Effects of Film Morphology and Surface Chemistry on the Direct Electrochemistry of Cytochrome c at Boron-Doped Diamond Electrodes

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Abstract

The effects of film morphology and surface termination on the direct electron transfer of horse heart cytochrome c on boron-doped ultrananocrystalline (B-UNCD) and microcrystalline (B-MCD) diamond thin-film electrodes were investigated. Quasi-reversible, diffusion-controlled cyclic voltammetric responses were observed on oxygen-terminated (atomic O/C ~0.015), but not hydrogen-terminated (atomic O/C ~0.02) diamond thin films. The effect of the surface termination was the same for both the nanostructured B-UNCD film with sp²-bonded carbon atoms in the grain boundaries and the well faceted B-MCD film with micron-sized grains and largely devoid of sp² carbon. Stable cyclic voltammetric *i-E* curves were recorded with cycling for both oxygen-terminated films indicating the absence of protein denaturation and electrode fouling. The peak currents increased linearly with the square root of the scan rate and the protein concentration; both indicative of a reaction rate limited by semi-infinite linear diffusion of the protein. Similar heterogeneous electron-transfer rate constants were observed for oxygen-terminated B-UNCD (3.48 (± 1.25) × 10⁻³ cm/s) and B-MCD films (2.38 (± 0.72) × 10⁻³ cm/s). The results clearly reveal that the oxygen-terminated surface is more active for electron-transfer with this soluble redox protein than is the hydrogen-terminated surface. The film

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4 morphology does not influence the diffusion-controlled response of the redox
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6 protein.
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1. Introduction

Mammalian cytochrome *c* is a small (~12 kDa), water-soluble heme protein that functions as an electron carrier between cytochrome *c* reductase and cytochrome *c* oxidase; components of oxidative phosphorylation [1,2]. The redox partners of this protein are membrane bound, whereas the protein resides in the cytosol. The redox process of this protein involves its heme cofactor shuttling between the ferric (Fe^{+3}) and ferrous (Fe^{+2}) oxidation states. Cytochrome *c* electron transfer is believed to occur at the solvent-exposed edge of the heme group by an outer-sphere mechanism [1-6]. Appropriate “docking” of the cytochrome *c* with these redox enzymes is critical for rapid electron transfer [3]. For rapid electron transfer, appropriate chemical and electrostatic interactions with an electrode are necessary to properly orient the heme edge of the redox protein close to the surface [3-7]. Electrostatic effects are important for properly orienting the protein with the redox partners in the “docked” state to facilitate electron transfer [1-7].

The redox behavior of cytochrome *c* at various solid electrodes has been studied extensively over the years [7-17]. One goal for this work has been to learn about the importance of electrostatic effects and electrode interfacial structure (hydrophobic/hydrophilic interactions) on electron transfer with the protein. This protein is a good model system for investigating the fundamental aspects of biological electron transfer both when the protein is dissolved in solution and when it is confined to a chemically-modified electrode surface [8-17].

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4 In general for all electrodes, two factors are necessary for rapid cytochrome c
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6 electron transfer: (i) a highly purified form of the protein dissolved in solution and
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8 (ii) electrode pretreatment and or chemical modification to produce a clean,
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10 hydrophilic/oxophilic and or negatively charged surface [18,19]. For carbon
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12 electrodes, Armstrong and coworkers reported that the electron-transfer rate for
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14 cytochrome c on the edge plane of pyrolytic graphite is much faster than on the
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16 basal plane sites due to the higher oxygen/carbon (O/C) ratio of edge plane
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18 [10,17]. Chemical interactions are possible with the “oxygenated” edge plane
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20 sites more so than with the oxygen-poor basal plane sites. The key functional
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22 groups are carboxylates that are deprotonated at physiological pH. Electrostatic
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24 interactions between the positively charged heme edge and the negatively
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26 charged electrode surface lead to proper orientation and positioning of the
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28 protein such that the activation barrier for electron-transfer is lowered. The redox
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30 protein’s orientation and distance from the electrode are critical factors.
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41 In recent years, boron-doped diamond has emerged as a useful new
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43 carbon electrode for electroanalytical measurements because of some unique
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45 properties [20-24]. Boron-doped diamond electrodes can be single crystal or
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47 polycrystalline. Polycrystalline diamond films can be subdivided into three types:
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49 microcrystalline, nanocrystalline or ultrananocrystalline [25]. Microcrystalline
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51 diamond thin-films are grown in a hydrogen-rich chemical vapor deposition (CVD)
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53 environment and are composed of well-faceted crystallites that are micrometers
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55 in dimension [26]. Nanocrystalline diamond thin films are also deposited in a
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4 hydrogen-rich CVD environment. Preparing the substrate surface to produce a
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6 high initial nucleation density is necessary to form nanocrystalline diamond.
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8 Such substrate preparation leads to a continuous film in a short deposition time
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10 with grains or crystallites having dimensions of tens of nanometers [23]. Finally,
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12 ultrananocrystalline diamond thin films are grown in an Ar-rich CVD environment,
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14 being pioneered by Gruen and coworkers at Argonne National Laboratory [27].
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16 These films consist of 50-100 nm aggregates of diamond grains (~15 nm diam.)
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18 and are grown under conditions where a high rate of renucleation exists. Unlike
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20 nanocrystalline diamond, ultrananocrystalline diamond films have high-energy,
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22 high-angle grain boundaries that contain π -bonded carbon [27,28]. There have
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24 been a few reports of direct electron transfer with redox proteins, namely
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26 cytochrome c, at unmodified and chemically-modified diamond (polycrystalline)
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28 electrodes [29-38]. Diamond's unique microstructure and resistance to electrode
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30 fouling have been drivers of this work.
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41 Our original work with dissolved horse heart cytochrome c utilized a
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43 boron-doped microcrystalline diamond thin-film electrode that was supposed to
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45 be low in surface oxygen due to growth in a hydrogen-rich plasma [29].
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47 Regardless of the electrode, chromatographically-purified protein is absolutely
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49 necessary to realize relatively large heterogeneous electron-transfer rate
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51 constants, $k_{app}^0 \sim 1 \times 10^{-3}$ cm/s [18,19,29,38]. At about the same time, Marken
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53 and coworkers [30] reported on studies of dissolved cytochrome c. In their paper,
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55 a boron-doped diamond microcrystalline diamond thin-film electrode, polished
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4 with alumina and oxidatively pretreated, was used. Reversible electron transfer
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6 for horse heart cytochrome *c* was observed. In contrast, little redox activity was
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8 observed on the oxidatively pretreated-only electrode. Evidence was provided
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10 that the electron transfer occurred with the protein in a surface confined state.
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12 Clearly, the alumina played a role in facilitating the electron transfer and one can
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14 surmise that residual grit on the electrode surface possibly functioned as an
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16 adsorption site for the protein.
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24 Since the publication of these initial two papers, several others have been
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26 emerged in the literature, all showing that the most reversible electrochemistry
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28 for cytochrome *c* is observed at diamond electrodes functionalized with carbon-
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30 oxygen groups or molecular linkers that are oxygenated and or charged [31-38].
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32 Reversible electrochemistry on these modified surfaces has been reported for
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34 both dissolved and immobilized protein. One paper by the Nebel group, for
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36 example, showed protein denaturation on a hydrogen-terminated diamond
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38 electrode with no observed electrochemical activity [32].
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45 Given the conflicting findings between our original work on low-oxygen,
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47 hydrophobic, hydrogen-terminated diamond and the literature reports for
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49 oxygenated or chemically-modified diamond, we decided to revisit this research.
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51 Support for our original finding of quasi-reversible electron transfer on a
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53 hydrophobic, low-oxygen diamond film was the reported importance of
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55 hydrophobic interactions for properly orienting the heme group of cytochrome *c*
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57 by Rivas *et al.* [39]. The authors reported rapid electron transfer ($k^0 \sim 10^{-3}$ cm/s)
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for cytochrome *c* immobilized on a Ag electrode modified with a hydrophobic self-assembled monolayer (SAM), *n*-alkanethiol HS-(CH₂)_{*n*}-CH₃ [39]. Their work showed that hydrophobic interactions with the electrode are also important for properly orienting the protein to promote electron transfer. Compared with electrostatically-bound cytochrome *c*, the hydrophobic interactions lead to the same structural changes of the heme and relatively large rate constants [39].

