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Separation and Enrichment of the Active Component of Carbon Based Paramagnetic Materials for Use in EPR Oximetry

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Carbon based paramagnetic materials are frequently used for EPR oximetry, especially *in vivo,* but the EPR spectra of these materials often have more than one paramagnetic center and/or relatively low signal intensity. To determine whether the multi-components of carbon based materials could be separated and enriched in the active component, we used density gradient centrifugation to separate the materials into several fractions. We studied two types of coals, gloxy and Pocahontas, and found these materials to have large density distribution. The separated density fractions had very different EPR spectra and intensities. The active component from the coal material had a more homogeneous EPR signal and significantly increased EPR signal intensity, whereas for India ink, only slight changes were observed. This result can be very useful in the development of better probes for EPR oximetry.

INTRODUCTION

Electron paramagnetic resonance (EPR) oximetry is a rapidly developing technique that has been used to measure the concentration of oxygen (or partial pressure of oxygen) in solutions, cell suspensions, and in tissues of living animals (*1–12*). This technique has the potential to be applied clinically, where repeated measurement of tissue oxygenation may be useful, to guide radiation therapy, and design better treatment strategies for treatment of vascular diseases and wound healing. The technique is based on the fact that molecular oxygen can interact with paramagnetic materials, resulting in reproducible changes in their EPR spectra. The spectral change is a function of pO_2 (or concentration of oxygen, depending on the type of material used), and therefore can be calibrated with pO_2 and then used to measure pO_2 (*13, 14*).

Recently, the discovery that the carbon based paramagnetic materials can be used as EPR oximetry probes has significantly advanced the technique of EPR oximetry. These particulate materials include some types of naturally occurring coal, such as fusinite and gloxy (*15, 16*), synthesized chars (*1*), and India ink (*17, 18*), all of which have been utilized successfully in a variety of systems. These materials have an EPR signal which is highly sensitive to oxygen, and also is very stable physicochemically, resulting in little or no toxicity *in vitro* and *in vivo* (*19*). These desirable properties, together with recent advances in low frequency EPR instrumentation which have made *in vivo* EPR feasible, have made EPR oximetry capable of measuring pO_2 in tissues of living animals with high sensitivity, accuracy, and repeatability. In addition, these materials have been reported to be used for three-dimensional or four-dimensional spectral-spatial imaging (*20, 21*).

There is room for improvement, however, in regard to many of these carbon based materials. Most of these materials can be viewed as consisting of three major components: nonparamagnetic (EPR invisible), paramagnetic but nonsensitive to oxygen, and paramagnetic and sensitive to oxygen (the "active" component). A combination of the last two components can produce a complex EPR spectrum, with different centers which can have different oxygen sensitivity. This makes the extraction of the spectral parameter (such as linewidth) used to calculate pO_2 values very complicated, and sometimes subject to large error. The presence of the nonparamagnetic component reduces the overall spin density of the material, which is already low for the carbon based material (in the range of 10^{18}–10^{19} spin/g), as compared to pure free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), which has a spin density of 1.5×10^{21} spin/gram. It would be very desirable, therefore, to increase the EPR signal intensity and simplify the EPR spectral lineshape by enriching the active component and eliminating or reducing the nonactive components. The increase in signal intensity would improve signal-to-noise ratio, which would increase the accuracy of measurement and expand the useful applications of EPR oximetry. The purpose of this study is to investigate (1) whether the multi-components of carbon based materials could be separated and result in enrichment of the active components, and (2) whether the isolated active components have improved EPR oximetry qualities compared to the original material in terms of increased responses to oxygen and increased EPR signal intensities. To separate the active components from the rest of the material, we used density gradient centrifugation (DGC) since many of the materials are known to have a relatively large density range.

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MATERIAL AND METHODS

Materials

Gloxy is a type of coal from a mine in South Wales, UK. Because of the nature of the naturally occurring material, native gloxy (defined as coal material retrieved from the coal mine without any treatment) is very heterogeneous, having a large range of density and conductivity, as well as variation in sensitivity of its EPR signal to changes of oxygen (*16*). The native material is initially washed and separated by a sink/float procedure using a mixture of tetrachloroethylene and petroleum ether at a density of 1.55 g/cm^3. Generally, 1–2% of the native material sinks at this density and is collected. This sink portion of the material, termed *selected gloxy*, has high conductance and is more sensitive to oxygen (*16*).

A1 gloxy nuggets are small pieces of gloxy manually picked from the selected gloxy, which have very high conductivity, high EPR signal intensity per unit weight, a narrow EPR linewidth in nitrogen (less than 600 mG), and an EPR spectrum which is very sensitive to oxygen. The A1 gloxy generally comprises about 10% of the selected gloxy.

