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Organophosphonate Biofunctionalization of Diamond Electrodes

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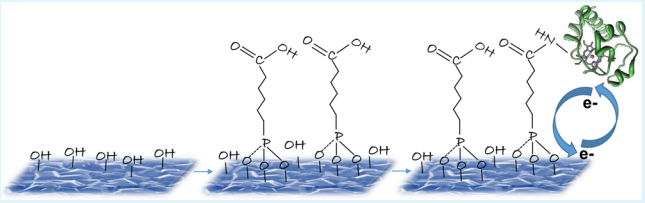
Supporting Information

ABSTRACT: The modification of the diamond surface with organic molecules is a crucial aspect to be considered for any bioapplication of this material. There is great interest in broadening the range of linker molecules that can be covalently bound to the diamond surface. In the case of protein immobilization, the hydropathicity of the surface has a major influence on the protein conformation and, thus, on the functionality of proteins immobilized at surfaces. For electrochemical applications, particular attention has to be devoted to avoid that the charge transfer between the electrode and the redox center embedded in the protein is hindered by a thick insulating linker layer. This paper reports on the grafting of 6-phosphonohexanoic acid on OH-terminated diamond surfaces, serving as linkers to tether electroactive proteins onto diamond surfaces. X-ray photoelectron spectroscopy (XPS) confirms the formation of a stable layer on the surface. The charge transfer between electroactive molecules and the substrate is studied by electrochemical characterization of the redox activity of aminomethylferrocene and cytochrome *c* covalently bound to the substrate through this linker. Our work demonstrates that OH-terminated diamond functionalized with 6-phosphonohexanoic acid is a suitable platform to interface redox-proteins, which are fundamental building blocks for many bioelectronics applications.



KEYWORDS: *surface functionalization, diamonds, organophosphonates, electrochemistry*

INTRODUCTION

Diamond as a semiconductor material is known for its wide range of extreme properties. It offers high thermal conductivity and optical transparency in a wide spectral range and is wear resistant and chemically inert. Its biocompatibility makes it an ideal material for biomedical and bioelectronic applications.[1−3] In particular, p-type boron-doped polycrystalline and nanocrystalline diamond electrodes combine high conductivity, a low background current, and a wide electrochemical potential window, together with the possibility of being optically transparent as well as producible at low cost.[4] As a result, in the past decade diamond has attracted attention for the development of applications requiring electrodes with improved electrochemical performance.[5] In contrast to metal electrodes, widely used for the immobilization of redox molecules and proteins,[6,7] diamond electrodes have shown higher stability and durability.[8] Moreover, the wider potential window and low background current offered by diamond electrodes with respect to gold or platinum enable the study of a wider range of chemical processes taking place in electrolytes.[9] The lower background current allows higher sensitivity and lower detection limits, which can be very relevant in bioelectrochemical applications.

However, a fundamental requirement for using diamond electrodes in bioelectrochemical applications is the ability to immobilize tailored molecular and biomolecular layers on the surface of diamond.[4] Despite many reports in the literature about noncovalent immobilization of biomolecules on different electrodes,[10,11] bare electrodes are usually not ideal interfaces to obtain direct electron transfer with most proteins: a better performance in term of charge transfer efficiency and stability is often obtained by modifying the electrode surface with self-assembled monolayers (SAMs).[12] In this respect, interfaces based on covalent immobilization can offer higher stability and permit conformational control of surface-bound receptors with higher target molecule binding efficiencies. In particular,