We report herein on a systematic investigation of the electron-transfer kinetics for horse heart cytochrome *c* at boron-doped diamond electrodes as a function of the (i) film morphology (ultrananocrystalline (B-UNCD) and microcrystalline (B-MCD) thin films), and (ii) surface chemistry (oxygen- and hydrogen-termination). Cyclic voltammetry was used to study the redox activity of dissolved protein as a function of scan rate and pH, and XPS was used to quantify the surface chemistry of the electrodes.

2. Experimental

2.1. Diamond Thin-Film Deposition. The boron-doped microcrystalline (B-MCD) and ultrananocrystalline (B-UNCD) diamond thin films were deposited on highly conductive *p*-type Si (100) substrates ($\sim 10^{-3}$ Ω-cm, 0.05 cm thick × 1 cm² area, Virginia Semiconductor Inc., Fredricksburg, VA) using a commercial microwave-assisted chemical vapor deposition (CVD) system (1.5 kW, formerly ASTeX Inc., now Seki Diamond (JAPAN)). The substrates were pretreated by mechanically scratching with dry 0.1 μm diameter diamond powder (GE Superabrasives,

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4 Worthington, OH) for 5 min on a felt polishing pad. The scratched substrates
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6 were then ultrasonically cleaned in ultrapure water, isopropyl alcohol (IPA),
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8 acetone, IPA, and finally, ultrapure water for 10 min each to remove polishing
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10 debris from the surface. The clean scratches and residual powder particles
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12 serve as the initial nucleation sites for diamond growth.
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19 The B-MCD films were deposited using 1.0 sccm (standard cubic
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21 centimeters per minute) of CH_4 and 197.5 sccm of H_2 (0.5% v/v) as the source
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23 gas mixture, 1 kW of microwave power, a system pressure of 45 Torr, a
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25 substrate temperature of $\sim 750^\circ\text{C}$ (estimated via a single filament optical
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27 pyrometer) and a growth time of 7-10 h. The B-UNCD films were deposited using
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29 gas flow rates of 1.0, 92.5 and 5 sccm for CH_4 , Ar, H_2 , respectively. Deposition
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31 was carried out using 800 W of plasma power under system pressure of 140 Torr
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33 and a substrate temperature of $\sim 750^\circ\text{C}$ for 2 h. All gases were ultrahigh purity
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35 ($\geq 99.999\%$) grade (CH_4 and H_2 from AGA Specialty Gas, Cleveland, OH; Ar from
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37 BOC Group, Inc., Murray Hill, NJ). Both the B-MCD and B-UNCD films were
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39 doped during deposition by adding 10 ppm B_2H_6 in H_2 at 1.5 sccm to the source
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41 gas mixture (0.1% B_2H_6 diluted in H_2 , Matheson Gas Products, Inc.). Total source
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43 gas flow rates for the B-MCD and B-UNCD films were 200 and 100 sccm,
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45 respectively. Following the deposition period, the CH_4 and B_2H_6 gas flows were
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47 stopped and the films remained exposed to the H_2 (MCD) or Ar/ H_2 (UNCD)
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49 plasma for an additional 5-10 min. The plasma power and pressure were then
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51 slowly reduced over a 15-30 min period to cool the samples to a temperature
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4 around 400 °C with the plasma ignited (*i.e.*, in the presence of atomic hydrogen).
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6 *Post growth annealing in atomic hydrogen is essential for removing adventitious*
7 *nondiamond carbon impurity via gasification, minimizing dangling bonds, and*
8 *ensuring full hydrogen termination.* Importantly, this annealing procedure is
9 different from that used in our prior work [29], a point that will be discussed
10 further below. The plasma power was then turned off and the films were cooled
11 to room temperature under a flow of H₂ (MCD) or Ar/H₂ (UNCD). Both film types
12 had a nominal boron dopant concentration between 5×10^{20} and 5×10^{21} B cm⁻³
13 and a film resistivity of ~0.01 Ω-cm, or less. The dopant concentration was
14 determined by boron nuclear reaction analysis (Surface Characterization Facility,
15 Case Western Reserve University).
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33 *2.2. Surface Termination.* After growth, the diamond films were chemically
34 treated to remove adventitious metal and sp²-bonded nondiamond carbon
35 impurities from the surface by (i) exposure to 3:1 HCl/HNO₃ (v/v) for 30 min at
36 ~50 °C followed by a rinse with ultrapure water and then (ii) exposure to 30%
37 hydrogen peroxide (Fisher Scientific) for 30 min ~50 °C followed by a rinse with
38 ultrapure water. This chemical oxidation introduces surface carbon-oxygen
39 functionalities, which we refer to as “oxygen-terminated” in the text. This
40 treatment increases the O/C atomic ratio from ~0.02 to 0.14-0.23, as determined
41 from X-ray photoelectron spectroscopy (XPS) measurements. To regenerate the
42 low oxygen, hydrogen-terminated surface, the diamond films were placed back
43 into a dedicated CVD reactor for a 30-min hydrogen-plasma treatment under a
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4 microwave power of 1000 W, a system pressure of 35 torr, and H₂ gas flow of
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6 200 sccm. The films were slowly cooled in the atomic hydrogen plasma over a
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8 15-30 min period, as described above. We refer to these films as “hydrogen-