Pocahontas is another type of coal and was obtained from the Argonne Premium Coal Samples Program (*22*). The original material was collected from the Pocahontas #3 seam in Buchanan County, Virginia. After transfer to a nitrogen-filled enclosure to prevent potential oxidation by oxygen, the native coal material was crushed, pulverized to about 100 mesh, mixed, and packaged in sealed amber borosilicate ampoules. The samples in the ampoules were used as received without further treatment.

The India ink (black India 4415, Higgins Brand, Newark, NJ) was purchased from a local bookstore. It is made from carbon black of oil, coated with gelatin to obtain an aqueous suspension. The particles in the India ink are homogenous in size and less than 1 μm in diameter (*18*).

Sample Preparation and Density Gradient Centrifugation

The coal materials were ground to less than 20 μm using a planetary ball milling attached to a dental amalgam mixer (Crescent dental MFG. CO., WIG-L-BUG model 3110-3A). Grinding the carbon material to fine particles is a crucial step for the effective separation into different density fractions. The India ink was spun down by centrifugation at 10,000 rpm in water, and the carbon particles were collected.

For separation by DGC, the samples were prepared following the procedure described by Dyrkacz *et al.* (*23*). A slurry made up of fine particles of coals or India ink was prepared in an aqueous CsCl (Aldrich Chemical Co.) solution. CsCl was used because this chemically stable salt has very high solubility in water, and the precise density of the solution can be easily adjusted by the concentration of CsCl. The slurries were prepared by adding a CsCl solution of known density to an appropriate amount of coal to achieve a final carbon particle concentration of 10–20 g/L. In order to reduce the surface tension and prevent the fine carbon particles from aggregating, a surfactant, Brij-35 (Aldrich Chemical Co.), was added to the CsCl solution at 10% of dry weight of carbon material. The separation process usually started with a CsCl solution at a lower density. For example, an aqueous solution of 2.57 M CsCl produces a density of 1.30 g/cm^3. After the centrifugation, the carbon particles with density less than the CsCl solution will float, while particles with higher density will sink. The sink portion of each separation was then added to a new CsCl solution with a higher density, usually in increments of 0.05, and subjected to further repetitive separations so that many fractions of carbon particles within specific density ranges were obtained from each original sample.

A Sorvall RC2-B centrifuge with swinging bucket rotors (HB-4010) was used for this work. Centrifugation of the slurry was performed at 13,000 rpm (R_{max} = 140 mm, the relative centrifugal force being 26,533*g*) for 2 h at 20°C. Standard glass centrifuge tubes (Corex 30 mL) were used for all of the centrifugal separations.

Following the centrifugation of the slurries, the speed was reduced without braking in order to reduce the possibility of mixing during deceleration.

EPR Measurements

Calibrations of the EPR linewidth to oxygen and measurements of spin density of the carbon based materials were performed using a Varian E-109E EPR spectrometer (9.5 GHz, X-band). Typical settings for the spectrometer were: magnetic field, 3240 G; incident microwave power, 10 mW; modulation frequency, 100 kHz. Modulation amplitude was set at one-third of the EPR linewidth. The EPR spectra were collected, stored, and manipulated using the software EW (Scientific Software Inc., Normal, IL) installed on an IBM compatible computer.

An aqueous slurry of the carbon based material to be tested was drawn into a gas permeable Teflon tube (Zeus Industrial Products, Raritan, NJ, 0.623 mm i.d., 0.038 mm wall thickness). This Teflon tube was folded twice and inserted into a quartz EPR tube open at both ends. The sample then was equilibrated with different oxygen:nitrogen gas mixtures. The pO_2 in the perfusing gas was monitored and measured by an oxygen analyzer (Sensor Medics, Model OM-11, Anaheim, CA) calibrated with air and nitrogen. The quantitative dependence on pO_2 of the EPR spectrum was obtained by measuring linewidth (LW) as a function of pO_2 in the perfusing gas, with LW defined as the difference in magnetic field between the maximum and minimum of the first derivative of the recorded signal. For measurement of the spin density of the materials, DPPH was used as the standard, and the spin density was calculated by comparing the double integration of the EPR spectra.

Computer Simulation of the EPR Spectrum

The computer program EWVOIGT (Scientific Software Inc., Normal, IL) was used to analyze and simulate the EPR spec-

