/type of suspension) were injected in the gastrocnemius muscle with a 50 μl suspension of particles (100 mg/ml). The EPR measurements were started the day after the injection and repeated for 3 months to determine the reproducibility and the stability of the EPR linewidth of the paramagnetic material. The mice were anesthetized by intraperitoneal injection of a mixture containing xylazine (8 mg/kg) and ketamine (80 mg/kg). Local hypoxia was induced by restriction of the blood supply in the muscle; the base of the thigh was reversibly tied with elastic to restrict flow through the femoral arteries. Measurements were started 1–2 min after inducing the ischemia. The muscle under study was placed in the center of an extended loop resonator (1-cm depth sensitivity) (34). The linewidth of the EPR spectrum was measured using these typical spectrometer parameters: modulation amplitude, 0.023 mT; scan range, 1 mT; scan time, 1 min. Experiments were also carried out to study the responsiveness of the sensor in a tumor model during a carbogen breathing challenge. The experimental tumors were grown intramuscularly after injection of ascites cells of a transplantable mouse liver tumor model (TLT) into the gastrocnemius muscle (35). These tumors have been characterized for pO_2 and blood flow (23,25). Tumors were injected with 25 μl (2.5 mg) of carbon blacks when they reached 7–8 mm in diameter (7–8 days after inoculation). The breathing challenge (20 min air breathing / 10 min carbogen breathing / 25 min air) was carried out 2 days after implantation of the sensor.

Cytotoxicity Assay

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The carbon black suspension at 50 mg/ml in saline water was diluted 250 times in DMEM and autoclaved (121°C, 20 min). The effect was evaluated using the tetrazolium salt MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltatrazolium bromide; Aldrich, Milwaukee, WI) colorimetric method based on the cleavage of the reagent by mitochondrial dehydrogenase in viable cells. Briefly, 5000 cells/well were seeded in 100 μl of medium in 96-well microculture plates for 24 h. After 24 h, the medium was replaced by 100 μl of suspension of carbon black. Each sample was tested in 8 wells. After 24 h incubation, the medium was replaced by 100 μl DMEM containing 10 μl of MTT solution (3 m/ml in PBS). After 45 min in the incubator, the medium was removed and 100 μl of DMSO was added to each well. The plates were shacked and absorbances were recorded at two wavelengths (570 nm and 650 nm; Molecular Devices, Eugene, OR, Vmax Kinetics Microplate Reader), against a background blank as control (DMEM plus 10 μl of MTT solution in the absence of cells). Camptothecin (Sigma, St. Louis, MO) at 10μM was used as positive control. The relative absorbances were expressed as percent of the control.

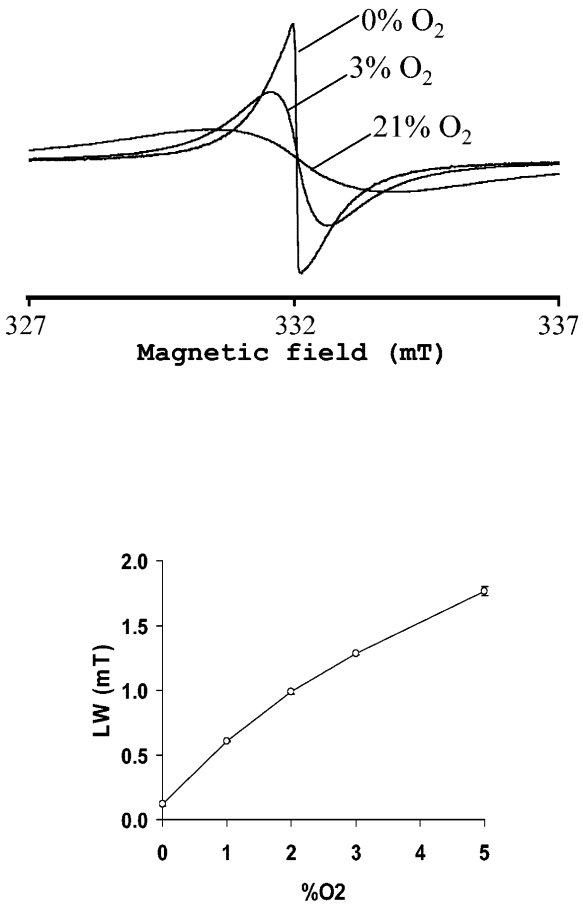

FIG. 1. Sensitivity of the EPR linewidth to the oxygen environment. Results obtained using Printex U. Top: EPR spectra recorded in X-Band (9 GHz) in different oxygen environments. Bottom: Calibration curve of the Printex U in a muscle homogenate.

Histology

Male NMRI mice were injected with 50 μl (50 mg/ml) of a suspension of carbon black (Printex U and Printex 140) in the gastrocnemius muscle. Two mice per compound and time were injected. On days 2, 7, and 28 the mice were sacrificed by cervical dislocation and the muscle was removed carefully. The muscles were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin.

