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Development and evaluation of biocompatible inks for the local measurement of oxygen using *in vivo* EPR

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ABSTRACT: *In vivo* EPR oximetry is a powerful minimally invasive method that allows the measurement of oxygen in tissues through the use of a paramagnetic probe. In the present study, we investigated new strategies for preparing biocompatible inks containing carbon black particles (Printex U), which could be used as oxygen sensors. The carbon black particles were dispersed in solutions of biocompatible polymers of carboxy methyl cellulose (CMC), hydroxypropyl methyl cellulose (HPMC) or polyvinyl pyrrolidone (PVP). A total of 12 polymers with different molecular weights were tested. A physico-chemical characterization of the inks was carried out to assess the sedimentation of the particles, the rheological behavior of these inks, and the relative diffusion of the inks. The preparations with CMC and PVP had the highest viscosity and stability. The presence of the polymers did not modify the calibration curves (EPR linewidth as a function of the pO_2) of the carbon black. *In vivo*, the oxygen sensors were stable for at least one month in muscles as the EPR linewidth remained fully sensitive to induced ischemia or carbogen challenge. The calibration curve was not modified after this period of implantation. A first study of biocompatibility was carried out *in vitro* (hemolysis and cytotoxicity assay) and *in vivo* (histological examination). No sign of toxicity was observed using these inks. These preparations are good candidates for future *in vivo* studies including clinical trials. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: EPR; oximetry; carbon black; ink; *in vivo*; biocompatibility; oxygen

INTRODUCTION

The measurement of oxygen in tissues is of great physiological and pathophysiological interest because of the fundamental role played by oxygen in numerous biochemical reactions.[1] Oxygen is also a key parameter in cancer therapies, since the tumor response to radiotherapy is strongly dependent on the partial pressure of oxygen (pO_2).[2] Therefore, numerous tools have been developed for the assessment of the pO_2 in biological systems. These methods include invasive ones, such as the polarographic electrodes[3] or techniques based on the fluorescence quenching of a ruthenium dye (Oxylite®).[4] Some non-invasive NMR methods have also been developed and used. These include ^{19}F NMR spectroscopy[5,6] and blood oxygen level-dependent (BOLD) imaging.[7,8] In addition, a highly sensitive and non-invasive measurement of oxygen can be achieved using EPR and Overhauser-based methods. These methods involve the use of a paramagnetic sensor that reports on its oxygen environment. Two kinds of paramagnetic materials can be used: particulate materials and soluble materials such as nitroxides or trityl radicals.[9–12] Particulate materials are usually inert and possess a higher sensitivity for oxygen than soluble materials. Once particulate materials are implanted in tissue, it is possible to make repeated localized measurements over long periods of time. Recently, several new paramagnetic particulate materials have been found to exhibit a pO_2-dependent EPR linewidth: lithium phthalocyanine crystals,[13] lithium naphthalocyanine crystals,[14] natural charcoals such as fusinite[15] or gloxy,[16] analytical charcoals,[17,18] and synthetic carbohydrate chars.[19]

Another type of particulate material that is particularly interesting can be found in India inks, which are suspensions of carbon black (CB) particles. The first EPR oximetry experiments in humans were in fact carried out using these inks.[20,21] These experiments raised great hope for immediate application of Indian ink as an oxygen sensor for clinical use, since these inks were already used for tattoos and surgery markings. Unfortunately, the EPR characteristics of these first inks were not

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Abbreviations used: CB, carbon black; CMC, carboxy methyl cellulose; HMPC, hydroxypropyl methyl cellulose; PVP, polyvinyl pyrrolidone.

optimal (low density of spins, multi-component signal). This stimulated a considerable amount of research that was carried out by us and HM Swartz's group to identify new sensors among commercially available materials. Some interesting compounds were identified. However, it turned out that EPR properties varied from one batch to another within the same trademark. Another unexpected problem with some inks was the significant inflammatory response of the host tissue after injection. In preliminary studies, we found that extraction procedures to remove the adjuvant in the India ink were able to abolish the inflammatory response. We were therefore convinced that the toxic reaction observed with some inks was not dependent on the CB particles but could be ascribed to the adjuvants that are present to stabilize the dispersion of the particles without any purpose of biocompatibility. In the old-fashioned India inks, the CBs were embedded by gelatin or arabic gum.