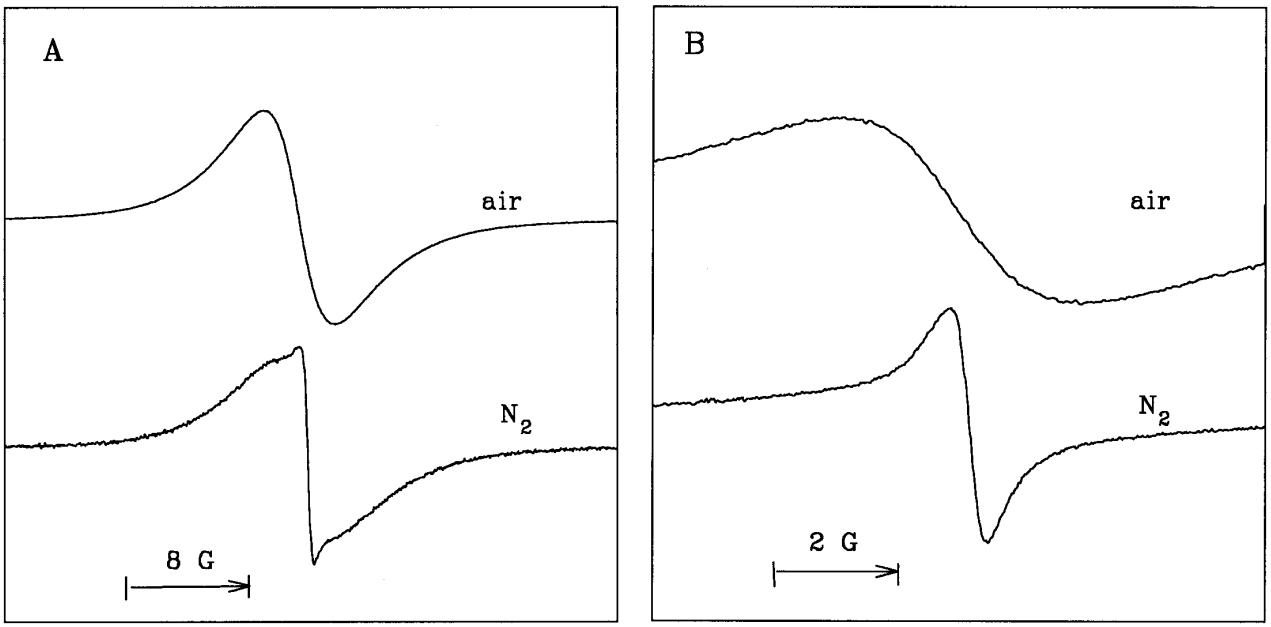

FIG. 1. EPR spectra of (A) native gloxy and (B) selected gloxy in air and nitrogen. The spectra were obtained from aqueous slurries of the gloxy samples, using a 9.6 GHz EPR spectrometer. The spectrometer conditions are given in the Methods section.

trum. This program, based on the use of a convolution algorithm of Lorentzian and Gaussian lineshapes, provides a good fit for the complex EPR signals which have two or more components, using a sum of two or three general EPR analytical functions. The computer fitting procedure directly gives EPR parameters such as linewidth, signal intensity, and field center, including the standard error of these measurements. The program indicates the Chi square for the fit to the simulated line and the residual part of the spectrum which could not be accounted for by the procedure, and the Chi square is used as the criterion to determine whether an EPR spectrum is homogeneous (i.e., one paramagnetic center), or consists of more than one center.

RESULTS

Gloxy

The EPR spectra of native gloxy in air and N_2 are shown in Fig. 1A. The spectra indicate that the native material has two (or more) paramagnetic centers, with a very small fraction sensitive to changes of oxygen, and the majority being insensitive. After initial separation of the native gloxy by the float/sink method using tetrachloroethylene/petroleum ether at a density of 1.55 g/cm^3, the EPR signal of the sink portion (termed selected gloxy, about 1–2% of the initial material) is much more responsive to changes of oxygen. This is indicated by the overall narrowing of the EPR spectrum of the sample in N_2, as shown in Fig. 1B. Computer simulation of the spectrum in N_2, however, reveals that the selected gloxy also consists of at least two paramagnetic centers, but the percentage of the oxygen sensitive material is greatly increased compared with the native gloxy.

The technique of DGC was employed to separate the selected gloxy into five fractions, with density ranging from less than 1.30 g/cm^3 to more than 1.65 g/cm^3. Figures 2A–E show the EPR spectra of each density fraction in air and N_2. The corresponding linewidths of the spectra are shown in Table 1. The different fractions had very different responses to oxygen. The fractions with density less than 1.45 g/cm^3 did not respond to oxygen at all (Figs. 2A and B), while the material with density between 1.45 and 1.65 g/cm^3 was very sensitive to oxygen. Computer simulation of the spectra in this sensitive range indicated that these fractions were quite homogeneous, i.e., consisted mostly of one type of paramagnetic center. The spin density of the fractions varied modestly, ranging from 1.1 to 2.2 $\times$ 10^{19} spin/g, with the most oxygen sensitive fractions (d = 1.45–1.65 g/cm^3) having a spin density nearly two times higher than the nonsensitive fractions ($d <$ 1.45 g/cm^3) (Table 1). Similar results were obtained for the separation of the native gloxy using the DGC technique.

Figure 3 shows the comparison of the density fractions of native and selected gloxy, as percentage of weight of the starting material. Since the most oxygen sensitive component is at a density between 1.45 and 1.65 g/cm^3, it appears that in native gloxy, about 10% potentially is useful material, whereas in the selected gloxy, the percentage of useful material increased to over 50%, suggesting that the initial float/washing process carried out on the native coal as retrieved from the mine was successful.