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tailored chemical modification of diamond electrodes may substantially improve the electron transfer rate between proteins and electrode surfaces, which is usually slow due to the buried electroactive groups of most proteins in the electrically insulated peptide backbones. A variety of methods for a controlled surface functionalization of diamond have been reported in the literature.[13−19] Most of them are based on the introduction of hydrogen, oxygen, halogen, and amino functionalities on the surface, which can be further extended by covalent grafting of organic and bioorganic molecules.[20] However, less work has been carried out on the specific covalent immobilization of proteins on diamond.[21] Initial attempts carried out on H-terminated diamond surfaces have shown that even small proteins like cytochrome *c* are very sensitive to surface hydropathicity and cannot retain their functional conformation on hydrophobic diamond surfaces.[22] Oxidized diamond surfaces, containing different oxygen surface groups, have been shown to be more suitable for applications involving biomolecules. Thanks to the hydrophilic character of the carboxyl and hydroxyl groups present on the surface, they can serve as a platform for the immobilization of enzymes without loss of functionality.[22] Following this approach, Hernando et al.[23] have successfully measured the catalytic properties of horseradish peroxidase covalently immobilized on oxidized nanocrystalline diamond. For the covalent immobilization on oxidized diamond, oxophilic reagents such as trialkoxysilanes are usually used to react with the OH-terminal groups present on the surface.[24] However, it is known that siloxane layers are quite unstable in saline solutions, especially for short-chain aminosilanes. The diamond−O−Si bond, in particular, is quite sensitive to hydrolysis, especially at lower pH values.[20] Moreover, multiple grafting and homocondensation in siloxane-mediated functionalization may lead to multilayer formation and low reproducibility. For electrochemical applications, where an effective charge transfer is typically desired by using defined short and hydrolytically stable interfacial layers, siloxane chemistry can be disadvantageous. Therefore, we have focused our work on the use of organophosphonate chemistry as a promising alternative route for the functionalization of OH-terminated diamond surfaces. Organophoshonate functional interfaces are hydrolytically more stable and do not exhibit homocondensation.[25] They have attracted interest due to the difference in surface reaction mechanisms between phosphorus and silane compounds.[26] The formation of organophosphonate SAMs occurs indeed in a way that is fundamentally different from silanization, which simply consumes surface OH groups.[25] Organophosphonates can instead utilize the OH groups already present on the surface as catalysts for surface bonding, enabling high surface coverage.[27] Hanson and co-workers have already reported that self-assembly of alkanephosphonates on oxides can occur by a simple deposition procedure (tethering by aggregation and growth method, T-BAG) followed by solvent evaporation;[28] subsequent heating produces strongly surface bound, ordered films of the alkanephosphonate species. Following this protocol a convenient route for the modification of different silicon devices has already been established.[29,30] However, comparatively, less attention has been paid to the use of organophosphorous molecules for the modification of semiconductor surfaces where the semiconducting substrate is in direct contact with the self-assembled organic layers, without the presence of an intermediate oxide. This is indeed possible and we have recently shown that organophosphonate monolayers can be successfully prepared directly on 6H-SiC surfaces.[31] A prerequisite for the successful formation of covalent bonds with organophosphonates is the generation of reactive OH groups on the surface by appropriate surface activation protocols. For diamond, a variety of methods are available to achieve O-termination, including thermal, electrochemical, UV ozone, and plasma oxidation.[20] A combination of oxygen moieties, including hydroxyl, ketone, and ether groups, is usually formed on the surface. On the basis of this, organophosphorus functional molecules should react straightforwardly on activated diamond surfaces. In reality, the atomic structure of the crystal facets, the extent of the surface oxygen coverage, and the roughness of the diamond surface may strongly influence the binding process. In this sense, a detailed study is needed in order to find out the optimal conditions for the grafting of organophosphonates on OH-terminated diamond. In this work a suitable functionalization protocol based on organophosphonate chemistry for the immobilization of electro-active proteins on OH-terminated diamond electrodes has been successfully developed. The short 6-phosphonohexanoic acid has been chosen as a model linker because of its peculiar characteristics: the presence of a short backbone structure and the high reactivity of the carboxylic group at the distal end in peptide coupling reactions. This novel functionalization process has been assessed using atomic force microscopy and spectroscopic techniques like X-ray photoelectron spectroscopy (XPS), confirming the formation of a stable layer on the surface. The charge transfer between electroactive molecules and the substrate has been studied by electrochemical characterization of the redox activity of aminomethylferrocene and cytochrome *c* covalently bound to this linker. The relatively low coverage presented in this work is not a disadvantage toward application, where the quality of the charge transfer and the surface properties are playing the crucial roles. Indeed, the priority for many applications is to avoid the formation of multilayers, which would inhibit the charge transfer.