In order to isolate pure oxygen-sensitive CB and to achieve a long-term availability of oxygen sensors, we recently carried out a very large screening experiment on these types of materials.[22] These experiments demonstrated that, within the 43 CBs analyzed, two compounds (Printex U and Printex 140 from Degussa-Hüls) had the most interesting EPR properties, including a high sensitivity of the linewidth to the changes in pO_2.

The aim of the present study was to develop a 'home made' ink based on the use of Printex U carbon black particles dispersed in biocompatible suspending agents which are already used in injectable pharmaceuticals. The role of the polymers will be: (1) to increase the viscosity of the samples in order to stabilize the suspension and to improve the reproducibility in sampling and injecting; and (2) to achieve a higher biocompatibility than commercial inks. Twelve polymers that were used in this study were based on the cellulose structure, such as carboxy methyl cellulose (CMC) or hydroxypropyl methyl cellulose (HPMC); a synthetic polymer, polyvinyl pyrrolidone (PVP) was also used. The steps used to develop the inks were: (1) to produce injectable suspensions of Printex U (synthetic inks); (2) to study the physico-chemical characteristics of the inks; (3) to test the sensitivity and the stability of responsiveness of the inks in animals; and (4) to test cell, blood and tissue toxicity of the inks.

EXPERIMENTAL

Chemicals

Carbon black Printex U was purchased from Degussa-Hüls (Frankfurt, Germany). The polymers used in the study, as well as their characteristics, are described in Table 1.

Ink formulation

Polymers were solubilized in NaCl 0.9%. The CB was then added and the suspension was homogenized using a vortex. The inks were sterilized by an autoclave (121°C, 20 min). The injectability of the solution was checked using a syringe and a 23 G needle. The highest possible concentration of polymer and CB which permitted injection was determined for these experiments.

Stability

Ten milliliters of freshly prepared inks were put into Falcon tubes (15 ml). The sedimentation was observed and noted over the course of 5 days. The effect of sedimentation was also simulated by centrifugation (Beckman J-21) at 115, 1030, 2850, 6430, 11430 and 16450 g for 10 min at room temperature. Sedimentation, if present, was noted. Resuspension properties and visual

Table 1. Polymers used in this study and their physical characteristics

	Viscosity (mPa s, w/v)	Molecular weight	Origin	Concentration (%)[a]
CMC 1	10–55 (4%)	n.m.[b]	Sigma-Aldrich	8
CMC 2	50–200 (4%)	90 000	Sigma-Aldrich	1.6
CMC 3	500–2000 (4%)	n.m.	Sigma-Aldrich	0.25
CMC 4	700–1500 (1%)	n.m.	Sigma-Aldrich	0.25
CMC 5	2500–6000 (1%)	700 000	Sigma-Aldrich	0.05
CMC 6	3000–6000 (1%)	n.m.	Sigma-Aldrich	0.32
HPMC 1	5 (2%)	10 000	Sigma-Aldrich	5
HPMC 2	4000 (2%)	86 000	Sigma-Aldrich	0.4
HPMC 3	100 000 (2%)	120 000	Sigma-Aldrich	0.08
PVP 1	350–600 (40%)	40 000	Sigma-Aldrich	40
PVP 2	n.m.	700 000	Vel	2
PVP 3	n.m.	1300 000	Sigma-Aldrich	0.8

[a] The concentration corresponds to the highest concentration of polymers that conferred the maximal viscosity to the suspension while remaining injectable through a 23 G needle. The concentrations are given as percentages weight/volume.
[b] n.m., not mentioned.

aspects were observed by inverting the tube 20 times after maximal centrifugation.

Diffusion

Experiments were performed at room temperature both in a protein hydrophilic gel (gelatin) and in isolated bovine muscle. The gelatin (2.5% W/V in distilled water) was prepared with water at 60 °C until homogenization, then cooled and used after gelification. An injection of 50 µl was performed with a micropipette in the gel, which was then covered with Parafilm®. The diffusion was noted after 48 h. Fifty microliters were injected into the muscle using a syringe with a 23 G needle. Ten minutes after injection, the bovine muscle was cut at the injection site along the axis of penetration of the needle, and the diffusion of the black material was measured.