Figure 4 compares the oxygen calibration curves of the EPR signals for three different gloxy materials: the selected

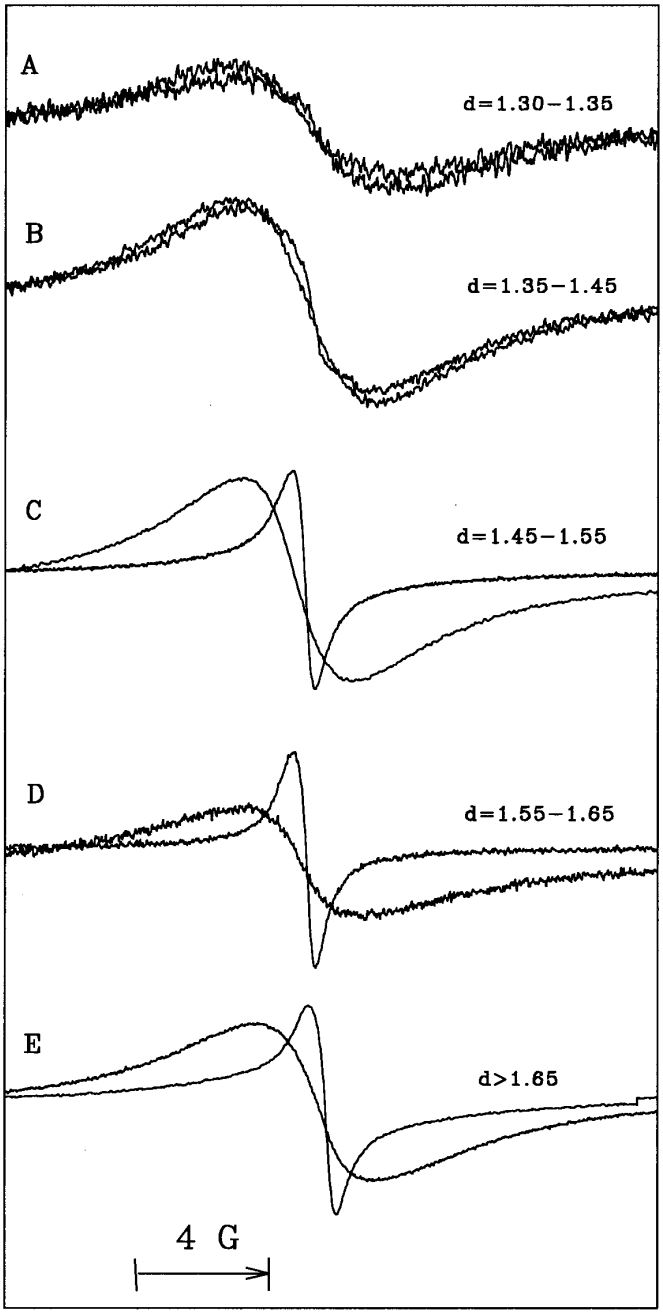

FIG. 2. EPR spectrum of each density fraction of selected gloxy in air and nitrogen. The spectrum with the narrower linewidth for each pair is in nitrogen. The spectra were obtained from an aqueous slurry of gloxy. The spectrometer conditions are given in the Methods section.

gloxy, one fraction of the selected gloxy (d = 1.5–1.6 g/cm^3), and the A1 gloxy. Oxygen sensitivity per se is almost the same for all three types of gloxy. This result was not surprising since the non-oxygen-sensitive portion of the paramagnetic material usually has a broader linewidth than the oxygen-sensitive portion, so changes in the latter are more readily observed. When the calibration curve of linewidth vs oxygen is obtained, the overall linewidth of the paramagnetic material reflects more of the oxygen-sensitive portion, resulting in similar correlation between the observed linewidth and oxygen for all three types of gloxy in Fig. 4. The benefit of the various separation procedures, however, becomes obvious when the changes in signal *intensity* are compared (Fig. 5). The signal intensities are normalized to intensity in air for the different forms of gloxy. The ratio of signal amplitude in air to that in N_2 was 78 for A1 gloxy, 18 for the "active" component of selected gloxy, 8 for nonseparated selected gloxy, and 2 for native gloxy.

We also used DGC to obtain similar density fractions using the A1 nuggets as the starting material. It was found that the A1 nuggets had a very high density with a narrow density range of 1.60–1.70 g/cm^3, which is consistent with the "active" components of the selected gloxy. All the fractions separated from A1 nuggets were sensitive to oxygen (Fig. 6).