RESULTS AND DISCUSSION

EPR Characterization / Oxygen Sensitivity of the Carbon Blacks

The results of the EPR properties of the carbon blacks are summarized in Table 1. Of 43 different carbon blacks studied, 19 did not contain paramagnetic centers detectable by 9 GHz EPR spectroscopy at 37°C. Twenty-four

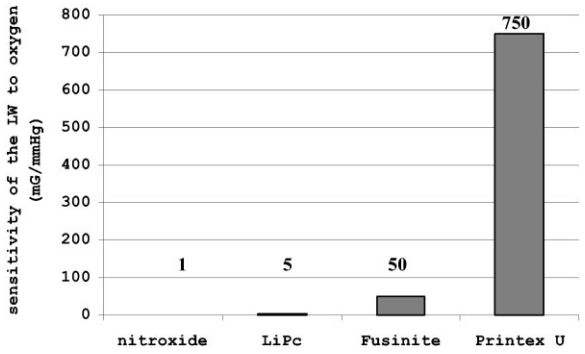

FIG. 2. Comparison of the sensitivity of several EPR oximetry probes at low pO_2. Results are expressed in terms of variation of the EPR linewidth per mmHg, assuming a linear response between 0% oxygen and 1% oxygen. Note the very high sensitivity of the Printex U compared to other paramagnetic probes.

carbon blacks presented EPR spectra that were typical of carbon-centered radicals (g-value around 2). Only three carbon blacks presented large variations of the linewidth dependent on the oxygen environment. The spin density for these three carbon blacks was $\sim 5 \times 10^{18}$ spins/g, which is a little bit lower than the amount found in other oxygen-sensitive materials such as chars and fusinite (36). The characteristics of these three carbon blacks are shown in Table 2. The presence of molecular oxygen broadened the EPR spectrum of these carbon blacks, as illustrated in Fig. 1. The calibration curve (linewidth as a function of the pO_2) of one carbon black (Printex U) is also shown in Fig. 1. This relationship is curvilinear, with a higher sensitivity to oxygen observed at low pO_2, a feature that has been observed using other carbon materials such as chars and charcoals (16–18). Printex 140 had very similar responsiveness to Printex U, while Specialschwarz 4 had a lower sensitivity (Table 2). The sensitivity of Printex U and Printex 140 is remarkably high. Assuming a quasilinear response between 0% oxygen and 1% oxygen, we can compare the sensitivity of different materials that have been used in EPR oximetry. In Fig. 2 the relative sensitivity, expressed in mG/mmHg is compared for nitroxides, lithium phthalocyanine, fusinite, and Printex U. Printex U is ~750 times more sensitive than nitroxides, 150 times more sensitive than lithium phthalocyanine, and 15 times more sensitive than fusinite. It has been reported that other materials such as glucose char (37) or wood chars (38) present an even higher sensitivity to oxygen. However, up to now, most chars described lose their oxygen sensitivity in vivo, except when an adequate coating covers the surface of the particles (30,31). In this study, the linewidth was simply measured as the peak-to-peak width of the first derivative spectra. When deconvoluted, the EPR spectrum at 0% oxygen (86% Lorentzian) suggests the presence of at least two types of paramagnetic centers, and the proportion of Lorentzian shape tends to decrease when oxygen increases in the environment of the sensor. The presence of two types of paramagnetic center has been described also in other materials such as wood chars (39). In this case, it was shown that the mechanism of oxygen response was dependent on a mutual and reversible transformation of these paramagnetic centers. In the future, it would be interesting to see if the oxygen sensitivity of the carbon blacks could be described according to the model of Atsarkin et al. (39) for carbon-based sensors.

Although we do not know yet the features that are necessary for a carbon black to be oxygen sensitive, it is interesting to note that the only carbon blacks that presented a very high oxygen sensitivity were produced by the gas blacks process. The carbon black types differ in their production process, which results in a very specific and distinctive set of characteristics that make them suitable for a very narrowly defined purpose. The gas black process is a thermal-oxidative decomposition of hydrocarbons where the main raw materials are distillates from coal tar. The raw material used for pigment black production evaporates directly before pyrolysis during gas black production. Due to unrestricted contact of flames with air, the gas black process always yields oxygenated and acidic surfaces. The hydrophilic surface character of this pigment class exhibits good wetting properties. The gas blacks are characterized by a loose structure and a high dispersibility, and they are now used almost exclusively in pigment applications. In the future, it would be interesting to compare the production procedures as well as the characteristics of the carbon blacks and the carbohydrate chars in order to improve the rationale for producing oxygen-sensitive materials.