Rheology

Measurements were made using a rotary rheometer (Rheomat RM 180). The inks were poured into two cylinders. One of these cylinders was rotated around its axis with different velocities, producing variable shear stresses and resulting in liquid deformations. Ten minutes after the inks were set into the rheometer, the shear rates of the sample were measured at 25 ± 2 °C using a thermostatic bath.

X-band EPR spectroscopy

Calibration measurements (EPR linewidth as a function of the pO_2) were performed at 9.3 GHz using a Bruker EMX EPR spectrometer equipped with a variable temperature controler BVT-3000. The modulation amplitude was less than one-third of the linewidth in all experiments. The inks where placed in a gas-permeable Teflon tube (0.625 mm inner diameter; 0.05 mm wall). This tube was folded at both ends and placed in a quartz tube open at both ends. Gas with known concentrations of nitrogen and air (Aalborg gas mixer, Orangenburg, NJ, USA) was analyzed by a Servomex Oxygen Analyzer OA 540, equilibrated at 37 °C, and flushed (400 l/h) over the samples. Calibration curves of inks were obtained from freshly prepared muscle homogenates (1 ml) doped with inks (100 µl), and from muscle homogenates which had been doped *in vivo* 1 month before. To ensure the equilibrium inside the sample, the spectra were acquired after 10 min of equilibrium.

L-band EPR spectroscopy

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 equipped with an extended loop resonator. Typical spectrometer parameters were as follows: modulation amplitude less than one-third of the linewidth (measured peak-to-peak); a scan range of 2–5 mT; and a scan time of 1 min. The NMRI male mice (Animalerie Facultaire, UCL, Brussels; five mice per ink) were injected in the gastrocnemius muscle with 100 µl of ink. Measurements were done under anesthesia induced by an intra-peritoneal (i.p.) injection of a mixture of ketamine (80 mg/kg) an xylazine (8 mg/kg). The EPR measurements started the third day following the injection and were repeated for 1 month to determine the reproducibility and the stability of the EPR linewidth of the paramagnetic probe. Local hypoxia was induced by restriction of the blood supply in the muscle. The base of the thigh was reversibly tied with a rubber elastic in order to restrict the blood flow in the femoral arteries. The muscle under study was placed in the center of the extended loop resonator (1 cm depth sensitivity). Measurements were started 2–3 min after inducing ischemia. Experiments were also carried out to study the responsiveness of the sensor in a muscle during a carbogen breathing challenge. This was done one month after injection of the sensor (100 µl suspension) in the gastronemius muscle of NMRI male mice. The breathing challenge (20 min air breathing, 10 min carbogen breathing, 30 min air) was done under anesthesia. Measurements were performed every 10 min during the first air breathing and then every 5 min after the beginning of the carbogen breathing.

Hemolysis assay

The protocol used is adapted from the American Society for Testing Materials (ASTM) Test F 756-00 'Standard Practice for Assessment of Hemolytic Properties of Materials'. Blood samples mixed with PBS buffer (pH = 7; 1/8 blood; 7/8 PBS) were prepared and placed (8 ml each) in glass tubes (1.6×12.5 cm). Three tubes were used for each sample, blank and controls. Inks (2 ml/tube), blank (PBS, 2 ml/tube), positive control (copper plate $1.4 \times 7.5 \times 2$ cm) and negative control (polyethylene (USP 88) $1.4 \times 7.5 \times 2$ cm) were added to the tubes. The tubes were closed with Parafilm® and incubated at 37 °C for 3 h. The tubes were gently inverted every 30 min. The content of each tube was transferred into a Falcon tube (15 ml) and centrifuged at 740 g (Hermle Z 320 K) for 10 min at room temperature. Six milliliters of supernatant were removed and then centrifuged at 27 000***g*** (Kontron Instruments Centrikon T-1160) for 15 min at room temperature. One milliliter of supernatant was taken and the optical density (OD) of the samples was measured with a spectrophotometer at 540 nm (Kontron Instrument Uvikon 940).