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10 terminated” in the text. The atomic O/C ratio of this surface is ≤ 0.02 .
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15 *2.3. Cyclic Voltammetry.* All electrochemical measurements were performed at
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17 room temperature in a single compartment glass cell using a computer-controlled
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19 potentiostat (CH Instruments, Inc., Austin, TX) [20]. A standard three-electrode
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21 configuration was employed. The electrochemical cell was housed inside a
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23 grounded Faraday cage for electrical shielding. The working electrode was
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25 clamped against a clean Viton o-ring at the bottom of the cell. Backside electrical
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27 contact was made to the diamond working electrode by scratching the
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29 conducting Si substrate with abrasive paper and then cleaning and coating the
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31 area with graphite from a standard pencil or a dab of Ag paste. This procedure
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33 produces good ohmic contact between the electrode and a copper current-
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35 collector plate. The geometric area of the electrode exposed to the solution was
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37 0.2 cm². A silver/silver chloride reference electrode (saturated KCl, $E^{\circ} = -45$ mV
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39 vs. SCE) was used, which was placed inside a cracked-glass capillary filled with
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41 1 mol dm⁻³ KCl and positioned in close proximity to the working electrode. A large
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43 area carbon rod served as the counter electrode and was positioned normal to
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45 the working electrode. Solutions were typically deaerated with N₂ gas bubbling
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47 for at least 20 min prior to any voltammetric measurement. Solutions remained
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49 blanketed with the gas during a measurement.
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4 2.4. *X-ray Photoelectron Spectroscopy (XPS)*. XPS was used to determine the
5 oxygen and carbon atomic ratios (O/C) on the diamond film surfaces [40-45].
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7 XPS spectra were obtained using a PHI 5400 XPS spectrometer (Physical
8 Electronics, Inc.) with an Mg X-ray source. A take-off angle of 45 degrees was
9 used for all measurements at a base pressure of *ca.* 5×10^{-9} Torr. The atomic
10 percentage of each element was determined from peak areas divided by
11 appropriate bulk sensitivity factors specific for the PHI 5400 instrument and the
12 supplied deconvolution software.
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26 2.5. *Chemicals*. All solutions were prepared with ultrapure water from a
27 Barnstead E-pure water purification system (Fisher Scientific, Inc.) with a
28 resistivity of $\sim 18 \text{ M}\Omega \times \text{cm}$. Stock solutions of cytochrome *c* (in 0.3 mol dm^{-3} NaCl,
29 10 mmol dm^{-3} Tris-HCl buffer, pH 7) were prepared by chromatographically
30 purifying and concentrating horse heart cytochrome *c* (Sigma-Aldrich Corp.)
31 using a published procedure [46-48]. Cytochrome *c* concentrations were
32 determined spectrophotometrically at 550 nm from the difference in the
33 absorbance of the ferrous and ferric forms of the protein using $\Delta\epsilon_{550\text{nm}} = 21 \text{ cm}^{-1}$
34 mM^{-1} [49,50]. Ferricytochrome *c* was reduced by a small amount of sodium
35 dithionite. Cytochrome *c* in potassium phosphate buffers was prepared by
36 repeated dilutions by buffers of desired pH and concentration followed by
37 centrifugations using an Amicon[®] Ultra-15 centrifugal filter (Millipore).
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All glassware and storage bottles were cleaned in a three-step manner: KOH/ethanol bath, Alconox/ultrapure water solution, and ultrapure water rinse. The cleaned glassware was then dried in an oven at ~50 °C.

3. Results

3.1. XPS Measurements. In our prior report, data on the oxygen content of the diamond film surface were not reported [29]. It was presumed that the surface was low in oxygen given the relatively high contact angle for water and the fact that the film was grown in a hydrogen-rich plasma, which favors a low oxygen, hydrogen surface termination [29]. Presently, we used XPS to characterize the elemental composition in the near-surface region of B-MCD and B-UNCD films “as deposited”, after oxygenation, and after rehydrogenation. Typical XPS spectra for “as deposited”, oxygen-terminated and hydrogen-terminated B-UNCD surfaces are presented in Figure 1. The spectrum for the as-deposited film showed a sharp C 1s peak near 284.5 eV and a small, but quantifiable, O 1s peak at ca. 532 eV with an O/C atomic ratio of 0.05. The plasmon loss feature at 318.5 eV results from energy losses of the outgoing photoelectrons upon excitation of the bulk and surface plasmons of diamond [51]. Its presence indicates high purity of the diamond film [51]. After chemical oxidation, the O 1s peak intensity increased considerably to an O/C value of 0.17. This is typically the maximum ratio obtainable on diamond films and is similar to the value of 0.18 reported for a diamond electrode treated in an oxygen plasma or exposed to acidic environments [40-45]. The rehydrogenation step converted the diamond

surface back to a hydrogen-termination by removing surface oxygen and reducing the O/C ratio to 0.02. Identical spectra were obtained for “as deposited”, oxygen-terminated, and hydrogen-terminated B-MCD films (data not shown).