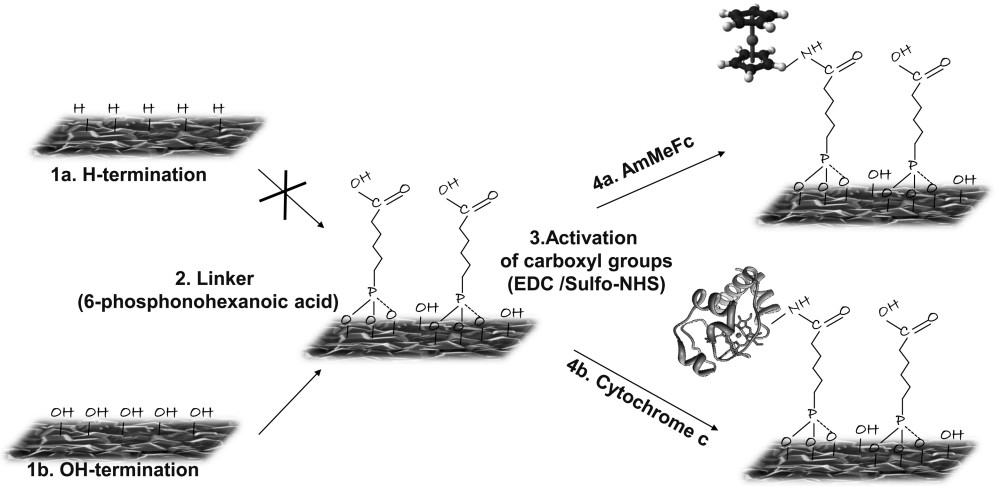
EXPERIMENTAL PROCEDURES

Diamond Substrates. Polished polycrystalline boron-doped diamond substrates (B-PCD) were used for the optimization of the functionalization process in order to take advantage of their low roughness. Further, XPS and FTIR experiments were also conducted with these substrates. B-PCD substrates with an estimated boron concentration of 10^{20} atoms per cm^3 and a surface roughness of 0.1 nm were purchased from Diamond Detectors Ltd.

Highly boron-doped nanocrystalline diamond (NCD) substrates were employed for the electrochemical experiments. NCD substrates are more suitable for these applications because they exhibit metallic behavior through high incorporation of boron, they are low cost, and they can be easily grown in thin films on many different substrates, including transparent substrates for photoelectrochemical applications.

Growth of Nanocrystalline Diamond Films. Highly boron-doped nanocrystalline diamond films were grown on Si as reported before. In short, a 2 in. highly doped p-type silicon wafer was cleaned in ammonium hydroxide and hydrogen peroxide and then in hydrochloric acid and hydrogen peroxide, followed by rinsing with deionized water. Subsequently, the silicon wafers were immersed in an aqueous nanodiamond colloid,[32] rinsed with deionized water, and blown dry with nitrogen. Diamond growth was performed in an ASTeX 6500 series microwave plasma-enhanced chemical vapor deposition reactor. Growth conditions were as follows: 1% methane in hydrogen, 5000 ppm trimethylboron, 5300 Pa process pressure, 3500 W microwave power, and 850 °C substrate temperature. The thickness of the grown layer was monitored during growth by laser

Scheme 1. Description of the Functionalization Process: (1) Surface Preparation in H-Plasma (a) or O-Plasma (b), (2) Exposure to a Solution of the Linker Molecule, (3) Activation of Carboxyl Groups, and (4) Coupling of Aminomethylferrocene (a) and Cytochrome *c* (b) via a Peptide Bond



interferometry and growth was stopped after 60 min when a thickness of 150 nm was obtained. The process was followed by cooling down in pure hydrogen plasma. After growth, the wafer was cut in 7 × 7 mm^2 pieces. The used B/C ratio was found to produce a diamond layer with resistivity values of 10−50 mΩ·cm.[33]

Surface Cleaning and Oxidation. Before functionalization, the samples were cleaned in a 3:1 mixture of hydrochloric acid (HCl) and nitric acid (HNO_3) at 80 °C for 60 min, followed by 30 min in a 3:1 mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). After each step, the samples were thoroughly rinsed with deionized water and blown-dry with nitrogen.