Cytotoxicity assay

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) 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 inks (50 mg/ml in saline water) were diluted 250 times in DMEM and autoclaved (121°C, 20 min). The cytotoxic effect was evaluated using the tetrazolium salt MMT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Aldrich) colorimetric method based on the cleavage of the reagent by mitochondrial dehydrogenase in viable cells. Briefly, 5000 cells per 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 diluted inks. Each sample was tested in eight wells. After 24 h incubation, the medium was replaced by 100 µl DMEM containing 10 µl of MTT solution (3 mg/ml in PBS). After 45 min in the incubator, the medium was removed and 100 µl of DMSO were added to each well. The plates were shacked and absorbances were recorded at 570 and 650 nm light (Molecular Devices, Vmax Kinetics Microplate Reader), against a background control (DMEM plus 10 µl of MTT solution without cells). Camptothecin (Sigma) at 10 µm was used as the positive control. The relative absorbances were expressed as the percentages of the control.

Histology

Male NMRI mice were injected with 40 µl (50 mg/ml) of artificial ink in the gastronemius muscle. For comparison, other mice were also injected using Pelikan A®, a commercial ink that contains the same carbon black. This ink was also sterilized by autoclave before injection. Two mice per compound and per time interval were injected. On day 2, 7, 28 the mice were sacrificed by cervical dislocation, and the muscle was removed carefully. The muscle was fixed in 10% formalin, embedded in paraffin and stained with hematoxylin–eosin. Histology analyses were performed on a microscope (Nikon Alphashot-2 Y52) with magnification 100, 200 and 400 times.

RESULTS

Characterization of inks

The concentration of 50 mg/ml Printex U proved to be a good compromise between ease of injection (using a 23 G needle) and sufficient spin density (good signal-to-noise ratio, SNR). We used the highest concentration of polymers that conferred the maximal viscosity to the suspension while remaining injectable through a 23 G needle. The concentrations of polymers are indicated in Table 1.

The stability of the suspensions was examined macroscopically by examining the sedimentation of the particles. The suspension of Printex U without suspending agents was not stable: a sedimentation was observed in the first 24 h. A complete sedimentation (phase separation) was also observed after a low speed centrifugation (115 g). Almost all inks prepared with biocompatible polymers were stable for at least 5 days without any sign of sedimentation, and were resistant to centrifugation with acceleration higher than 6430 g. The exceptions were the inks that used HPMC 2, which sedimented after 24 h without centrifugation.

The results of the diffusion experiments are presented in Fig. 1. We observed that inks which used low-molecular-weight (MW) polymers exhibited a large diffusion in the gelatin as well as in the muscle. The inks containing high-MW polymers showed less diffusion, similar to the diffusion observed with a suspension of Printex U alone. The rheological characteristics of the inks are presented in Fig. 2. Inks containing CMC and PVP polymers displayed a pseudo-plastic behavior: they

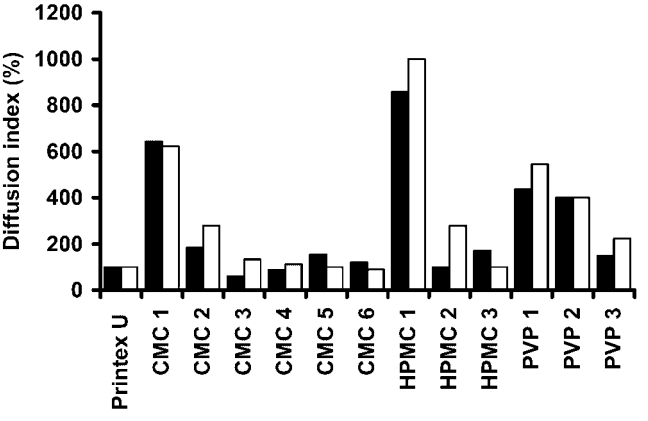

Figure 1. Diffusion index of inks (compared to Printex U) after injection into gelatin (black) and into muscle (white)