Figure 1. X-ray photoelectron survey spectra of B-UNCD electrodes (A) as-deposited, (B) after oxygenation (oxygen terminated) and (C) after rehydrogen (hydrogen terminated).

Figure 2. X-ray photoelectron survey spectra of oxygen-terminated (A) B-MCD and (B) B-UNCD electrodes after immersion and potential cycling in a solution of 0.1 mmol dm^{-3} cytochrome *c* in 0.1 mol dm^{-3} NaCl + 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). The electrodes were rinsed with ultrapure water after the cycling.

Measurements were performed to probe for residual protein adsorption on both diamond electrodes. The electrodes were subjected to multiple cyclic voltammetric scans in a 0.1 mM cytochrome *c* solution over the heme Fe(II/III) redox transition between 0.3 and -0.2 V . XPS survey spectra shown in Figure 2 for oxygen-terminated B-UNCD and B-MCD electrodes cycled and rinsed reveal the presence of a weak surface nitrogen signal (N 1s, ca. 400 eV) in addition to the C1s and O1s signals. The increased N, an element normally not detected on the diamond film surface, is consistent with some residual protein on the surface. Interesting, however, is that fact that no signals for Fe ($2p_{3/2}$, ca. 710 eV) or S ($2p$, ca. 165 eV) were detected. It is unclear why no Fe or S was detected. It is possible that regions of the adsorbed protein containing these elements were buried in adsorbed protein on the surface deeper than the escape depth for the

photoelectrons. Most importantly, this small amount of residual and presumably denatured protein did not adversely affect electron transfer with dissolved

Figure 3. Cyclic voltammetric *i-E* curves for a hydrogen-terminated B-UNCD film showing (A) the initial background response in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7), (B) a repeat background scan after exposure to a cytochrome *c* solution and (C) the response in 0.1 mmol dm^{-3} cytochrome *c* plus 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). Scan rate = 20 mV s^{-1} . Electrode area = 0.2 cm^2 . The scan was initiated at 0.3 V and the potential swept initially in the negative direction.

cytochrome *c* molecules. The absence of electrode fouling is clearly evident in the voltammetric data presented below.

3.2. Cyclic Voltammetric Studies. Figure 3A-C shows a series of cyclic voltammetric curves for a hydrogen-terminated B-UNCD thin-film electrode. Background curves in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7) before (A) and after (B) potential cycling in a cytochrome *c* solution are presented along with a curve for 0.1 mmol dm^{-3} cytochrome *c* plus 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7) (C). XPS confirmed the low oxygen content on this electrode ($\text{O/C} \leq 0.02$). In contrast to our previously reported results [29],

there is no clear cycling of the redox protein between the ferric (Fe^{+3}) and ferrous (Fe^{+2}) states. Similar inactivity was observed for low oxygen, hydrogen-

Figure 4. Cyclic voltammetric i - E curves for 0.1 mmol dm^{-3} cytochrome c plus 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7) at oxygen-terminated (A) B-UNCD and (B) B-MCD electrodes. Curves are shown for different scan numbers (1-10). The scans were initiated at 0.3 V and the potential swept initially in the negative direction.

terminated B-MCD electrodes. This inactivity on the hydrogen-terminated surface is consistent with the results reported by the Nebel group [33]. There is an increase in the cathodic current at -0.2 V that develops after a couple of cycles. This current is attributed to the electrochemically irreversible reduction of ferricytochrome c (III) to ferrocyanochrome c (II). The fact that the background curves before and after exposure to the protein solution show no current at this potential is confirmation that the current is associated with the protein. This lack of reversibility may be due to protein denaturation as has been previously reported for hydrogen-terminated diamond [33].

A completely different response was observed for oxygen-terminated B-UNCD and B-MCD electrodes. Figure 4 shows cyclic voltammograms for both

electrodes during repeated cycles in a 0.1 mmol dm^{-3} cytochrome *c* solution. The oxygen termination was confirmed by XPS ($\text{O/C} = 0.15\text{-}0.23$). Well-resolved oxidation and reduction peaks (heme Fe(II/III)) are seen for both electrodes with no decrease in peak current or change in ΔE_p over 10 consecutive cycles. This indicates that there is no significant protein denaturation or electrode fouling during short term cycling at the oxygen-terminated electrode. This result also indicates the residual adsorbed protein, evidenced by the weak N1s signal in the XPS survey scan (Fig. 2), does not inhibit the electron-transfer kinetics. Consistency of voltammetric traces over multiple cycles is indicative of a stable interfacial structure at the electrode surface.

Figure 5. Cyclic voltammetric background i - E curves for an oxygen-terminated B-UNCD electrode in 0.1 mol dm^{-3} NaCl plus 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). Scan rate = 20 mV s^{-1} . Electrode area = 0.2 cm^2 . The curves were recorded in buffer before (black, solid line) and after (red, dashed dot line and blue, dashed line) cycling in a cytochrome *c* solution. The black and blue curves were recorded in solutions deaerated with N_2 while the red curve was recorded in naturally aerated electrolyte solution. The scan was initiated at 0.3 V and the potential swept initially in the negative direction.

Figure 5 shows background cyclic voltammograms of an oxygen-terminated B-UNCD electrode before and after potential cycling in a cytochrome *c* solution. The absence of current peaks in the 0.2 to -0.1V region indicates there is no detectable adsorption of electroactive protein following the redox cycling and subsequent washing of the electrode. There is, however, an increase in the background cathodic current at -0.2 V seen for the cytochrome *c*-cycled electrode in the naturally-aerated electrolyte (red curve). This current is reduced after deaerating the solution with N₂. In this electrolyte, there would normally be little current for dissolved oxygen reduction at this potential. This result suggests the oxygen reduction reaction may be catalyzed by low levels of residual protein on the surface (see Fig. 2), possibly a denatured protein residue with 5-coordinated, catalytic Fe-porphyrin [52,53].

