JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 3 (1992) 418–425

Electron microscopic and voltammetric analysis of carbon fibre electrode pretreatments

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The comparison between the differential pulse voltammetric capability of untreated or treated 12 μm or 30 μm diameter carbon fibre electrodes (CFE) to detect electroactive compounds *in vitro* has been analysed. Their affinity for ascorbic acid (AA), catechols (dopamine (DA); 3,4-dihydroxyphenylacetic acid (DOPAC)), indoles (serotonin (5HT); 5-hydroxyindoleacetic acid (5HIAA)) and uric acid (UA) has been quantified. It appeared that only the 12 μm CFE, when electrically pretreated, can selectively and simultaneously detect the oxidation of AA, catechols and indoles, while the electrically pretreated 30 μm CFE are specifically sensitive to the indoles. However, both electrodes displayed highest sensitivity for serotonin.

To avoid the detection of acids and selectively monitor the oxidation of DA and 5HT the 12 μm CFE and the 30 μm CFE were chemically (chromic acid) and electrically pretreated then electrically coated with Nafion. However, the 30 μm CFE alone selectively measured these compounds *in vitro* as the 12 μm CFE did not accept the coat of Nafion.

Using transmission electron microscopy (TEM) and scanning electron microscopy (SEM), we have investigated the effect of electrical and/or chemical pretreatments on the structural characteristics of the CFE. It appeared that both pretreatments increased the surface area of the carbon fibres by pitting, roughening and fracturing the carbon. Holes were also observed on the surface of the 30 μm dia. carbon fibres following the electrical pretreatment. The possibility of a correlation between these modifications and the pretreatment-induced voltammetric high sensitivity of the CFE is discussed.

1. Introduction

Voltammetry with various types of working electrodes is an electrophysiological technique allowing continuous detection of oxidizable chemicals in buffered solutions *in vitro* and in brain extracellular fluid (ECF) *in vivo* without recourse to perfusion-based or post-mortem analysis [1].

Differential pulse voltammetry (DPV) and carbon fibre electrodes (CFE) have been recently developed to detect *in vivo* electroactive amines and/or their metabolites [2–4]. The main advantages of the CFE are their temporal and spatial resolution. They are smaller than either push–pull cannulae or dialysis tubes or even carbon paste electrodes, the diameter of the CFE varying between 8 and 30 μm. Thus very small cerebral nuclei, such as the suprachiasmatic nucleus and/or the dorsal raphe, can be studied with minimal tissue damage [5, 6].

The properties of CFE can be substantially altered by electrical pretreatments, which result in electrodes with high sensitivity and high resolving power between electroactive compounds. By using CFE made with 12 μm diameter carbon fibres specifically electrically pretreated, we are able to detect simultaneously and in the same cerebral structure AA, DOPAC, 5HIAA, UA, homovanillic acid (HVA), 3-methoxytyramine (3MT) and somatostatin [7–9]. Furthermore, with the new 30 μm dia. CFE chemically (with chromic acid) and electrically pretreated, then electrically coated with Nafion (a sulphonated polymer which repels anions but is selectively permeable to cations [10, 11] we are now able to selectively detect dopamine (DA) and serotonin (5HT) rather than their metabolites [12].

Nevertheless, the cause of the elevated sensitivity and resolution following pretreatments for the CFE are still unclear.

In the present study, an *in vitro* comparison between untreated and treated 12 μm CFE and 30 μm CFE has been carried out to analyse their selectivity and sensitivity for detecting electroactive compounds, i.e. AA, catechols (DOPAC or DA), indoles (5HIAA or 5HT) and UA. In addition, with transmission electron microscopy (TEM) and scanning electron microscopy

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(SEM) [13, 3], the structural modifications of the surface and the section of the carbon fibres following the pretreatments described above were studied. This is the first step in a complex examination which will be pursued with the analysis of chemical modifications induced by the pretreatments to the carbon fibre, which could hopefully help to elucidate why and how they allow the CFE to be selectively sensitive to the presence of oxidizable compounds.

2. Methods

2.1. CFE pretreatments

(a) Six 12 μm and six 30 μm CFEs were used without pretreatment.

(b) Six 12 μm CFEs and six 30 μm CFEs were electrically pretreated as previously described [7]. Briefly, the treatment consisted of a 70 Hz triangular waveform applied in three stages: from 0 to + 3 V for 15 s; 0 to + 2.5 V for 20 s and 0 to + 1.5 V for 30 s. Then three successive continuous potentials were applied: + 1.5 V, − 0.9 V and + 1.5 V each for 10s.

(c) Six 12 μm CFEs and six 30 μm CFEs were cleaned by immersion in chromic acid (5 g sodium dichromate in 5 ml H_2O + 95 ml concentrated sulphuric acid) followed by distilled water.

(d) Six 12 μm CFEs and six 30 μm CFEs were chemically treated as in (c) then electrically treated as in (b).

(e) Six 12 μm CFEs and six 30 μm CFEs were prepared as in (d) and then washed in distilled water for 60 min three times.