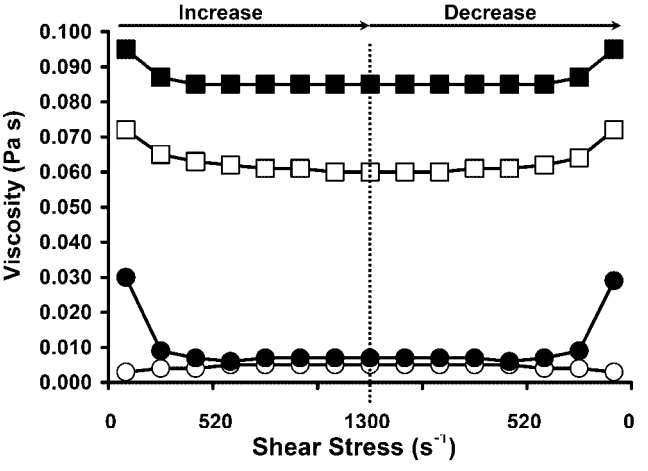

Figure 2. Rheological behavior of the different polymer classes. (■) PVP 1; (□) CMC 1; (●) Printex U; (○) HPMC 2

showed a decrease in viscosity when the shear stress increased. Interestingly, we observed that the viscosity of a suspension of Printex U (around 10 mPa s without polymers) was increased several times with polymers such as CMC and PVP. A suspension of Printex U without suspending agent was also apparently pseudoplastic. Unexpectedly, inks containing the HPMC polymers displayed a Newtonian behavior: the viscosity did not change with the fluctuating shear stress. HMPC did not confer any advantage in terms of stability compared to the water suspension of Printex U without dispersing agent.

EPR characterization

In our previous screening on CBs, we learned that the EPR linewidth of Printex U has a very high oxygen sensitivity. Here, we found that the calibration curves were not changed when the particles were dispersed in polymers (data not shown). More interestingly, the calibration curves were not different between the freshly prepared muscle homogenates doped with inks and the muscle homogenates in which the inks had remained for 1 month *in vivo* (Fig. 3).

In vivo experiments confirmed the stability of responsiveness of the inks. By repeating experiments for one month with temporarily induced ischemia, we found out a dramatic decrease in EPR linewidth after interruption of the blood flow. When a carbogen breathing challenge was applied to increase the muscle oxygenation, a significant increase in EPR linewidth was observed (Fig. 4).

Biocompatibility test

In the MTT assay, no significant cytotoxicity was observed for all preparations containing Printex U (with or without coating). Camptothecin, used as a positive control, induced the expected cytotoxicity (Fig. 5).

In the hemolysis assay, a slight hemolytic property was observed compared to the negative control (Polyethylene USP 88). However, this effect was very small compared with positive controls (Fig. 6). Interestingly, the hemolytic properties were significantly reduced by the use of some polymers, such as CMC (Fig. 6).

Typical results from the histology study are presented in Fig. 7. This shows the comparison between the muscle sections 2, 7 and 28 days after injection of Pelikan A, a commercial ink, and Printex U suspension. No sign of toxicity or necrosis surrounding the carbon black at the site of injection was observed using our preparation. A very slight inflammatory response was observed for some preparations. At day 28, few macrophages were observed surrounding the ink. By contrast, the commercial ink Pelikan A® induced a large inflammatory reaction observed at days 2 and 7, and even necrosis (cytoplasm