Figure 6 shows cyclic voltammetric *i*-*E* curves for cytochrome *c* on an oxygen-terminated B-UNCD electrode at different scan rates from 2-100 mV s⁻¹. The midpoint potential (*E*_{1/2}), measured as the average between the reduction and oxidation peak potentials, remained unchanged at 0.032 V vs. Ag/AgCl (0.229 V vs. NHE) over the probed scan rates. The peak currents and ΔE_p , as can be seen, both increased with increasing scan rate. Figure 7A and B show plots of the cathodic peak current, *i*_{pc}, and the scan rate^{1/2}, $v^{1/2}$, and the protein concentration, respectively. The Randles-Sevcik equation predicts a linear relationship between both the peak current, *i*_p, and $v^{1/2}$ and the analyte

Figure 6. Cyclic voltammetric *i*-*E* curves for 0.1 mmol dm⁻³ cytochrome *c* in 0.1 mol dm⁻³ NaCl and 0.01 mol dm⁻³ Tris-HCl (pH 7) on an oxygen-terminated B-UNCD electrode at different scan rates from 2 -100 mV s⁻¹. Electrode area = 0.2 cm². The scan was initiated at 0.3 V and the potential swept initially in the negative direction. The fifth cycle is shown for each scan rate.

concentration, C^* (mol cm⁻³), for a diffusion-controlled redox reaction according to:

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C^* v^{1/2}$$

The other variables are as follows: n (number of electrons transferred per mole), A (electrode area, cm²) and D (diffusion coefficient, cm² s⁻¹). The plots reveal a linear increase in i_{pc} with both $v^{1/2}$ and C . Similar trends were observed for the oxygen-terminated B-MCD electrodes (data not shown). The diffusion coefficient, D_{ox} , was determined from the slope of the i_{pc} vs. $v^{1/2}$ curve to be 2.63×10^{-6} cm² s⁻¹ for B-UNCD and 2.09×10^{-6} cm² s⁻¹ for B-MCD.

Figure 7. (A) Plot of the reduction peak current, i_{pc} , for 0.1 mmol dm^{-3} cytochrome *c* in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7) as a function of the $(\text{scan rate})^{1/2}$. (B) Plot of i_{pc} versus the cytochrome *c* concentration in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). Both sets of data were created from cyclic voltammetric *i*-E curves recorded with an oxygen-terminated B-UNCD electrode. The i_{pc} -concentration data are for a 20 mV s^{-1} scan rate.

The apparent heterogeneous electron-transfer rate constant, k_{app}^O , was calculated from the scan rate dependence of ΔE_p using Nicholson's theoretical treatment and assuming a charge-transfer coefficient $\alpha=0.5$ [54]. The nominal values of k_{app}^O were $3.48 (\pm 1.25) \times 10^{-3} \text{ cm s}^{-1}$ for B-UNCD and $2.38 (\pm 0.72) \times 10^{-3} \text{ cm s}^{-1}$ for B-MCD. The values are in good agreement, by order of magnitude, with the rate constant we reported in our original work ($1.0 (\pm 0.1) \times 10^{-3} \text{ cm s}^{-1}$) [29], and other rate constants reported in the literature [55,56]. The fact that the calculated k_{app}^O values are statistically indistinguishable for the different scan rates examined here, as shown in Table 1, indicates that uncompensated ohmic resistance within the electrode and the electrolyte solution are not significantly influencing the voltammetric curve shapes.

Figure 8. Plots of (A) the reduction peak current, i_{pc} , and (B) the heterogeneous electron-transfer rate constant, k^o_{app} , for cytochrome *c* as a function of the solution pH for oxygen-terminated B-UNCD (black) and B-MCD (red) electrodes. Cyclic voltammograms were recorded for 0.1 mmol dm⁻³ cytochrome *c* in 0.1 mol dm⁻³ phosphate buffer of different pH. The i_{pc} -solution pH data are for 20 mV s⁻¹. Average and standard deviation values are shown for $n \geq 3$.

The effect of solution pH on the cytochrome *c* cyclic voltammetric response was investigated between pH 6 and 9. The isoelectric point of the protein is ~10 [57], so at these pH values the protein is positively charged. The buffering capacity for phosphate buffers is generally from about 5.5 to 8 so the buffering capacity of the pH 9 solution is somewhat questionable. Nevertheless, Figure 8 A and B show plots of the reduction peak current, i_{pc} , and the heterogeneous electron-transfer rate constant, respectively, for the redox protein as a function of the solution pH. Data are shown for oxygen-terminated B-UNCD and B-MCD electrodes. The maximum reduction current for both electrodes is observed between pH 6.5 and 7.5. Overall, there is not a significant pH effect on the current magnitude. The same is true of the k^o_{app} – pH data. There is a relatively minor effect of pH on the rate constant and the values for the B-UNCD electrodes are slightly larger than the values for the B-MCD electrodes. Table 2 presents a summary of some cyclic voltammetric data ($E_{1/2}$ and ΔE_p) for an

oxygen-terminated B-UNCD and B-MCD electrode at different solution pH. In the absence of statistical analysis, the trend in the data for both electrodes is that the midpoint potential, $E_{1/2}$, and the peak potential separation, ΔE_p , are similar for both electrodes and relatively independent of the solution pH.

4. Discussion

The diamond electrode surface chemistry, but not the surface morphology, influences the electron-transfer kinetics of cytochrome *c*. A key finding from this work is that the hydrogen-terminated diamond surface is inactive for electron transfer with this redox protein, even though this surface shows highest kinetic activity for several other inorganic redox systems [20-26,28]. Rather, the oxygen-terminated diamond electrodes are the most kinetically active toward cytochrome *c* redox chemistry. This observation is in agreement with the other reports in the literature for this redox protein on diamond electrodes [30-37]. For example, work by the Nebel group has shown the importance of chemical interactions of the protein with the diamond surface that are promoted by an oxygen-terminated surface [32-35]. These chemical forces include hydrogen bonding and dipole-dipole interactions that are critical for orienting the heme edge appropriately for rapid electron exchange with the electrode. Nominal heterogeneous electron-transfer rate constants of $2-4 \times 10^{-3}$ cm/s (see Fig. 8B) are seen for both oxygen-terminated B-UNCD and B-MCD electrodes and are comparable to other values reported in the literature [29,55,56].