The final step of the sample preparation was exposure to an oxygen plasma, performed in a 100-E Technics Plasma system at 200 W microwave power and 1.4 mbar oxygen pressure for 5 min. This procedure leads to a clean and oxidized surface.

Functionalization with 6-Phosphonohexanoic Acid. The method of choice for the grafting of linker molecules was T-BAG. A 25 μM solution of the linker molecule was prepared in dry tetrahydrofuran (THF). The OH-terminated samples are positioned hanging inside the solution, far enough from the liquid/air interface so that the evaporation of the desired amount of solvent takes place in a fume hood at room temperature in about 2−3 h.[28,31]

Once the solvent has evaporated so far that the diamond substrates are completely hanging in air, the samples are annealed at 120 °C for 18−20 h.[31] The essential chemisorption of the anchor group of the linker to the diamond takes place during this heating step, while the molecules are only physisorbed after the evaporation step.

After annealing, some cleaning steps are applied to remove eventual multilayers physisorbed on the surface. These steps consist of two 10-min sonications in ethanol at 50% power of the ultrasonic bath, 10 min of the same cleaning in a solution of deionized water and THF (10 mL:3 mL), and finally 10 min in deionized water. Samples are then blown-dry with nitrogen and put in an oven for 5 min at 120 °C in order to remove as much waster as possible from the surface.

An additional T-BAG process, following exactly the same procedure described above, was done after these cleaning steps to improve the density of the monolayer and to obtain a higher coverage of the substrate.

The cleaning after this second process is almost identical to the previous one; the second step, however, is performed in deionized water, THF, and triethylamine in a ratio of 10:3:1.

Activation of Carboxyl Group with EDC-Sulfo NHS. After the diamond samples are modified with the SAMs of 6-phosphonohexanoic acid, the carboxyl group of the linker molecule has to be activated to enable covalent binding to the amino group of the redox molecule aminomethylferrocene (AmMeFc) or the protein cytochrome *c*. To this end, the samples are exposed to a 1:1 solution of 400 mM EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] and 100 mM Sulfo-NHS (hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid sodium salt) both in 100 mM MES [2-(*N*-morpholino)ethanesulfonic acid] buffer at acidic pH. The combination of these two water-soluble carbodiimides leads to a high yield of stable amide bondings.[34]

The chemicals used to activate the linker molecules are dissolved in buffer directly before the activation to avoid losses of the activity.

Grafting of Redox Molecules. The attachment of AmMeFc on diamond functionalized with linker molecules was done immediately after the EDC/NHS activation. Here, the samples were covered with a solution of 0.5 mg mL^{-1} of AmMeFc in acetonitrile for 15 h at 4 °C; alternatively, the process can be done for 4 h at room temperature. In order to passivate the carboxyl groups that are still reactive after the grafting process, the samples were put in a solution of 1 M ethanolamine in ethanol for 20 min. Nevertheless, no difference in the electrochemical behavior of the samples was observed when skipping this step. After functionalization and eventual passivation, the samples were cleaned in an ultrasonic bath in three different solvents to remove possible layers of physisorbed molecules, acetonitrile, ethyl acetate, and finally ethanol, after which the surfaces have been blown-dry with nitrogen.

Cytochrome *c* from equine heart (pure 99%) as purchased from Sigma-Aldrich was diluted in 10 mM monopotassium dihydrogen phosphate buffer (KH_2PO_4) pH 8 in a concentration of 0.5 mg mL^{-1}. The samples are exposed to this solution for 1 h at room temperature. In order to preserve the protein on the surface, no strong cleaning was applied but just rinsing with buffer.

X-ray Photoelectron Spectroscopy. The samples were measured in an XPS system at a base pressure of 5 × 10^{-9} mbar with non-monochromatic Al Kα radiation at an operating power of 20 mA at 12.5 kV.

Spectra were acquired using a SPECS Phoibos 100 hemispherical analyzer with an MCD-5 detector at a pass-energy of 25 eV with a 0.025 eV step size. The intensity of the signals has been normalized over the total number of counts for the specific measurement taking into account the differences in the sensitivity factor for each measured chemical element.