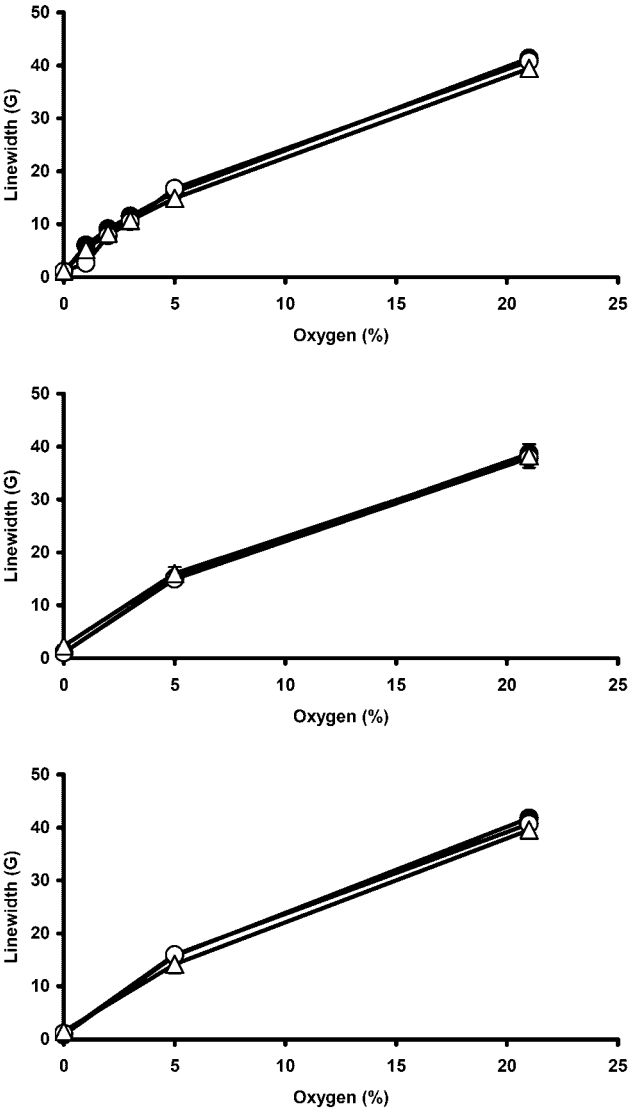

Figure 3. Calibration curves of the EPR linewidth as a function of the oxygen content. Top: Printex U without suspending agent. Middle: PVP 2 used as dispersing agent. Bottom: CMC3 used as dispersing agent. Calibration curves of inks were obtained from suspensions in water (●), from freshly prepared muscle homogenates doped with inks (○), and from muscle homogenates which had been doped *in vivo* 1 month before (Δ)

without structure, absence of nucleus, hypereosinophily). At day 28 there were areas with empty vacuoles replacing the dead cells. The responses observed in the muscles were quite similar from one polymer to another, showing that the chosen polymers are well tolerated by the organism. The diffusion observed inside the muscle corresponded well with the results obtained with the diffusion test.

DISCUSSION

There are at least four reasons for coating or encapsulating oxygen sensitive paramagnetic materials:[23] (1) to

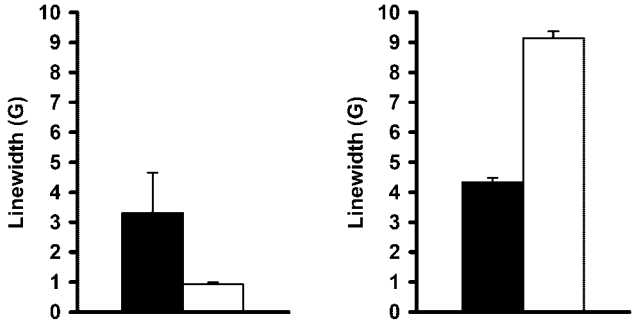

Figure 4. Typical *in vivo* EPR measurements carried out one month after the injection of the ink into a gastrocnemius muscle. Left: EPR linewidth recorded in muscle before (■) and after inducing an ischemia by interrupting the blood flow (□). Right: EPR linewidth recorded in muscle when the mouse is breathing air (■) and when the mouse is breathing carbogen (□)

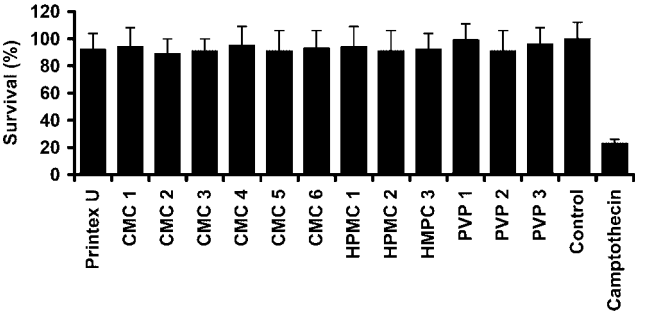

Figure 5. MTT assay. Survival of HeLa cells after exposure to different inks. Note the absence of toxicity, compared to the camptothecin used as positive control