(f) Six 12 μm CFEs and six 30 μm CFEs prepared as in (e) were electrically coated with Nafion as already described [12].

2.2. Voltammetric analysis

The sensitivity and selectivity of each CFE was determined *in vitro* by using DPV, perfomed with a Princeton 176A polarograph, applying the following scan parameters: potential range − 0.2 V to + 6.0 V, scan rate 5 mV s^{-1}, modulation amplitude 50 mV, pulse frequency 0.2 s, low pen filter 0.3. Phosphate buffered saline (PBS 0.1 M, pH 7.4) was used as blank and solvent for the electroactive compounds tested: AA, DOPAC, DA, 5HIAA, 5HT and uric acid (UA) at concentrations ranging from nM to μM (to mM for AA).

Peak current values (nA) were determined by constructing a tangent to the shoulders of the peak and measuring the perpendicular height between the tangent and the apex of the peak.

2.3. Electron microscopic observations

Five 12 μm and five 30 μm CFEs from each group (from (a) to (f)) were viewed in a Jeol JSM-35 scanning electron microscope. Micrographs of the fibres were produced at two final magnifications, 3176x and 15 176x. For the transmission electron microscopic investigation five fibres from each treatment group were placed in flat silicon moulds and embedded in epoxy resin. The resin was made up of three components, araldite CY212 (1 ml), dodecenyl succinic anhydride (1 ml) and benzyldimethylamine (6 μl). 100 nm sections were cut on a Reichert OMU3 ultramicrotome. Pioloform (plastic film) coated copper grids were held under the water at the knife edge to pick up the sections and any carbon which had become detached from them during cutting. The grids were then observed in a Philips EM 410 electron microscope.

3. Results

(a) The untreated 30 μm CFE presents a uniform surface when observed with the SEM at both magnifications (Fig. 1a, b). Only a few particles can be observed on the surface, probably electrostatically attracted.

The untreated 12 μm CFE presents a very different surface than the 30 μm CFE since numerous irregular longitudinal grooves are observed along the whole length of the fibre (Fig. 1c, d). Both 30 μm and 12 μm CFE act similarly in the DPV analysis performed in phosphate buffered saline (PBS) (Fig. 2). In PBS containing AA, DOPAC and 5HIAA a wide oxidation wave is obtained with both electrodes without any separated peak (see Fig. 2c, d).

(b) The electrically pretreated 12 μm CFE displays good selectivity and sensitivity as it allows the separation and simultaneous detection of AA (Peak 1), DOPAC (Peak 2) and 5HIAA (Peak 3) dissolved in PBS

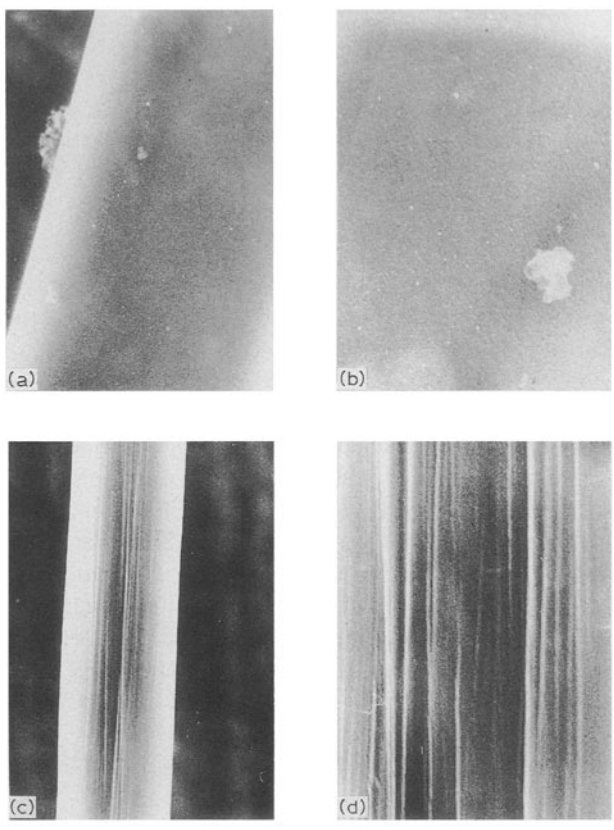


Figure 1 Micrographs showing the result of an SEM analysis at 3176x (a, c) and 15 176x (b, d) of an untreated 30 μm carbon fibre (top) and an untreated 12 μm carbon fibre (bottom) respectively.