Oxygen Responsiveness In Vivo

The typical EPR spectra of Printex U recorded in vivo after implantation in the gastrocnemius muscle of mice are

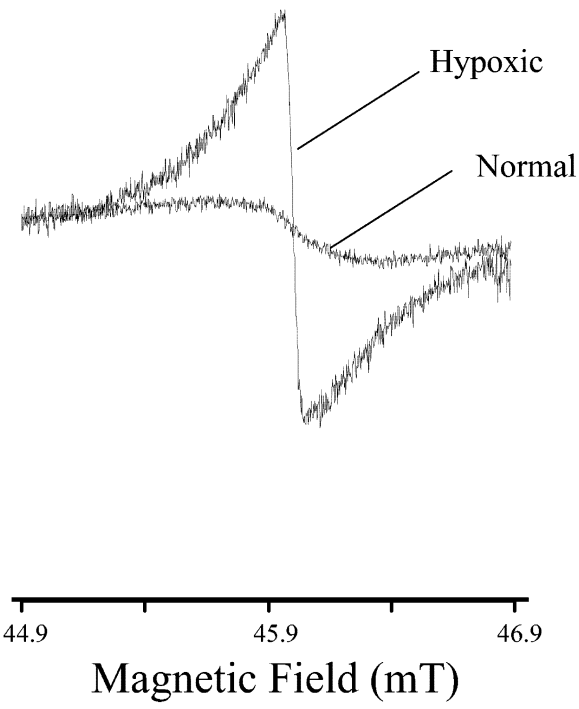

FIG. 3. EPR spectra recorded in vivo using 1.2 GHz spectrometer in the gastrocnemius muscle of an anesthetized mouse before (normal) and after restriction of the blood flow (hypoxic). Note the large variation in EPR linewidth.

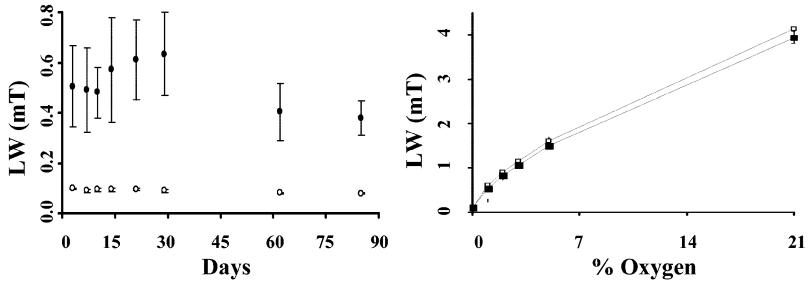

FIG. 4. Stability of the responsiveness to oxygen in vivo. The carbon black Printex U was implanted in the gastrocnemius muscle of mice. The EPR linewidth was recorded before (filled circles) and after temporary restriction of the blood supply (open circles). The results are expressed as mean linewidth ± SD. Note the constancy of the responsiveness over time (for 3 months). Note also that the SD is higher for the measurements recorded in normoxic muscles than for those recorded in hypoxic muscles. This could be explained by the lower SNR when the EPR linewidth is large, i.e., at high pO_2.

presented in Fig. 3. A dramatic change in the EPR linewidth was observed when the blood supply in the muscle was temporarily interrupted. We monitored the stability of the responsiveness of Printex U over 3 months by repeating experiments with temporary ischemia. The results are presented in Fig. 4. We observed that the responsiveness to oxygen was stable over time. We also observed no change in the calibration curve built using a muscle homogenate in which the carbon black stayed for 1 month, compared to a freshly prepared suspension of carbon black. This feature is very important in the context of new EPR oxygen-sensitive probes, as most compounds that have been characterized up to now are unstable in certain situations (14,15,30,31). The long period of stability that we observed makes carbon blacks excellent sensors for monitoring pO_2 in a chronic situation. In Fig. 4, it is also interesting to note that the standard deviation on the linewidth measurements was higher when they were recorded in normal muscles rather than in hypoxic muscles. This might be due to the very broad linewidth recorded in these conditions (higher than 5 Gauss), resulting in a lower SNR and a lower accuracy when measuring the linewidth. This feature is the natural consequence (and drawback) of the very large oxygen sensitivity. The variation in the pO_2 measured might also be due to variations in the depth of anesthesia. For future work, we believe it will be possible to modify the oxygen sensitivity by covering the particles with a convenient coating. We previously found that the calibration curve of some other carbon materials could be modulated by specific coating (29). It is likely that the most interesting applications for these materials will be in tissues where a low pO_2 is expected, for example, in tumors, as they are more sensitive and easy to detect in these conditions. Therefore, to illustrate a possible application, we also carried out experiments in tumors implanted in mice. A carbogen breathing challenge was applied in order to monitor the local responsiveness of the tumor when breathing this oxygen-enriched gas. A typical example is shown in Fig. 5, demonstrating the possible application of monitoring the changes in tumor pO_2 after treatments (i.e., the increase in pO_2 when breathing carbogen, and then the return to the initial pO_2).