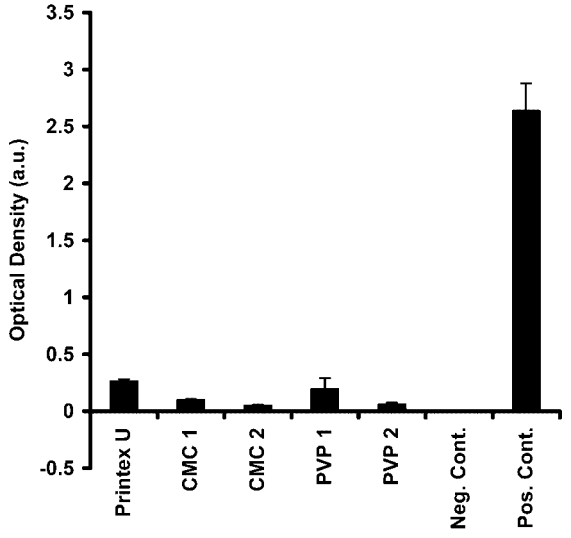

Figure 6. Hemolysis assay. Optical density recorded at 540 nm from plasma samples after exposure of the blood to different inks. Note a slight hemolytic effect, an effect that is small compared with the positive control

increase the sensitivity to oxygen, especially for soluble radicals;[24] (2) to stabilize the responsiveness of materials that progressively loose their sensitivity to oxygen in biological media;[25–27] (3) to increase the biocompatibility of the oxygen sensors;[27,28] (4) to get stable homogenized suspensions of fine particles.[29] In the present study, the two latter objectives were sought. Using the recently discovered CB (Printex U) that showed an EPR spectrum highly sensitive to oxygen (750 mG/mmHg at low pO_2), efforts were made to develop a 'home-made' ink using dispersing agents known for their biocompatibility.

Our results demonstrated that the use of biocompatible polymers such as CMC or PVP dramatically increased the physical stability of the CB suspensions. The use of high-MW polymers reduced the sedimentation of the Printex U particles. The inks prepared were autoclaved without changing the EPR properties of the oxygen sensors or modifying the injectability with 23 G needles. High diffusion was observed for inks containing low-MW polymers, in contrast to high-MW polymers, for which diffusion was minimal. Printex U, when used without dispersing agent, also showed a very low diffusion. Based on the observation that the CB particles aggregate quickly in water when used without stabilizing polymers, it is likely that the CB particles aggregate at the point of injection. The practical consequence is that high-MW polymers will be more desirable for localized measurements of oxygen while the low-MW polymers will allow measurement from particles dispersed over large areas in tissues. The explanation for the stabilization of the suspensions together with an injectability of the inks can be found in our rheological studies. Using CMC and PVP, the viscosity increased up to 10 times compared with the Printex U without suspending agent. This significantly reduced the sedimentation. The pseudo-plastic behavior that we observed for these suspensions conferred a practical advantage: the viscosity is high when the suspension is at rest (allowing the stability of the suspension) and the viscosity is decreased when the ink is mobilized for sampling or injection.

EPR studies showed that the use of polymers did not affect the responsiveness of Printex U to oxygen. Our *in vivo* studies with induced ischemia and carbogen breathing challenge showed that the CB particles are fully responsive to oxygen changes in tissues for at least 1 month. The calibration curves performed 1 month after implantation in tissues were identitical to those made in freshly prepared homegenates doped with inks, thereby confirming the stability of the responsiveness.

The motivation for using these specific polymers stemmed from their potential clinical applicability. These suspending agents are already used in injectable suspensions. Our first results from the biocompatibility assays are quite encouraging. *In vitro* tests (hemolysis and cytotoxicity assays) did not reveal any significant toxicity against blood components or against cells. Histological examination did not show toxicity or necrosis: at worst, a slight and localized inflammatory response may have been observed. This is in stark contrast with the commercially available ink containing the same CB, for which a