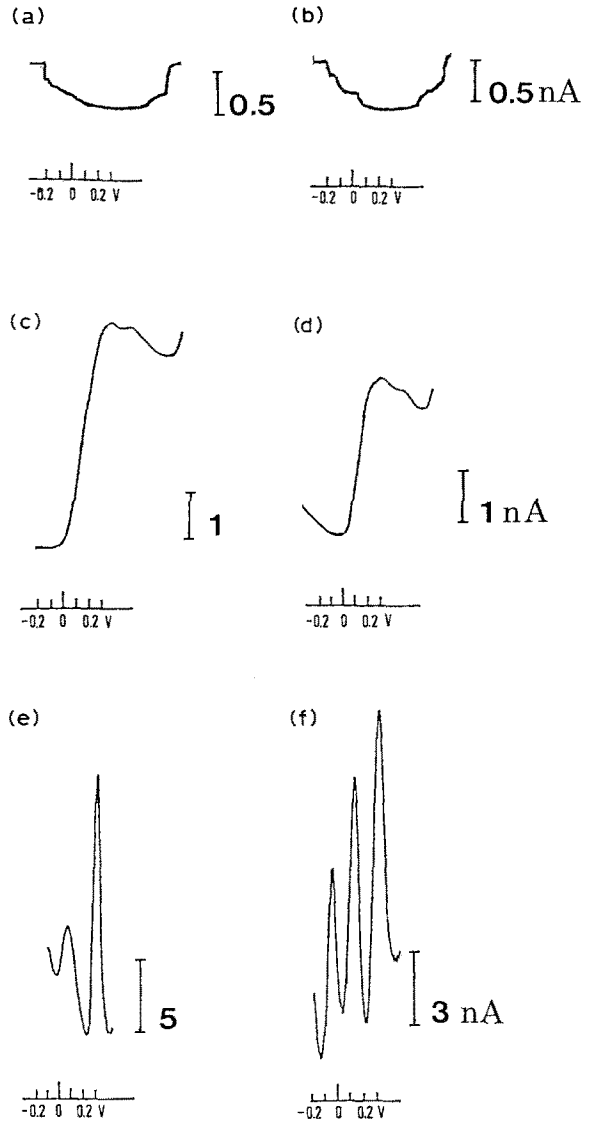


Figure 2 (a, b) Voltammogram obtained in PBS with an untreated 30 μm and an untreated 12 μm CFE respectively. (c, d) Voltammogram obtained in PBS containing AA 0.2 mM, DOPAC 50 μM and 5HIAA 25 μM with the same untreated electrodes as in (a) and (b). (e, f) As in (c, d) but after electrical pretreatment.

at concentrations of 0.2 mM, 50 μM and 25 μM respectively (Fig. 2f). The SEM analysis shows that the surface of these CFEs is covered by a large number of small crystals (Fig. 3a, b) which have not yet been characterized. These crystals disappear after cleaning the CFEs in distilled water (this suggests that they could be chloride or phosphate based) and the surface of the electrically treated 12 μm CFEs thus again resembles that of the untreated ones. However, their selectivity and the sensitivity towards AA, catechols and indoles (and UA) is not altered, the voltammogram obtained (after cleaning) in the solution described above appearing identical to that in Fig. 2f. In addition, it appears that the pretreated 12 μm CFE is more sensitive to the indoles *in vitro* (Fig. 4a), (with similar sensitivity for 5HIAA and UA) but with higher sensitivity for 5HT (Fig. 4b).

(c) The surface of the 30 μm CFE dipped in chromic acid appears cleaner and smoother than the untreated ones (Fig. 5a, b). This treatment enhances the *in vitro* voltammetric sensitivity but not the selectivity of these