Pocahontas

In order to determine whether the separation technique developed for the separation of gloxy also could be applied to other coal materials, we carried out a similar separation technique on another coal, named Pocahontas. Figure 7 shows the EPR spectra of native Pocahontas in air and N_2. Similar to the EPR spectra of gloxy, the spectra of Pocahontas consist of more than one paramagnetic center. In contrast to gloxy, the component which is not sensitive to oxygen has a narrower EPR linewidth, and the majority of the EPR active material is oxygen sensitive. After separation by DGC into four fractions, each had different oxygen sensitivity, as shown in Fig. 8. Similar to the gloxy, some components were not sensitive to oxygen, while others were. But in contrast, the component sensitive to oxygen was found in the density range of 1.3–1.5, with little oxygen-sensitive material above a density of 1.5.

India Ink

India ink has been shown to be sensitive to oxygen. Its EPR spectrum usually consists of more than one paramagnetic center (Fig. 9). The density range of India ink particles, in contrast to naturally occurring coal materials, was

TABLE 1
Density Fraction of Selected Gloxy

Density (g/cm^3)	LW (air) (G)	LW (N_2) (G)	Spin density (spin/g)
1.30–1.35	5.11	5.11	1.2×10^{19}
1.35–1.45	4.36	4.28	2.0×10^{19}
1.45–1.55	3.46	0.60	2.2×10^{19}
1.55–1.65	3.77	0.58	2.2×10^{19}
>1.65	3.58	0.85	1.1×10^{19}

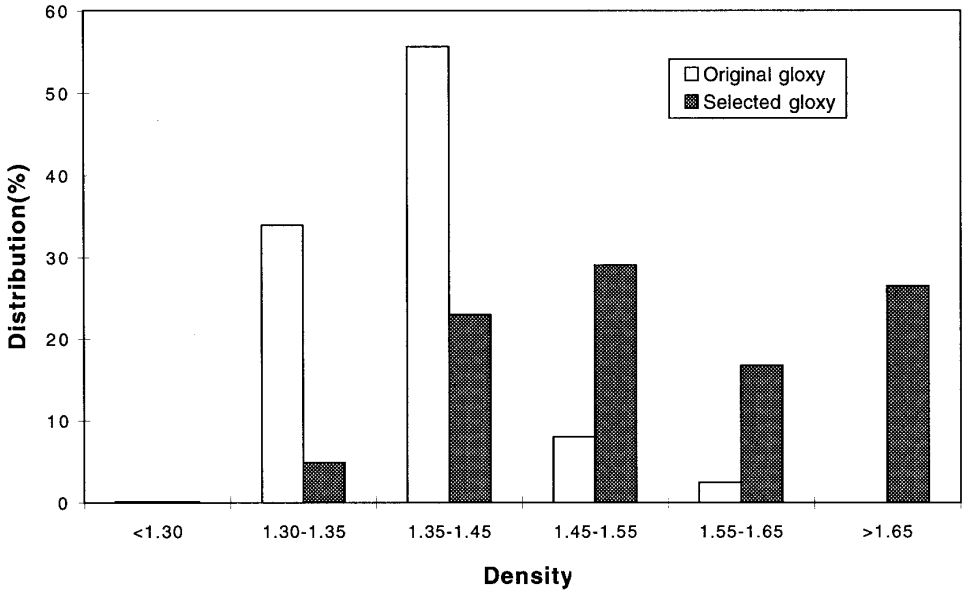

FIG. 3. Weight distribution of density fractions of native and selected gloxy. The separation of the density fractions was achieved by DGC.

found to be very narrow, in the range of 1.50–1.60 g/cm^3, with over 80% of the material centered around 1.55–1.58 g/cm^3. Although we separated the material into four fractions using the DGC technique, and each fraction showed a slightly different response to oxygen, the overall separation was not satisfactory: the fractions showed little improvement in the response to oxygen compared to the material before separation, and the spectrum of each fraction still appeared to contain more than one paramagnetic center (Fig. 9).

DISCUSSION

This study shows that components of certain types of carbon based paramagnetic materials can be separated by DGC, and that enriched active components can be obtained. The separated active components can have a single paramagnetic center and an EPR signal intensity and response to oxygen that are significantly higher compared to the native material. Our results suggest that the technique of separation by DGC is best applied to separate the fractions of multicomponent material