Over the years, metal electrodes coated with self-assembled monolayer (SAM) films have been routinely used to study the electron transfer of cytochrome *c* (both dissolved in solution and adsorbed), specifically the distance dependence of the electron-transfer rate constant [58] and the impact of specific surface chemical sites on electron-transfer [59-63]. SAMs composed of carboxylic acid-terminated thiols, which provide a negatively charged surface at physiological pH, have been used to study the electron-transfer properties of electrostatically complexed cytochrome *c*; a condition that mimics its natural binding with cytochrome *c* oxidase [63,64]. This interaction involves several lysine residues on the front surface of the protein that form a ring-like pattern around the exposed heme edge and matches the arrangement of negative charges on the binding domains of the partner proteins [39]. As indicated by Yue et al. [63], molecular dynamics calculation shows that van der Waal interactions and protein-solvation energy help to stabilize the cytochrome *c*-enzyme complex, whereas electrostatic forces play a guiding role in the initial stage of complex formation [65]. Overall, the most important aspects for controlling the heterogeneous electron-transfer rate constant for cytochrome *c* are the geometry or organization of the protein and how close the heme edge can approach the electrode surface [63]. The distance of closest approach and the protein structure can be controlled through manipulation of the electrode surface chemistry. Bowden et al. recognized early on the importance of electrode hydrophilicity/oxophilicity [56]. Recently, Baio et al. [66] probed the orientation of electrostatically immobilized cytochrome *c*. Using a COOH-SAM (negatively

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4 charged at neutral pH) on Au, the authors observed the protein with the heme
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6 group pointed toward the electrode. When a NH₂-SAM (positively charged) was
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8 used, the protein adopted the opposite orientation with the heme group pointing
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10 away from the electrode. This example shows that changing the surface charge
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12 can have a significant impact on how close the heme center can approach the
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14 electrode (i.e., reduced electron tunneling distance). Our results further confirm
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16 that for boron-doped diamond electrodes, like other electrodes, the surface
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18 hydrophilicity/oxophilicity, and presumably surface charge, are critical for
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20 achieving rapid rates of electron transfer with purified protein.
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29 An important question is, how do we rationalize these findings with our
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31 original report of quasi-reversible, diffusion-controlled redox chemistry for
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33 cytochrome *c* on an as-deposited, low-oxygen, hydrophobic boron-doped
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35 ultrananocrystalline diamond electrode [29]? In that work, we reported a
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37 heterogeneous electron-transfer rate constant of $\sim 1 \times 10^{-3}$ cm/s on electrodes
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39 that were presumed to be hydrogen-terminated (low in surface oxygen) because
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41 of the post-deposition hydrogen plasma treatment that was employed [29]. This
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43 post-growth treatment consisted of a 30-min hydrogen plasma treatment at the
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45 growth temperature and pressure. At the end of this treatment, the plasma was
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47 extinguished and the samples were allowed to cool from near that of the diamond
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49 growth (ca. 700-800 °C) down to room temperature under a flow of H₂ in the
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4 In light of the results presented herein, we now believe that post-growth
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6 hydrogen plasma treatment in our previous work did not produce a hydrogen-
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8 terminated surface as was supposed. Rather, surface oxygen was likely
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10 introduced upon exposure of the films to the air as a consequence of the cool-
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12 down procedure that was followed. A key point in the prior work is that the
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14 hydrogen plasma was extinguished with the substrates at or near the growth
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16 temperature of 700-800 °C. In the absence of atomic hydrogen, which occurs
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18 once the plasma is extinguished, surface hydrogen will desorb at the high
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20 temperatures creating free radical carbon sites that can (i) couple to produce π -
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22 bonded carbon dimers on the surface and or (ii) react with O₂/H₂O upon
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24 exposure to air to form surface carbon-oxygen functionalities [67-69]. Therefore,
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26 the procedure that was followed in the prior work likely produced more of an
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28 oxygen rather than a hydrogen surface termination. We have since learned that
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30 to obtain a hydrogen-terminated surface, atomic hydrogen must be present as
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32 the substrates are being cooled down. This is accomplished, as was the case for
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34 the electrodes used in the present work, by gradually reducing the power and
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36 pressure of the post-growth hydrogen plasma until the substrate temperature
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38 reaches of ca. 400 °C or lower [67-69]. Below this temperature, the surface
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40 hydrogen is sufficiently stable and oxygenation is avoided.
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53 Cyclic voltammetric measurements were made on the oxygen-terminated
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55 diamond thin-film electrodes to determine the diffusion coefficient for the protein,
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probe for protein denaturation and electrode fouling. The diffusion coefficients determined from the slope of the $i_{pc}-v^{1/2}$ curves ($2.63 \times 10^{-6} \text{ cm}^2/\text{s}$ for B-UNCD and $2.09 \times 10^{-6} \text{ cm}^2/\text{s}$ for B-MCD) are close to other reported values, $0.87 \times 10^{-6} \text{ cm}^2/\text{s}$ [1] and $1.1 \times 10^{-6} \text{ cm}^2/\text{s}$ [70,71]. There was some enhancement of the oxygen reduction current in naturally-aerated electrolyte solution after potential cycling in the presence of the redox protein. This appears to be due to a small amount of adsorbed residual protein. Evidence for the adsorbed protein was the increased N 1s signal in the XPS data. The reason there was no detectable signals for Fe or S is unclear but could be related to a high number of N atoms relative to heme Fe and S in the protein and the fact that the regions of the protein containing Fe and S are buried within the tertiary structure such that the probability of a photoelectron escaping from these two atoms is low. Most interesting though is the fact the residual adsorbed protein does not adversely affect the redox kinetics for dissolved cytochrome c. It is therefore likely that the residual adsorbed protein is at sites distinct from those where the diffusion controlled electrolysis of the protein occurs.