ATR-FTIR. Attenuated total reflection (ATR) spectra were recorded using a Bruker Vertex 70v FTIR spectrometer equipped with an MCT detector. All spectra were obtained with unpolarized light over the 900−4000 cm^{-1} spectral range by averaging 2000 scans with a resolution of 3 cm^{-1}. A VariGATR ATR unit from Harrick equipped with a single reflection Ge crystal plate was used to enhance surface

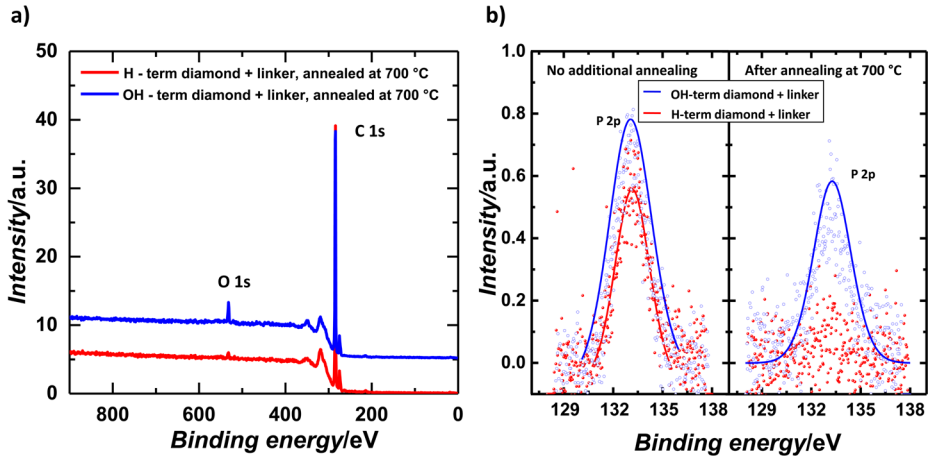


Figure 1. XPS characterization of functionalized diamond electrodes. (a) Survey scan of diamond surfaces after the functionalization process with 6-phosphonohexanoic acid and annealing at 700 °C starting from H-terminated surface (red curve) and OH-terminated surface (blue curve). The spectra show no contamination for both samples and a drastic reduction of the oxygen peak for the sample where the functionalization was performed on the H-terminated surface, indicating the absence of bound oxygen on this surface. (b) High-resolution spectra of phosphorus 2p recorded directly after functionalization (left panel) and after an additional annealing at 700 °C (right panel) for an OH-terminated surface (blue) and H-terminated surface (red). The curves are fittings to the experimental data (symbols).

sensitivity. The diamond samples were pressed against the ATR crystal to improve physical contact.

Electrochemical Experiments. The electrochemical characterization was performed with a Gamry ref 600 potentiostat in a three-electrode electrochemical cell consisting of the sample mounted as working electrode, a commercial Ag/AgCl reference electrode (DriRef-5SH from World Precision Instruments, 3 M KCl, 210 V against standard hydrogen electrode), and a Pt wire as counter electrode. The cell was filled with 25 mL of 100 mM ionic strength KH_2PO_4. The buffer was degassed before measuring and the atmosphere during measurements was saturated with nitrogen.

RESULTS AND DISCUSSION

Sample Preparation. The procedures to prepare the different samples studied in this work are presented in Scheme 1. Organophosphonates need the presence of hydroxyl groups to efficiently bind to the surface. XPS analysis presented below clearly shows that, as expected, H-termination (Scheme 1a) does not lead to a successful functionalization. In order to achieve OH-termination of the diamond surfaces for subsequent reaction with the 6-phosphonohexanoic acid, three different surface activation methods previously reported in the literature[24] were tested: UV ozone activation, O-plasma, and Fenton oxidation. Under our experimental conditions the ozone treatment turned out to be rather difficult to reproduce and gave very low yields of functionalization. Fenton oxidation[35] and O-plasma, however, were both effective in increasing the population of OH groups on the surface with an acceptable yield in subsequent functionalization with 6-phosphonohexanoic acid (see XPS characterization in the Supporting Information). In particular, O-plasma treatment was found to be more efficient in oxidizing samples of nanocrystalline diamond; for this reason, it was chosen as the standard oxidation method thorough out this work. After surface activation, a monolayer of organophosphonates can be assembled on the diamond surface via T-BAG.[28] In this work, 6-phosphonohexanoic acid has been used as a spacer between the diamond substrate and the redox molecule of interest, aiming at enhancing charge transfer between the functional molecules and the substrate. The distal carboxyl groups at the end of the organophosphonate linkers can be further functionalized by activation with EDC/sulfo-NHS (Scheme 1). This procedure allows one to covalently immobilize AmMeFc and cytochrome *c* on diamond, as confirmed by XPS, FTIR, and electrochemical characterization.