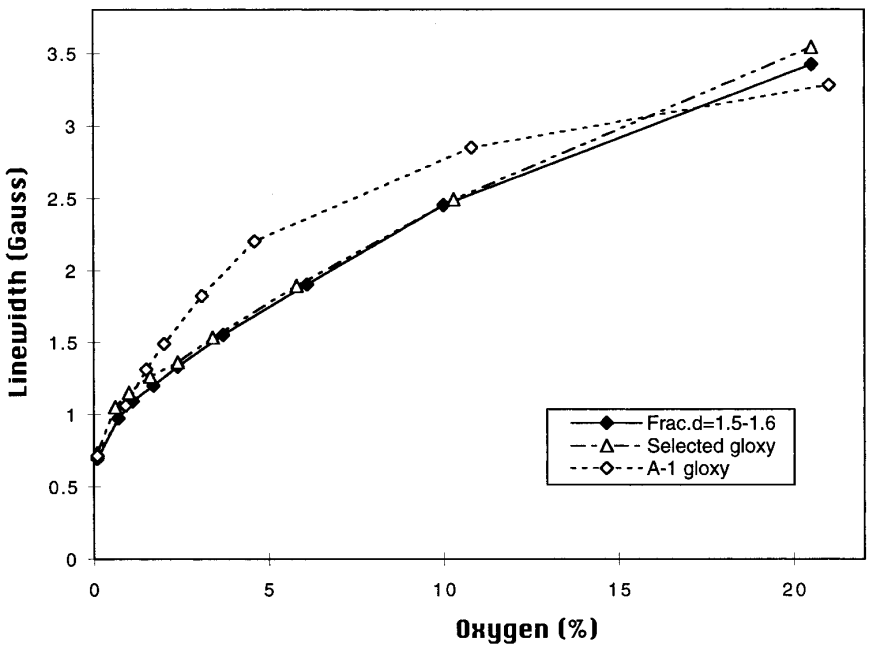

FIG. 4. Calibration curves of the linewidth of the EPR signal of various gloxy samples vs pO_2, in saline. The linewidth was measured after the sample was equilibrated with different O_2:N_2 gas mixtures in the EPR spectrometer cavity.

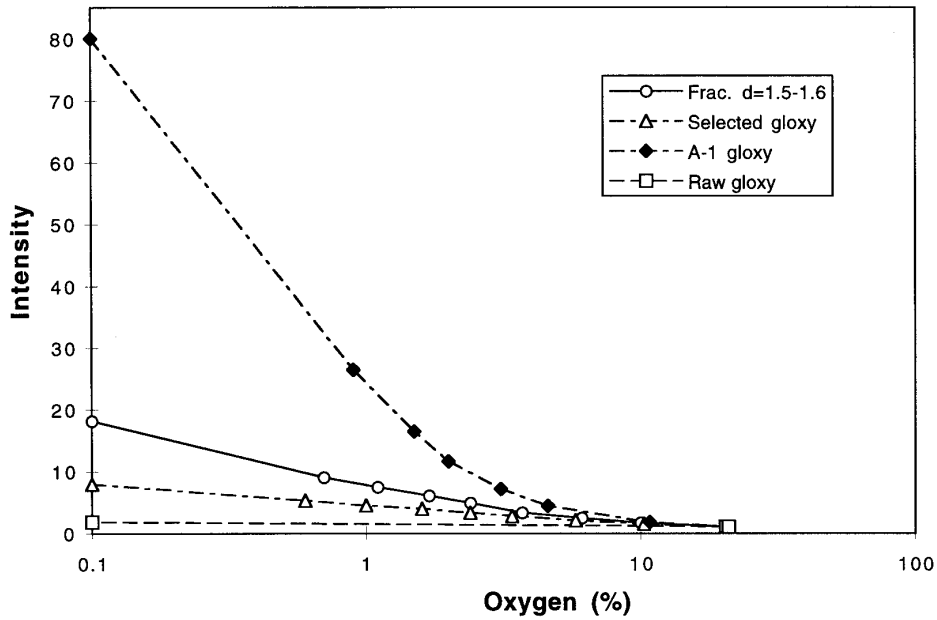

FIG. 5. Relationship of the EPR signal intensity to pO_2 of various gloxy samples in saline. The intensities were normalized to the intensity of each sample in air.

with very heterogeneous properties, such as the naturally occurring coal materials, which have a large density distribution. Indeed, our unpublished results have shown that the DGC method can also be used to separate the different density fractions of fusinite, another coal material which has been used for EPR oximetry.

One of the benefits of the successful separation for EPR oximetry is the achievement of a relatively homogenous EPR signal in the "active" component, as compared to the multicomponent EPR signal of the native material. Having a homogeneous EPR signal is very useful for EPR oximetry because calculation of the pO_2 is based on the accurate measurement of spectral parameters, usually the linewidth. A multiple center EPR spectrum makes computer simulation complex and sometimes yields erroneous results.