The peak current, i_{pc} , was observed to increase linearly with the protein concentration in solution from 0.02 to 0.2 mM and with the scan rate^{1/2} between 2 and 100 mVs at both oxygen-terminated diamond electrodes. These trends are expected for a semi-infinite linear diffusion-controlled redox reaction. On both oxygen-terminated B-UNCD and B-MCD electrodes, the cyclic voltammetric curve shapes were stable with potential cycling (10-20 cycles) consistent with

the absence of protein denaturation and electrode fouling. This fouling resistance is generally only observed with chromatographically-purified protein free of oligomeric contaminants [18,19]. Reproducible curve shapes were observed for different oxygen-terminated B-UNCD and B-MCD electrodes. While the reduction peak current, i_{pc} , and the heterogeneous electron-transfer rate constant, k_{app}^0 , exhibit maxima in the 6.5-7.5 pH range for both electrodes, there is a relatively minor pH effect over the range investigated (Fig. 8). The same holds true for the midpoint potential, $E_{1/2}$, and peak potential separation, ΔE_p , as both values are generally independent of the solution pH (Table 2). These are the first such ΔE_p -pH data (6-9) reported for this redox protein at diamond electrodes. In recent work with screen-printed graphite electrodes, researchers have shown that direct electron transfer in aqueous solutions is possible due to the electrostatic attraction between the positively-charged lysine groups on the heme protein and the negatively-charged carboxylate functional groups on the electrode surface [72,73]. The authors attributed the observed pH dependence of the electron-transfer kinetics to changes in the protein orientation and distance of closest approach at the electrode surface. Using three different horse heart cytochrome c mutants, researchers found good correlation between the measured rate constant and the separation distance for protein immobilized on Au [74]. Clearly, at these pH values, the protein has significant positive charge [57,75]. Research has also shown that the secondary protein structure, examined by circular dichroism, is not significantly changed over this pH range [75]. Therefore, we conclude that the absence of a significant pH effect for electron transfer at the

oxygen-terminated B-UNCD and B-MCD electrodes is due to the fact that the oxygenated surfaces contain few anionic carboxylate groups.

To confirm this, one would need to identify the types of carbon-oxygen functional groups formed on the B-UNCD and B-MCD surfaces after the acid washing and hydrogen peroxide treatments. In principle, this could be determined from deconvolution of the XPS C1s signal, however, this was not undertaken in the present work. Other researchers, however, have reported XPS results for oxygenated diamond films. For example, Fujishima and coworkers reported that carbonyl (C=O) and ether (C-O-C) groups form on diamond (100) surfaces [43] and hydroxyl (C-OH) groups form on diamond (111) surfaces after oxidation [44]. It is, therefore, likely that our oxygen-terminated B-UNCD and B-MCD electrodes contained C-OH and C-O-C functional groups with lower levels of COOH groups due to the absence of a significant effect of pH on the cyclic voltammetrically determined reduction peak current, k_{app}^0 , $E_{1/2}$ and ΔE_p . Xue et al. [76] reported how the UV/ozone treatment of carbon nanofiber electrodes improved the direct electron transfer of cytochrome c as compared to the untreated surface. The pretreatment increased the hydrophilicity of the surface, as measured by water contact angle, and the surface carbon-oxygen functional group content. Increases in the C-OH, C=O and COOH functionalities were observed by XPS based on deconvolution of the C1s peak. The authors suggested that the changes in surface chemistry serve to orient the redox protein in such a way as to shorten the distance for electron transfer. The same is likely

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4 the case for the oxidized diamond electrodes used in this work. The surface
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6 carbon-oxygen functional groups likely interact with the exposed heme edge of
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8 the redox protein to orient the protein close to the surface through a combination
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10 of dipole-dipole, ion-dipole and hydrogen bonding interactions with uncharged
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12 surface carbon-oxygen functional groups. The orientation of cytochrome c on the
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14 surface and the distance of the heme edge are probably the most important
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16 factors controlling the heterogeneous electron transfer rate. The hydrogen-
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18 terminated diamond electrodes, on the other hand, do not interact strongly with
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20 cytochrome c as the polarity of the surface is much lower. The weak interaction
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22 likely positions the Fe-heme in an orientation farther away from the electrode
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24 surface, thus decreasing the probability for electron transfer. The sluggish ET
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26 rate on the hydrogen-terminated surface is not due to any electrode electronic
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28 effect a fast ET kinetics are typically observed for many redox systems on this
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30 surface.
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41 Finally, it should be noted that solid-state electron transport studies using
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43 mutant forms of cytochrome c integrated into a molecular electronic device
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45 (contacted to two electrodes) have produced useful insights on the importance of
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47 the protein's electronic coupling to the electrode surface and orientation on the
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49 current that flows through the device [75,77,78]. Fundamentally, the current flow
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51 through the solid-state device is an electron flux across the protein without the
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53 need for a redox process, while the current measured in the electrochemical
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55 process is due to electronshuttling to and from the Fe(III) and Fe(II) oxidation
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states of the heme. Removing the Fe redox center from the porphyrin completely eliminates the redox peaks but has little effect on the current flow in the solid-state measurement [77]. In contrast removing the porphyrin ring irreversibly alters the protein conformation and reduces current flow in the solid-state measurement. Importantly, the solid-state measurements revealed a general trend of greater current flow when the heme is closest to the electrode (covalently or electrostatically bound) [77,78]. The results, which have relevance for solution-based electron transfer with the redox protein, suggest that the Fe heme cofactor-electrode proximity and likely the cofactor-electrode coupling are important for the most efficient current flow. Evidence for this was the fact that the mutant with its cofactor closest to the electrode had the lowest temperature dependent current flow.

5. Conclusions

The electroactivity of dissolved horse heart cytochrome c was investigated at hydrogen and oxygen-terminated microcrystalline and ultrananocrystalline boron-doped diamond thin films. The role of the surface chemistry and film morphology were investigated. The following are key findings from the work.