Characterization by XPS Analysis. After surface modification with 6-phosphonohexanoic acid, X-ray photoelectron spectroscopy was used to confirm the presence of organophosphonates on the surfaces (see Figure 1 and Supporting Information). To prove that the 6-phosphonohexanoic acids are not simply physisorbed on the surface and to investigate the nature of the interaction between the organic phosphonic groups and the OH groups present on the diamond surface, the functionalization protocol was applied to both a diamond substrate intentionally terminated with OH groups through exposure to an oxygen plasma (blue curve in Figure 1) and a substrate of the same type exposed to a hydrogen plasma, i.e., essentially free from OH groups at the surface[36] (red curve in Figure 1). Moreover, in order to discriminate between organophosphonates simply adsorbed on the surface and those effectively bound to the diamond, XPS spectra were further recorded immediately after ex-situ annealing in UHV at several temperatures for 30 min. The XPS surveys for both control and OH-terminated diamond samples after functionalization and annealing for 30 min at 700 °C are shown in Figure 1a. Beside the strong carbon signals and the weak phosphorus P 2p peak, the only additional visible contribution comes from oxygen. As expected, a small O 1s signal can be seen in the sample activated with O-plasma. The less evident oxygen signal observed in the sample activated with H-plasma is probably due to the presence of water molecules on the surface, since it was not possible to anneal the samples in situ in the XPS chamber. Figure 1b shows a high-resolution XPS scan of the P 2p peak: on the left side are the spectra of both control and OH-terminated diamond samples immediately after surface modification and on the right side those after subsequent ex-situ annealing at 700 °C. The measurements evidence a reduction of the phosphorus signal on both surfaces after annealing at 700 °C, indicating that a certain amount of organophosphonates is physisorbed on the sample and can be detached by thermal treatment. Interestingly, no P 2p signal can

be detected after annealing for the H-terminated surfaces treated with the 6-phosphonohexanoic acid, while a significant signal is still present for the OH-terminated diamond surface. The values of normalized peak area for the P 2p XPS signal after the annealing at different temperatures are reported in Table 1, and the trend is in accordance with the discussion of

Table 1. Peak Areas for P 2p XPS Signal after Annealing Ex Situ at Different Temperatures Normalized over the Value Measured for Each Sample at Room Temperature[a]

	P 2p normalized XPS signal (%)	
annealing temp (°C)	for OH-terminated diamond	for H-terminated diamond
25	100	100
200	80	100
300	70	90
400	90	40
700	70	20

[a]The values have to be considered affected by an error of at least 10%. Within the range of tolerance, the amount of phosphorous on the OH-terminated diamond can be considered fairly constant after an initial decrease due to the desorption of a small fraction of physisorbed molecules while on H-terminated substrates it constantly decreases to the complete desorption after annealing at 700 °C.

the spectra reported in Figure 1. The presence of phosphate species strongly bound to the surface at temperatures higher than 500 °C is compatible with the hypothesis of a covalent interaction of the organophosphonates on OH-terminated diamond with binding energies higher than 200 kJ mol^{-1}. This result is in good agreement with a previous thermogravimetric study of the thermal stability of organophosphonate monolayers on silica, alumina, titania, zirconia, and calcium hydroxyapatite particles, which have shown activation energy for the desorption process of ~265 ± 15 kJ mol^{-1}.[37]