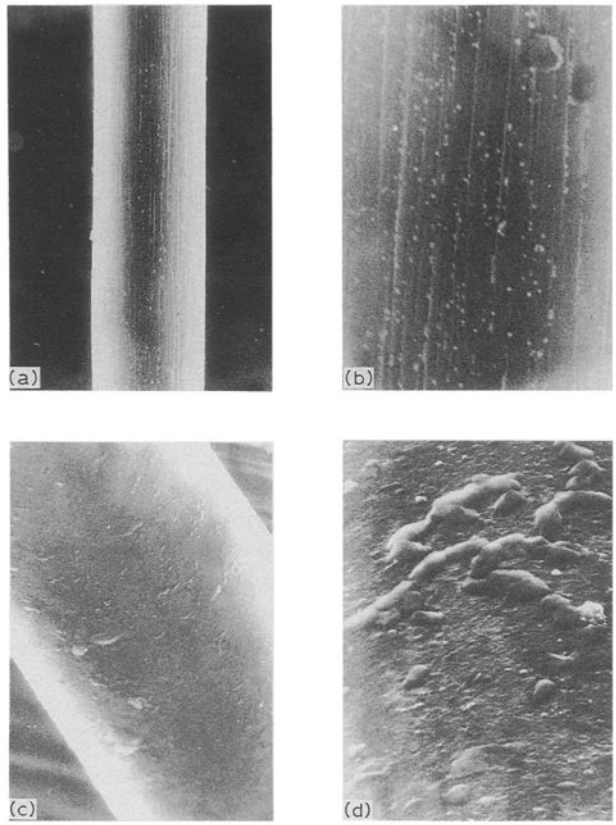


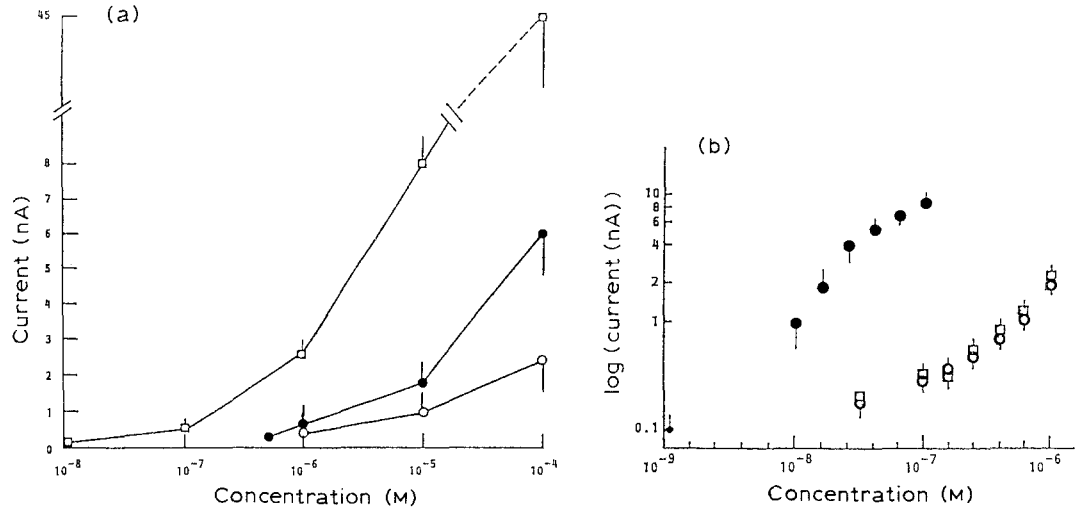
Figure 3 Micrographs showing the result of an SEM analysis at 3176x (a, c) and 15 176x (b, d) of an electrically pretreated 12 μm CFE (top) or 30 μm CFE (bottom).

electrodes towards the mixture of electroactive compounds described in (b) (Fig. 5c). Similar electrochemical results are observed when the chemically treated 12 μm CFE are used (result not shown).

(d) The surface of the electrically pretreated 30 μm CFE is blistered and uneven, small holes are also observed (Fig. 3c, d). These electrodes allow clear detection of Peak 3 (at + 300 mV) while Peak 1 and Peak 2 become merged (Peak 1 + 2 at ~ + 50 mV) (see Fig. 2e). This suggests that the electrical pretreatment increases, as already observed for the 12 μm CFE, the selectivity of the 30 μm CFE preferentially for the indoles.

(e) The chemical (chromic acid) and electrical pretreatments further modify the surface of the 30 μm CFE; groups of crystals are now regularly spaced along the fibre. In addition, at both SEM magnifications these crystals appear to be surrounding pits or craters (Fig. 6a, b). Holes, with a dia. of 100–150 nm are also evident (Fig. 6b). The voltammogram obtained with this CFE *in vitro*, in PBS containing AA 0.2 mM, DOPAC 50 μM and 5HIAA 25 μM is similar to that obtained after the electrical pretreatment alone (with possibly a slight increase in sensitivity for the indoles) (Fig. 6c).

The surface of the 12 μm CFE is difficult to interpret because of the large natural irregularities originally present; however, crystals are observed (as after the electrical pretreatment alone) and their sensitivity and selectivity for the electroactive compounds tested are maintained (unshown).



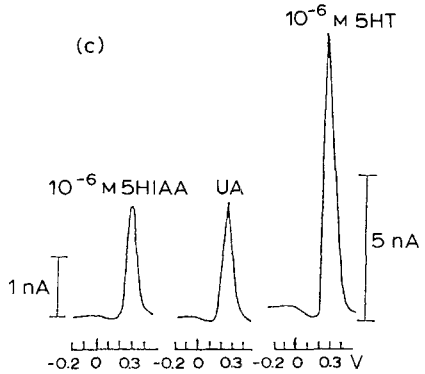


Figure 4 *In vitro* sensitivity of the electrically pretreated 12 μm CFE ($n = 5$) to mixtures of AA (○), DOPAC (●) and 5HIAA (□) (similar results are observed with mixtures of AA (μM to 0.1 mM), DA (nM to 10 μM) and 5HT (nM to 5 μM). The electrically treated 30 μm CFE are not used in this analysis because of their inability to separate AA from catechols. (b) Sensitivity of the electrically treated 12 μm CFE to 5HT (●) $n = 5$, UA (□) $n = 5$ and 5HIAA (○) $n = 5$ (similar results are observed with the 30 μm CFE). (c) Representative *in vitro* voltammogram ($n = 1$).

(f) Washing the CFE with distilled water removes the crystals leaving a smoother surface in which the holes are readily visible (Fig. 6d). However, the voltammogram obtained here is no different to that recorded in (e) (Fig. 6e).