1. The film morphology had little effect on the heterogeneous electron-transfer rate constant for cytochrome c. In contrast, the surface chemistry was much more influential. The highest redox activity was found on the oxygen-terminated electrodes. In contrast, little activity was observed on the hydrogen-terminated

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4 diamond electrodes. k_{app}^o values were similar for oxygen-terminated
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6 microcrystalline and ultrananocrystalline diamond thin films.
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10 2. Heterogeneous electron-transfer rate constants in the $2-4 \times 10^{-3} \text{ cm s}^{-1}$ range
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12 were observed on both diamond film types when oxygen terminated.
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15 3. Diffusion-controlled voltammetric behavior was observed on both diamond film
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17 types when oxygen terminated. The diffusion coefficient was calculated to be ~ 2
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19 $\times 10^{-6} \text{ cm}^2/\text{s}$. Stable cyclic voltammetric curves were observed with multiple
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21 cycles on both electrodes when oxygen terminated indicating the absence of any
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23 electrode fouling by denatured protein.
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28 4. The cyclic voltammetric peak current increased with the solution concentration
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30 of cytochrome *c* at concentrations from 0.02 to 0.2 mM. There was a minor effect
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32 of solution pH on the cyclic voltammetrically-determined reduction peak current,
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34 $E_{1/2}$, ΔE_p and k_{app}^o at either oxygen-terminated diamond electrode.
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39 5. The polar carbon-oxygen functional groups likely orient the redox protein with
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41 the Fe heme edge close to the electrode surface through dipole-dipole, ion-dipole
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43 and hydrogen bonding interactions. This leads to relatively rapid rates of electron
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45 transfer. In contrast, the low polarity, hydrogenated surface may interact with the
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47 protein in such a way as to position the the Fe heme farther from the electrode.
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49 The greater distance between the redox center and the electrode reduces the
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51 electron tunneling probability and results in a significantly reduced rate of
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53 electron transfer.
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Figure 1. X-ray photoelectron survey spectra of B-UNCD electrodes (A) as-deposited, (B) after oxygenation (oxygen terminated) and (C) after rehydrogen (hydrogen terminated).

Figure 2. X-ray photoelectron survey spectra of oxygen-terminated (A) B-MCD and (B) B-UNCD electrodes after immersion and potential cycling in a solution of 0.1 mmol dm^{-3} cytochrome *c* in 0.1 mol dm^{-3} NaCl + 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). The electrodes were rinsed with ultrapure water after the cycling.

Figure 3. Cyclic voltammetric *i-E* curves for a hydrogen-terminated B-UNCD film showing (A) the initial background response in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7), (B) a repeat background scan after exposure to a cytochrome *c* solution and (C) the response in 0.1 mmol dm^{-3} cytochrome *c* plus 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). Scan rate = 20 mV s^{-1} . Electrode area = 0.2 cm^2 . The scan was initiated at 0.3 V and the potential swept initially in the negative direction.

Figure 4. Cyclic voltammetric *i-E* curves for 0.1 mmol dm^{-3} cytochrome *c* plus 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7) at oxygen-terminated (A) B-UNCD and (B) B-MCD electrodes. Curves are shown for different scan numbers (1-10). The scans were initiated at 0.3 V and the potential swept initially in the negative direction.

Figure 5. Cyclic voltammetric background *i-E* curves for an oxygen-terminated B-UNCD electrode in 0.1 mol dm^{-3} NaCl plus 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). Scan rate = 20 mV s^{-1} . Electrode area = 0.2 cm^2 . The curves were recorded in buffer before (black, solid line) and after (red, dashed dot line and blue, dashed line) cycling in a cytochrome *c* solution. The black and blue curves were recorded in solutions deaerated with N_2 while the red curve was recorded in naturally aerated electrolyte solution. The scan was initiated at 0.3 V and the potential swept initially in the negative direction.

Figure 6. Cyclic voltammetric *i-E* curves for 0.1 mmol dm^{-3} cytochrome *c* in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl (pH 7) on an oxygen-terminated B-UNCD electrode at different scan rates from $2 - 100 \text{ mV s}^{-1}$. Electrode area = 0.2 cm^2 . The scan was initiated at 0.3 V and the potential swept initially in the negative direction. The fifth cycle is shown for each scan rate.

Figure 7. (A) Plot of the reduction peak current, i_{pc} , for 0.1 mmol dm^{-3} cytochrome *c* in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7) as a function of the (scan rate)^{1/2}. (B) Plot of i_{pc} versus the cytochrome *c* concentration in 0.1 mol dm^{-3} NaCl and 0.01 mol dm^{-3} Tris-HCl buffer (pH 7). Both sets of data

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4 were created from cyclic voltammetric i-E curves recorded with an oxygen-
5 terminated B-UNCD electrode. The i_{pc} -concentration data are for a 20 mV s^{-1}
6 scan rate.
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9 **Figure 8.** Plots of (A) the reduction peak current, i_{pc} , and (B) the apparent
10 heterogeneous electron-transfer rate constant, k_{app}^0 , for cytochrome *c* as a
11 function of the solution pH for oxygen-terminated B-UNCD (black) and B-MCD
12 (red) electrodes. Cyclic voltammograms were recorded for 0.1 mmol dm^{-3}
13 cytochrome *c* in 0.1 mol dm^{-3} phosphate buffer of different pH. The i_{pc} -solution pH
14 data are for 20 mV s^{-1} . Average and standard deviation values are shown for $n \geq 3$.
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TABLES

Table 1. Apparent heterogeneous electron-transfer rate constant, k_{app}^o , calculated from cyclic voltammetric data (ΔE_p vs. scan rate trends).

Scan rate V/s	k_{app}^o ($\times 10^{-3}$ cm/s)*	
	B-UNCD	B-MCD
0.005	3.55 ± 1.18	1.98 ± 0.31
0.01	2.90 ± 0.58	2.26 ± 0.34
0.02	3.28 ± 0.72	2.55 ± 0.35
0.05	3.29 ± 0.94	2.33 ± 0.34
0.1	3.46 ± 1.23	2.80 ± 0.30
Avg.	3.48 ± 1.25	2.38 ± 0.72

Calculations were performed with $T=298$ K, $D_o=D_R=1.1 \times 10^{-6}$ cm²/s and $\alpha=0.5$ [54]. Oxygen-terminated B-MCD and B-UNCD thin-film electrodes were used for the measurements.

Table 2. pH dependence of the cyclic voltammetric midpoint potential, $E_{1/2}$, and peak potential separation, ΔE_p , for cytochrome *c* on an oxygen-terminated B-UNCD and B-MCD electrode.

pH	$E_{1/2}$ (mV) vs. Ag/AgCl	ΔE_p (mV) B-UNCD	ΔE_p (mV) B-MCD
6	40	82	98
7	33	72	79
7.5	32	73	77
8	32	71	77
9	30	77	82

The data were determined from cyclic voltammetric recordings of 0.1 mmol dm⁻³ cytochrome *c* in 0.1 mol dm⁻³ phosphate buffer of different pH. Scan rate=20 mV/s. Electrode area=0.2 cm².