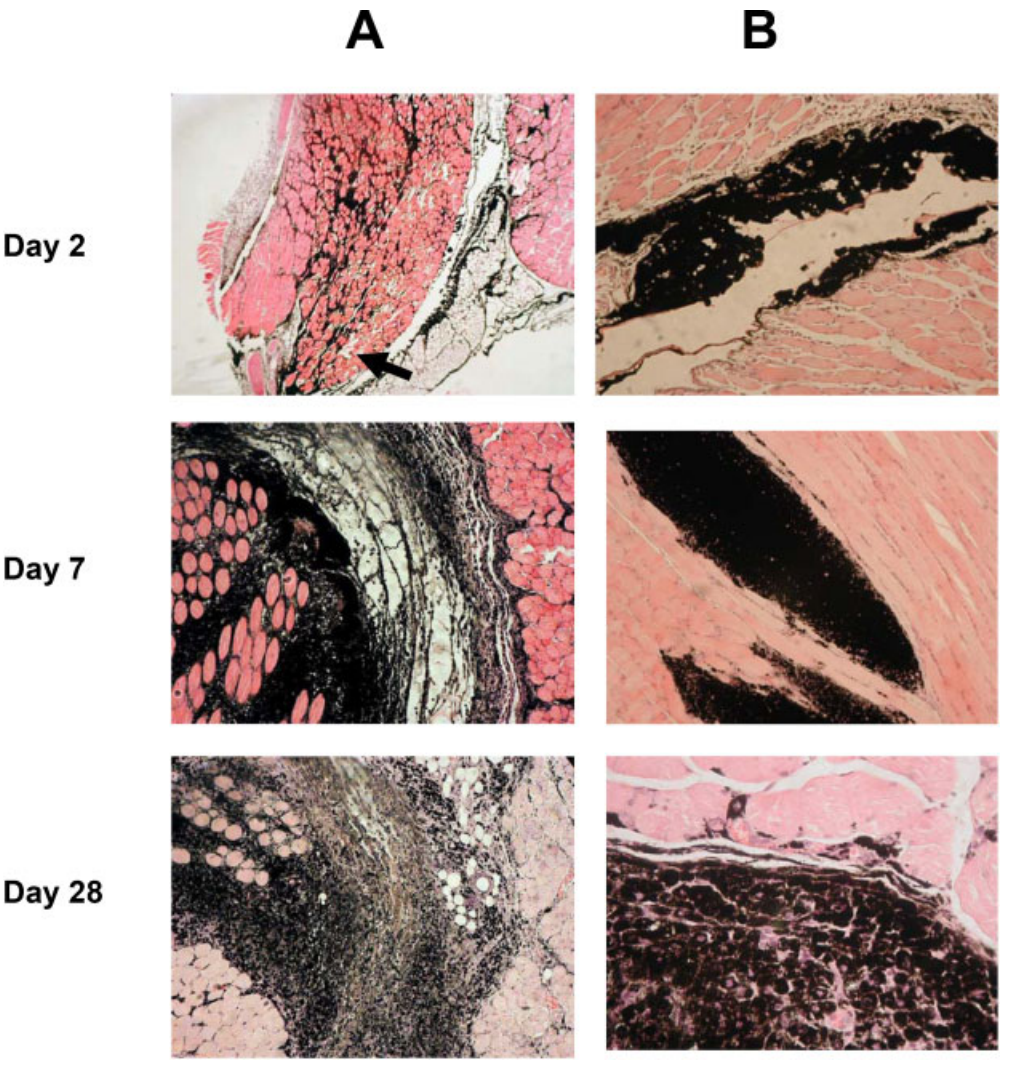

Figure 7. Typical histological sections from muscles 2 days (top), 7 days (middle), and 28 days (bottom) after injection of inks. Left: Pelikan A®, a commercial ink. Right: home-made preparation of Printex U. Note the absence of toxic effect (very small inflammatory response) observed with preparation of Printex U. In contrast, note the presence of a large inflammatory reaction and necrosis after injection of Pelikan A®. The arrow indicates the presence of muscle cells without structure or nucleus

large inflammatory response and necrosis were observed during the first week after injection.

CONCLUSION

In conclusion, we have defined relatively facile ways to prepare inks containing oxygen sensitive carbon blacks (Printex U) dispersed in polymers. The use of high molecular weight CMC and PVP appears particularly effective for obtaining stable suspensions with low diffusion inside tissues. The EPR properties were not affected by the presence of the polymers or by month-long residence in tissues. The biocompatibility studies (hemolysis, cytotoxicity, histology) did not reveal any sign of suspected toxicity using these inks. These preparations are good candidates for future *in vivo* applications, both for animal studies and clinical trials.

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