Electrochemical Characterization and Charge Transfer. Cyclic voltammetry (CV) is a powerful technique that can provide valuable information on the charge transfer between the electrode and redox molecules. The current flowing between the working electrode and the counter electrode (Pt) is measured in a standard three-electrode setup while the voltage of the working electrode versus the reference electrode (Ag/AgCl) is swept in a cycle at a constant scan rate. A diamond electrode functionalized with the linker molecule and the redox molecule AmMeFc covalently grafted on it (as described above) was measured and compared to a sample prepared in the same way but lacking the linker layer. Figure 2a shows the resulting CVs and evidences how the presence of the linker layer is necessary to observe the peaks of direct charge transfer between the redox species at the electrode. For this sample, AmMeFc exhibits a formal potential of 220 ± 5 mV vs Ag/AgCl. The study of the peak current density as a function of the scan rate, summarized in Figure 2b on a double logarithmic scale, reveals a linear dependence (after background subtraction) on the scan rate, which supports the hypothesis of a stable immobilization of the species on the electrode through the linker molecule. A diffusion-limited process of AmMeFc in the electrolyte can be excluded because the peak current density would exhibit a square root dependence with the scan rate in this case.[38] On the basis of the electron transfer model developed by Laviron[39] for species adsorbed on an electrode, the standard rate constant k_0 can be derived using the peak

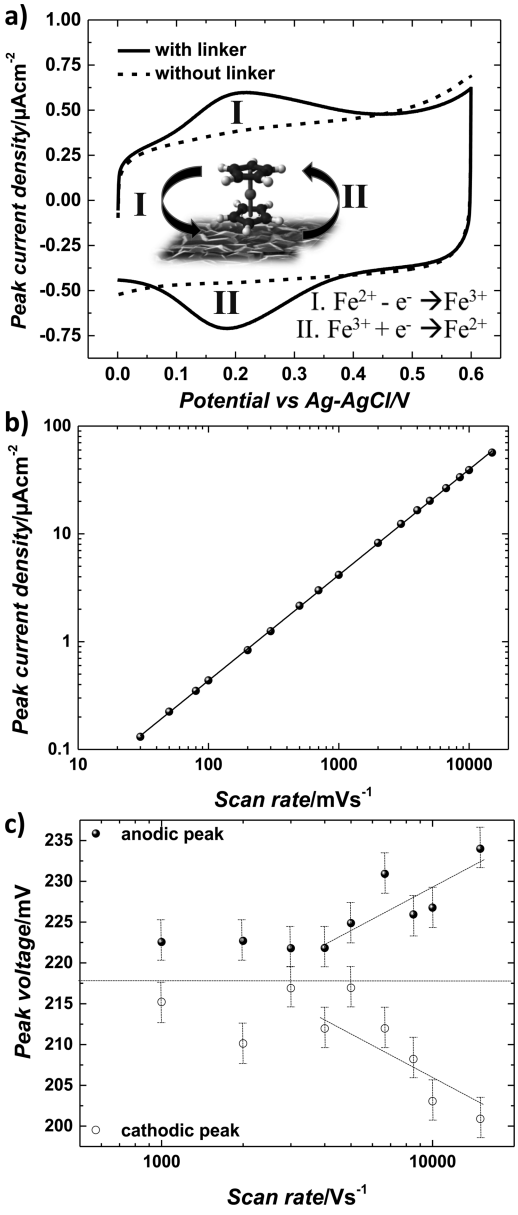


Figure 2. Electrochemical characterization of AmMeFc-modified diamond electrodes. (a) Cyclic voltammetry between 0 and 600 mV performed at a scan rate of 50 mV s^{-1} in KH_2PO_4 buffer of 100 mM ionic strength. Both diamond substrates have been functionalized with AmMeFc. One of the substrates was modified with 6-phosphonohexanoic acid as a linker layer while the other only underwent O-plasma treatment to obtain OH-termination. The direct charge transfer between the redox molecule and the substrate is detected only in the presence of the linker molecule acting as anchor to covalently tether the redox probe. (b) Peak current densities extrapolated from cyclic voltammetry at different scan rates after background subtraction (data not shown) present a linear dependency on the scan rate, indicating that the process is not limited by the diffusion of the redox species in the electrolyte. (c) Voltage of the anodic and the cathodic peaks plotted against the scan rate. From this plot it is possible to observe how the peak separation starts to depend on the scan rate after 4 V s^{-1}. At high scan rates the system is kinetically limited and the linear relation between the peak separation and the scan rate (see dashed line) can be used to calculate the electron transfer rate constant. The error bars on the experimental points are considered as an upper error upon taking into account that, in the range of low scan rate, the system is limited by scan rate and the peak potential is constant.