(g) The chemically, electrically treated and Nafion coated 30 μm CFE has a very smooth surface when examined by SEM, neither holes nor protuberances are now detectable (Fig. 7a, b). The Nafion coated CFE is not sensitive to the anions AA, DOPAC, 5HIAA in the μmolar concentration range, while it presents selective sensitivity for DA and 5HT in the nmolar concentration range (Fig. 7c, d).

By contrast, the 12 μm CFE appears to be uncoatable with Nafion even at a higher or lower potential than that used to coat the 30 μm CFE.

(h) All the chemically and/or electrically pretreated carbon fibres appeared to be very fragile and shattered during cutting on the ultramicrotome. In the TEM analysis, particulate carbon was seen adhering to the

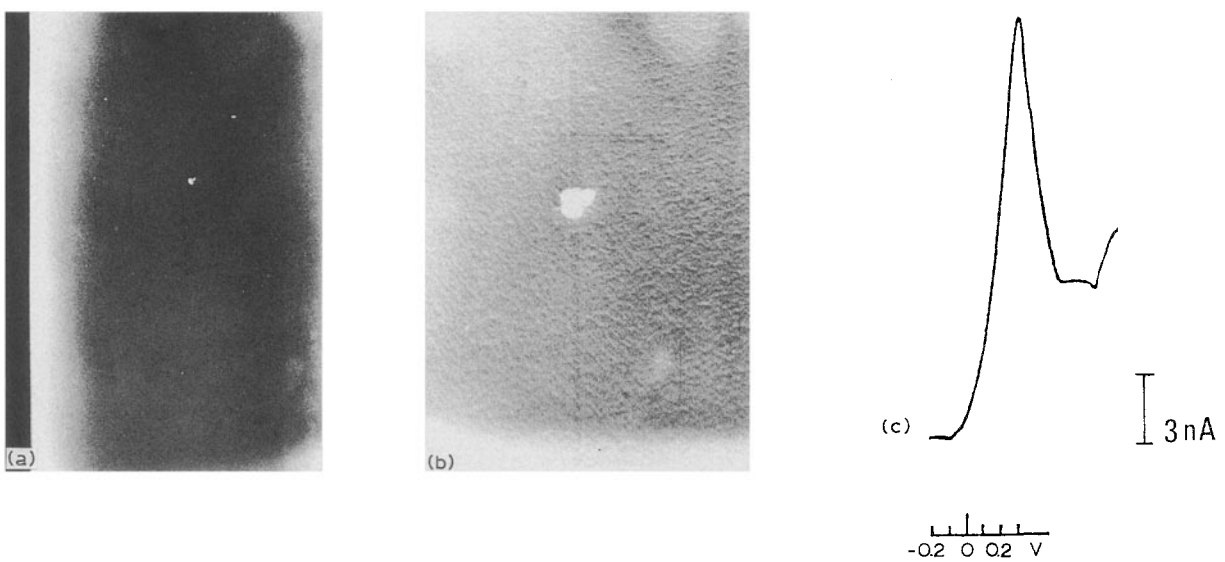


Figure 5 Micrographs showing the result of an SEM analysis at 3176x (a) and 15 176x (b) of a 30 μm carbon fibre pretreated with chromic acid. (c) Voltammogram obtained with a chromic acid treated 30 μm CFE in PBS containing AA 0.2 mM, DOPAC 50 μM and 5HIAA 25 μM (similar results are observed with a chromic acid cleaned 12 μm CFE).