Another benefit is the separation of the active component from the rest of the nonactive (and/or less active) component, resulting in increased signal intensity per unit mass. There are two factors contributing to the increase in intensity: elimination/reduction of the nonactive components, and an increase in the spin density of the active component. The EPR linewidth of the nonactive compo-

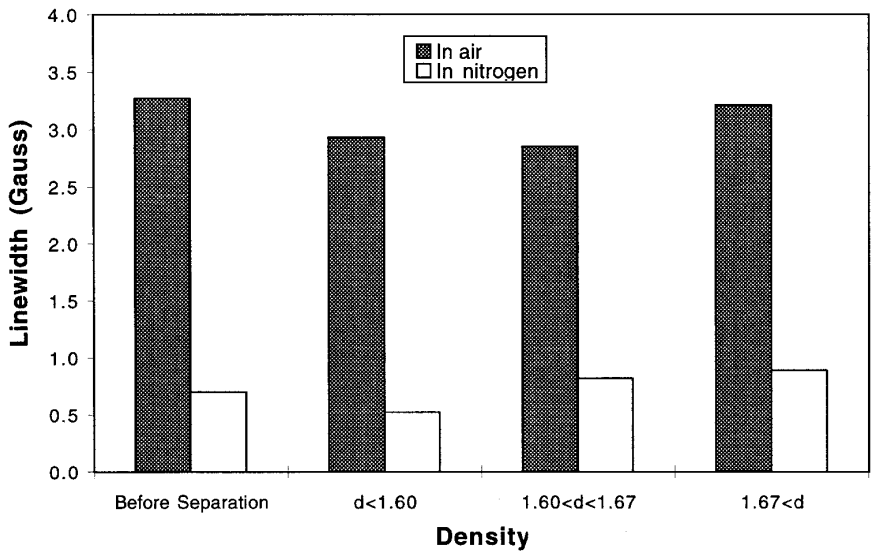

FIG. 6. EPR linewidth of separated fractions of A1 gloxy in air and nitrogen.

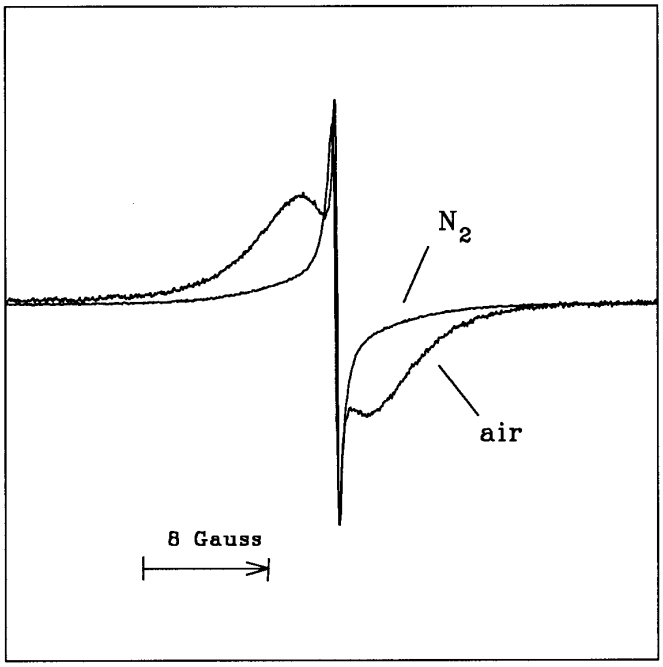

FIG. 7. EPR spectra in air and nitrogen of an aqueous slurry of a sample of native Pocahontas coal.

nent in gloxy was wider, in the range of 5 G or more with or without the present of oxygen, while the active component had a linewidth of about 0.5 G in N_2, and about 3–5 G at 5% O_2. Because the relationship between the intensity and linewidth varies as the square of the linewidth, for the same number of spins, narrower line will have a much greater intensity. Therefore, the enrichment of the active component produces an increased observable EPR signal. This increased intensity is very desirable, and indeed much needed, particularly for measurement of pO_2 in tissue in living animals. Increased intensity per unit weight of material also implies that smaller amounts of the material can be introduced into the systems, minimizing disturbance caused by the presence of the oxygen sensitive probes.

Our attempt to separate the multiple component of India ink was less successful and suggests that where the density range of the original material is narrow, as was the case with India ink (1.54–1.62 g/cm^3), the chance of obtaining an effective separation is very small. This conclusion, however, does not exclude the possibility that the failed attempt for India ink is due to some special property of India ink, and it remains to be tested whether other materials with a narrow density range can be separated by DGC into useful fractions. As for India ink, it is still possible that the observable multiple component could be separated by exploiting other physical properties, such as size, magnetic susceptibility, or electric susceptibility (or electroaffinity).

Although successful separation of the active component of coal material was achieved, it is interesting to note that the A1 gloxy nugget (manually picked from the selected gloxy according to the conductance and EPR spectral properties of each piece) appears to offer higher signal intensity as compared to the active component separated from the selected or native gloxy (Fig. 5). This result suggests that through some unknown mechanism, the conditions under which the coal material was produced nature concentrates a relatively pure active component, which is very similar in density to the active component from the native material. The phenomenon also implies that the "active" component collected by the DGC technique may not