Biocompatibility of Carbon Blacks

As the materials are very small (25 nm), they may be internalized in cells. Therefore, it was necessary to check their potential toxicity. We used the MTT test at the highest concentration possible for carbon black compatible with this colorimetric assay. At higher concentrations, several washing cycles were needed and cells detached from their support, leading to a high variability in the absor-

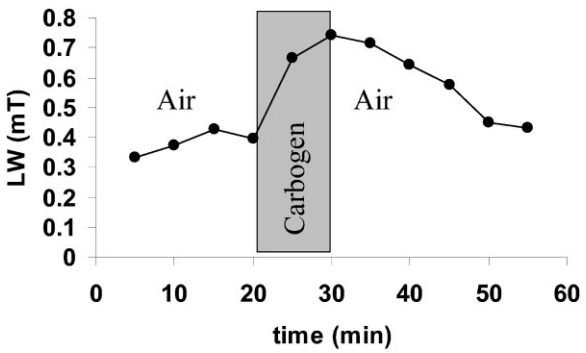

FIG. 5. Modulation of the EPR linewidth in an TLT tumor by breathing carbogen. The breathing gas was switched from air to carbogen (95% O_2 / 5% CO_2) and then switched back to air.

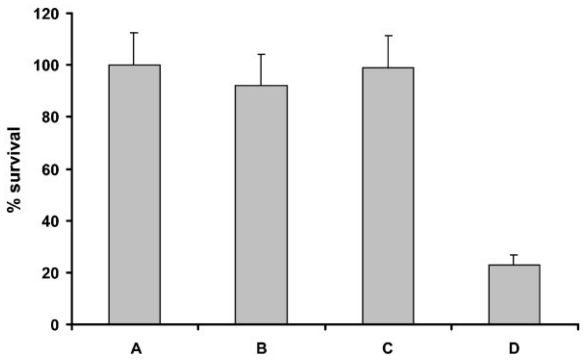

FIG. 6. Cytotoxicity assay (MTT test). Percentage of cell survival after incubation of the HeLa cells in the presence of: (**a**) DMEM medium (control); (**b**) Printex U; (**c**) Printex 140; (**d**) camptothecin (positive control). See Materials and Methods for the conditions of the test.

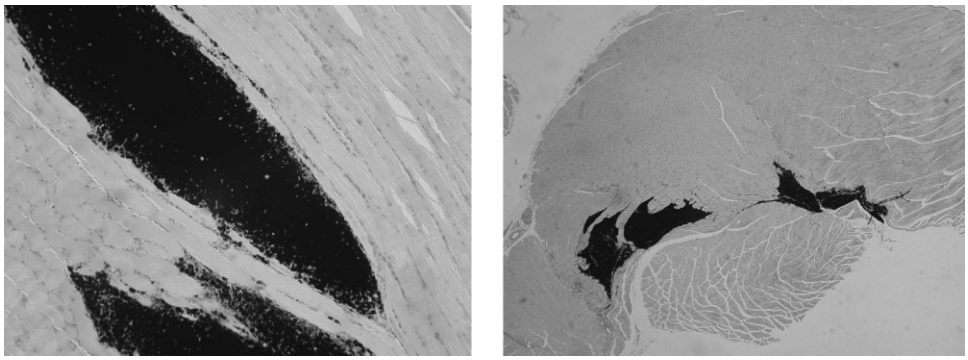

FIG. 7. Histological section of a mouse muscle after implantation of the carbon black Printex U. On the left: Muscle studied 2 days after injection (enlargement ×100). On the right: Muscle studied 28 days after injection (enlargement ×25). Note the absence of toxicity (intact muscle cells) and the very small (if any) inflammatory reaction.

bance values (for carbon blacks and controls). Under the conditions of the experiments, we did not find any significant difference (*t*-test, $P > 0.05$) between control cells and cells exposed to Printex U or Printex 140 (Fig. 6), while the positive control (camptothecin at 10 μM) induced the expected toxicity. A complementary histological study was performed. Figure 7 shows the muscle tissue 2 days and 28 days after implantation of the carbon black Printex U inside the tissue. A very small stromal inflammatory reaction was observed. No sign of toxicity or necrosis was observed in the muscle cells surrounding the carbon black. Overall, both tests indicate that these carbon blacks are well tolerated by living systems.

CONCLUSION

In conclusion, we identified new particulate EPR oxygen sensors in the class of carbon blacks. The most interesting characteristics of these compounds include a very high oxygen sensitivity, long-term stability of responsiveness in tissues, a well-characterized production procedure that makes them available on a large scale for the EPR community, and an absence of toxicity. These features make them especially useful for monitoring the oxygen level inside tissues that possess a low partial pressure of oxygen, i.e., tumors.

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