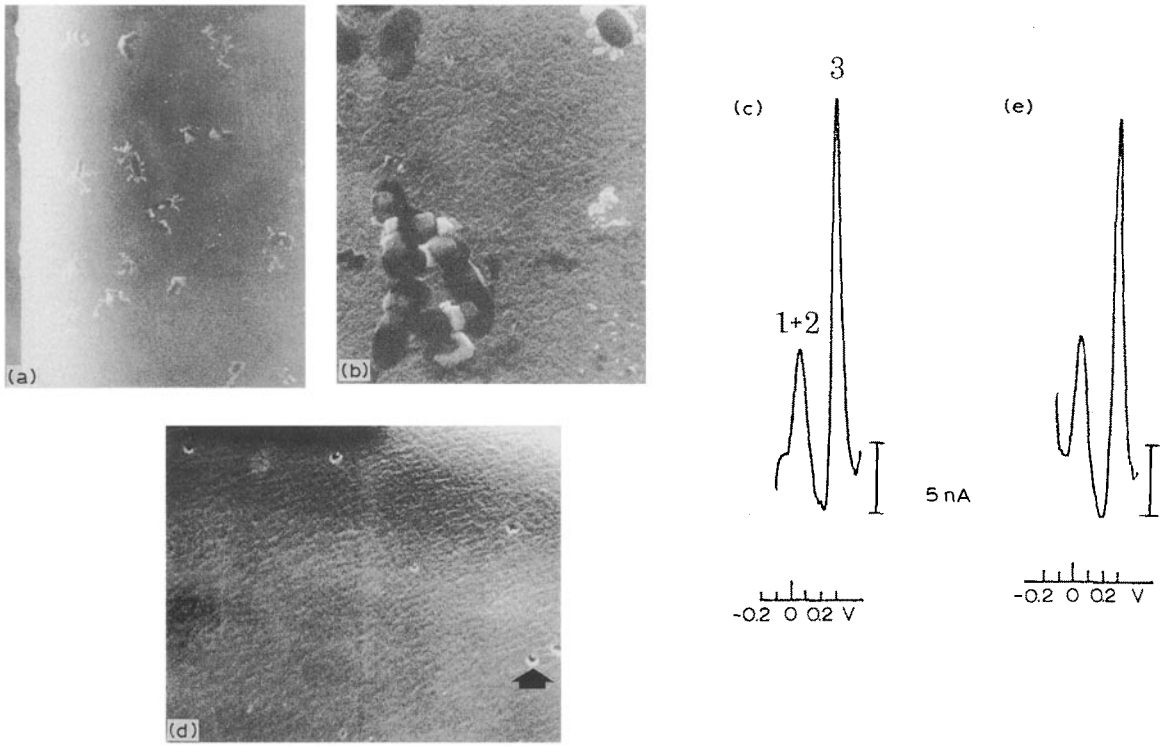


Figure 6 Micrographs showing the result of an SEM analysis at 3176x (a) and 15 176x (b) of a 30 μm carbon fibre which has received both chromic acid and electrical pretreatments. (c) Voltammogram obtained with a chromic acid and electrically pretreated 30 μm CFE in PBS containing AA 0.2 mM, DOPAC 50 μM and 5HIAA 25 μM (Peak 3). Note that AA + DOPAC oxidations merged in Peak 1 + 2 at + 50 mV. (d) Micrograph showing the result of an SEM analysis at 3176x of a 30 μm carbon fibre pretreated as above and then washed in distilled water 3 times, 1 h each. Note the presence of holes (arrows). (e) As in (c) but after cleaning with water as in (d).