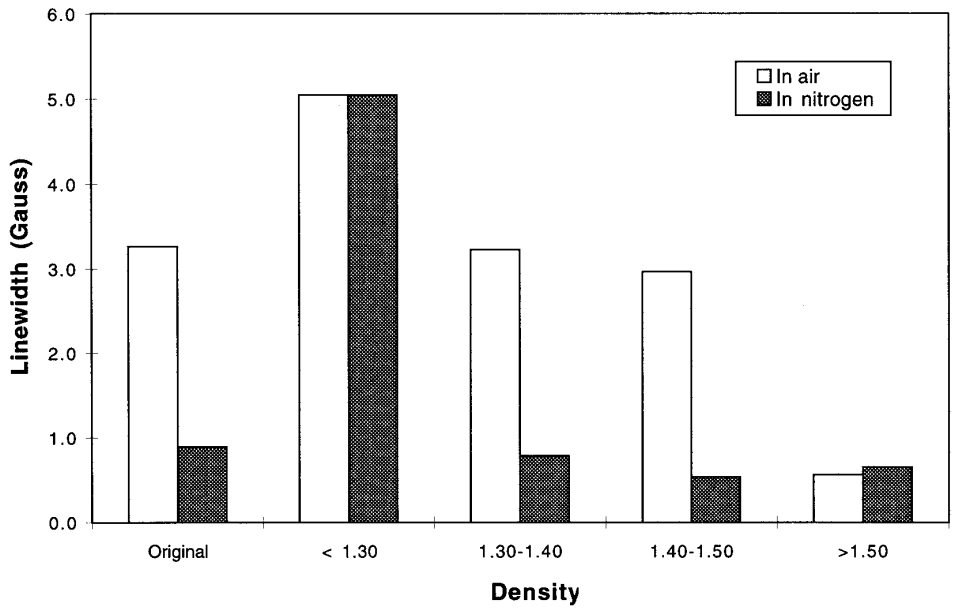

FIG. 8. EPR linewidth of separated density fractions of native Pocahontas coal in air and nitrogen.

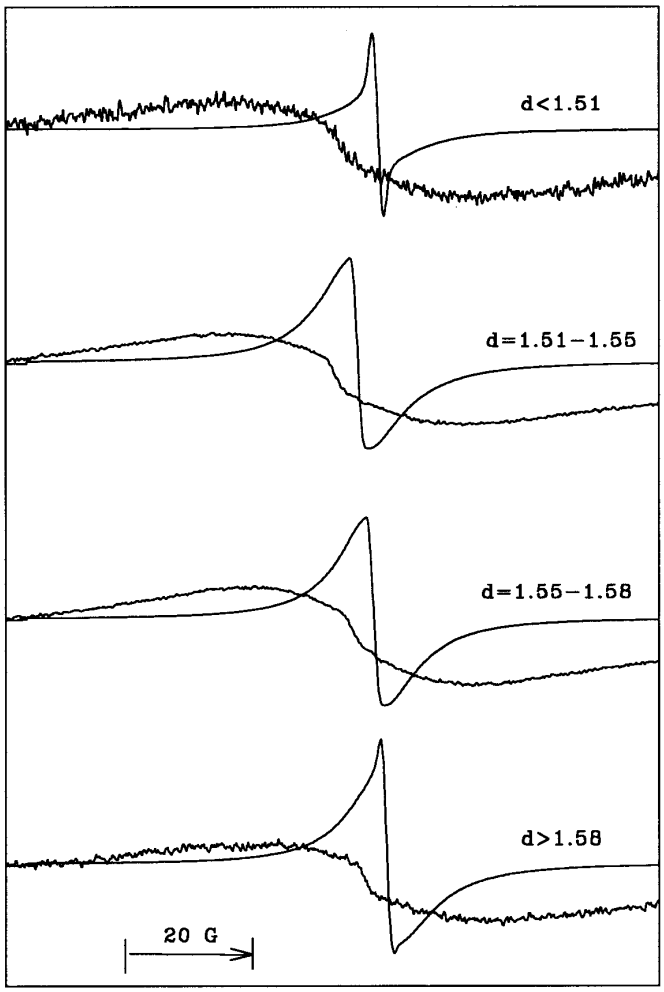

FIG. 9. EPR spectra of density fractions of aqueous slurries of India ink in air and nitrogen. For each density fraction the spectrum with a narrower linewidth for density fraction is in nitrogen.

really be homogeneous and potentially could be further purified to obtain even higher signal intensities.

While DGC clearly is capable of separating and enriching the active component for use in EPR oximetry, the technique also has several drawbacks. First, the starting material has to be ground to particles with diameter of less than 20 μm to achieve effective separation by the DGC method (*21*). This would seem to exclude the possibility of making pO_2 measurement using a larger piece of gloxy, say 0.1–1.0 mm in size, which is desired in certain situations, particularly *in vivo* where one may wish to localize the site of observation. With the use of the proper binding material or a coating/embedding procedure, however, the fine particles could be made into desirable physical sizes, overcoming this limitation. Second, the process can be tedious and time consuming. Under laboratory conditions, only a limited quantity of material can usually be processed at a time. Third, the material to be separated needs to have a relatively large density range.

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