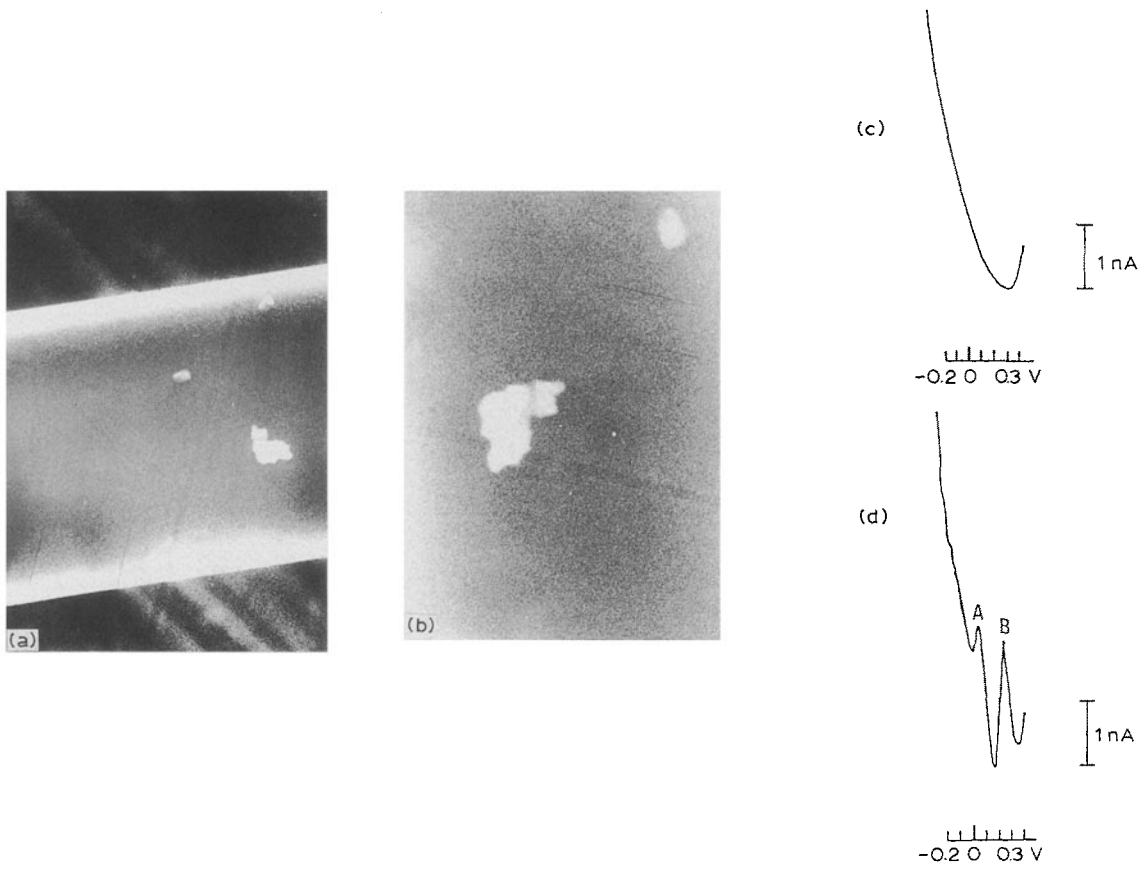


Figure 7 Micrographs showing the result of an SEM analysis at 3176x (a) and 15 176x (b) of a 30 μm carbon fibre chromic acid and electrically pretreated then coated with Nafion. (c) Voltammogram obtained with a pretreated and Nafion coated 30 μm CFE in PBS containing AA 0.2 mM, DOPAC 50 μM and 5HIAA 25 μM (or UA 10 μM) or in (d) PBS containing DA (Peak A at + 70 mV) and 5HT (Peak B at + 250 mV) both 20 nM. (the selective detection of DA and 5HT is not possible with the 12 μm CFE which does not accept the coat of Nafion).

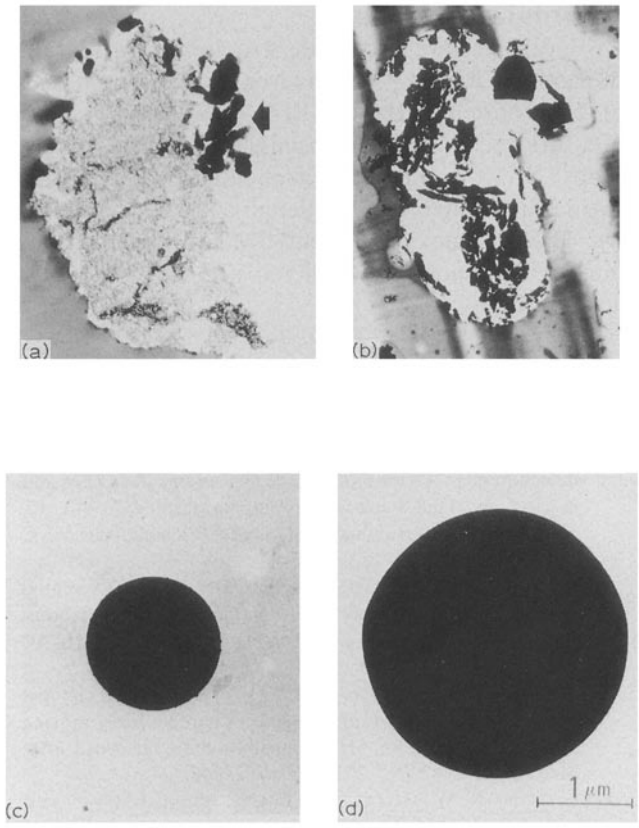


Figure 8 Micrographs showing the result of a TEM analysis in (a) a treated 12 μm carbon fibre showing a spongy internal structure with few crystallized particles (arrow, 23 100x) and (b) an untreated 12 μm carbon fibre showing crystals of carbon adhering to the side of the section hole (6060x). The untreated and treated 30 μm carbon fibres show similar carbon crystals but no spongy internal structure. (c, d) Small circular discs of approximately 1 μm (left) and 2–3 μm (right) on the sections (38 818x) from 12 μm or 30 μm untreated carbon fibres respectively.

sides of the hole resulting from the section. However, it has been possible to observe a more spongy structure for the treated 12 μm CFE when compared with the crystallized aspect of the untreated fibre section (Fig. 8a, b respectively). Vice versa, the crystalline appearance of the section of the 30 μm CFE is not altered by pretreatments (not shown). Finally, discs (approximately, 1 μm or 2–3 μm in diameter for the 12 μm or 30 μm carbon fibres respectively) uniform in shape and very dense are observed on the section of the untreated fibres (Fig. 8c, d).

4. Discussion

This study demonstrates two main points.

1. Differences in sensitivity and selectivity for electroactive compounds are also correlated with differences in the type of carbon fibre used (i.e. 12 μm or 30 μm dia.). The electrically pretreated 12 μm CFE can separate the oxidation of AA from that of the catechols and that of the indoles (Peaks 1, 2 and 3 respectively) while the 30 μm CFE can only selectively detect the indole signal (Peak 3; Peak 1 and 2 merged). However, both sensors displayed better sensitivity to the indoles and in particular to 5HT. Nevertheless, another difference between 12 μm and 30 μm CFE is the fact that it appeared impossible to electrically coat the 12 μm CFE with Nafion, while this was feasible for the 30 μm CFE. While no clear explanation is to date available for this phenomenon, the fact that the reaction to identical pretreatments results in different voltammetric specificities, may support further the influence of structural differences upon the electrochemical behaviour of these two kinds of CFE.

2. Any chemical and/or electrical pretreatment of the micro-CFEs alter not only the surface, as already suggested [15, 16], but probably the entire structure of the carbon fibre. The higher fragmentation of the pretreated carbon fibres also manifested by the disappearance of the dense disc present before treatment (which perhaps corresponds to the core of the untreated fibres [17, 18]), indicates that a larger surface of carbon is now available for interaction with electroactive compounds (which for instance could penetrate the carbon fibre through the pits, the holes and other induced fractures, thus further enhancing this interaction).

Taken together with the increased presence of redox couples [19] and that of oxides [20] on the surface of the carbon fibre following anodic treatments, this could be suggested as one of the possible explanations for the increased sensitivity of the pretreated CFE.

The TEM observations indicate that the internal structure appears to be more spongy for the treated 12 μm carbon fibre while more crystalline for the treated 30 μm carbon fibre. In association with the SEM analysis, which showed a very different surface between these two types of fibres, this supports the suggested presence of a structural difference between them [14, 21] and could explain their different electrochemical behaviours. We have observed that while the electrical pretreatment allows the 12 μm CFE to separate oxidation of AA from that of catechols and that of indoles, this is not the case when the 30 μm CFE is used, which presented selective sensitivity for the indole compounds only and in common with the 12 μm CFE, highest sensitivity for serotonin.

The large irregularities observed with SEM on the surface of the untreated 12 μm carbon fibres could be responsible for the lack of Nafion coating, a process which by contrast succeeded when performed on the smoother surface of the 30 μm carbon fibres.

Chromic acid is used in industry to enable carbon fibres to key to resins and other materials. It is also used to improve the sensitivity of the glassy-carbon electrode used in electrochemical detectors connected to an HPLC column [22]. In the present study, chromic acid markedly cleaned the surface of the carbon fibres, possibly by removing surface film and/or chemicals deposited during carbon fibre and CFE manufacture and storage. This process may expose a larger carbon surface for interaction with electroactive compounds and allow a more accurate Nafion coating, which could explain the greater selectivity for neurotransmitters observed in comparison with uncleaned Nafion CFEs [23]. In addition, in the TEM observations the solid core of the fibre appeared disrupted after the chromic acid treatment. This suggests that, like the electrical treatment, chromic acid

enhanced the fragility of the fibre, resulting in a more particulate carbon available for chemical exchanges, thus in an increased sensitivity. However, the holes on the surface of the fibre are obtained only after electrical pretreatment, thus suggesting a particular role for the holes in the resulting selectivity.

The newly reported crystals present on the surface of the pretreated CFEs did not modify the sensitivity and the selectivity as the voltammogram obtained was not changed after their removal. However, this observation underlines the importance of cleaning the tip of the treated CFEs before implantation, to avoid the introduction of unknown chemicals into the brain areas studied. The suggested presence of a film on the surface of the Nafion coated CFEs [11] seems to be confirmed by the SEM analysis; their surface appears very clean and very smooth, neither holes, pits or other fractures being observable, probably because they are covered by a layer of this sulphonate polymer. This observation also supports the view that it is the Nafion film that allows specific measurements of cations (i.e. dopamine and serotonin), while anions (i.e. AA, DOPAC, 5HIAA, UA) are repulsed [12].

Carbon fibres are made from a precursor, the two most widely used being polyacrylonitrile (PAN) or pitch (derived from coal tar or as a by-product in petroleum production). Manufacturers submit the precursor to progressive heat (up to 300–400 °C) in an oxidizing atmosphere, thus driving off impurities to obtain a liquid crystal called "graphene" made of hexagonal rings of carbon linked at the edge. The graphene is then heated to a final temperature of 1200 or 2500–3000 °C while it is stretched, causing the chains of carbon to line up in a regular and orientated structure [13, 14, 21].

In conclusion, the present results indicate that the chemical and electrical pretreatments induce friability of the linear structure of the carbon fibres with: (i) a rise in interacting fragments of carbon, with thus a possible concomitant rise in active chemicals on the surface of each fragment of carbon [19, 20], and (ii) the possible increase in penetration of fluids (i.e. the extracellular fluid *in vivo*) into the fibre through holes, pits and other fractures. These effects may offer a key to understanding the development of sensitivity and selectivity following pretreatments.

Further extension of this investigation is required with the analysis of the carbon fibres with a link energy dispersive system combined with a Jeol 35C microscope. This will allow the identification of the chemicals present on the surface of the carbon fibre before and after each pretreatment, therefore permitting a more precise interpretation of the chemical interactions between electroactive compounds and carbon.

Acknowledgements

We thank Mr T. Gray for kind assistance with the scanning microscope, and Dr S. J. Harris and Mr A. S. Pyper for their valuable advice. F. C. is a Medical Research Council Fellow. We thank AVCO-TEXTRON, USA, for generous supplies of the carbon fibres.

Abbreviations used:

CFE, carbon fibre electrodes; AA, ascorbic acid; DA, dopamine; DOPAC, 3,4-dihydroxy-phenylacetic acid; 5HT, serotonin; 5HIAA, 5-hydroxyindoleacetic acid; UA, uric acid; TEM, transmission electron microscopy; SEM, scanning electron microscopy ; ECF, extracellular fluid; DPV, differential pulse voltammetry; HVA, homovanillic acid; 3MT, 3-methoxytyramine; PBS, phosphate buffered saline; PAN, polyacrylonitrile.

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Received 5 March
and accepted 8 July 1991