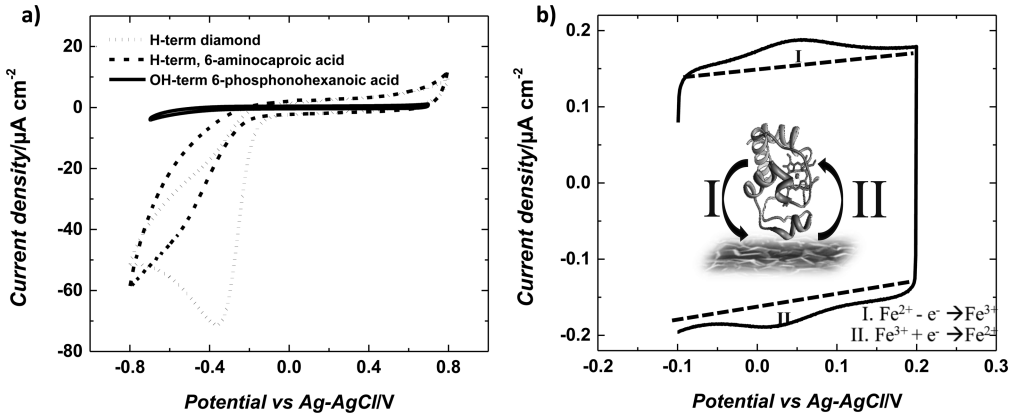


Figure 3. (a) Cyclic voltammetry for cytochrome *c* immobilized on differently treated diamond surfaces. H-terminated surfaces, even after passivation with monolayers, present evidence of cytochrome *c* denaturation. The sample functionalized with 6-phosphonohexanoic acid provides a suitable environment to avoid protein denaturation. (b) Cyclic voltammogram recorded in a smaller range of voltage at 50 mV s^{-1}. It evidences the presence of the redox peaks from cytochrome *c*, proving the direct charge transfer between the protein and the electrode. The dashed lines are guides for the eye to separate the redox contribution from the background current.

separation between the reduction and the oxidation peaks. Laviron's method is widely used for determining the electron transfer rate constan,t even though it is based on many assumptions, like the value of the transfer coefficient here assumed to be $\alpha = 0.5$ for all overpotentials. Figure 2c shows the peak potential as a function of the scan rate: when scanning at a rate faster than 4 V s^{-1}, the peak separation starts to depend on the scan rate, as expected for the kinetically limited electron transfer regime.[38] Following the theory of Laviron for values smaller than 200 mV, the separation between the reduction and the oxidation peak should resemble the dashed line in Figure 2c; the resulting electron transfer is $k_0 = 620 \pm 70$ s^{-1}, derived from the average of the values of peak separation calculated between 4 and 15 V s^{-1} and excluding the measurement at 5 V s^{-1}. The standard deviation is used to calculate the error. This value of the electron transfer rate is comparable with the values reported in the literature for ferrocene immobilized on conductive diamond through alkyl monolayers.[40] The k_0 reported in our work indicates an efficient charge transfer between the redox species and the diamond electrode modified with 6-phosphonohexanoic acid. The total charge transferred between the redox probe and the electrode, calculated through the integration of the current density over time after background subtraction for a scan rate of 1 V s^{-1}, is 0.9 μC cm^{-2}, which corresponds approximately to 0.06 active molecules of ferrocene/nm^2. If one considers hexagonal packing[41] and a radius of 0.33 nm for the AmMeFc, the maximum density of molecules in a monolayer is 2.7/nm^2. This result indicates a coverage of AmMeFc of about 2% of the theoretical fully packed film. Interestingly, it has been demonstrated that low coverage can facilitate electron charge transfer.[40] In this sense, the obtained low yield in surface immobilization is acceptable for our particular application, in which a compromise between an effective electron charge transfer and sufficient loading yield is needed.

Immobilization and Direct Charge Transfer from Cytochrome *c*. In order to assess the suitability of our functionalization protocol for the development of more complex interface systems, immobilization of the model redox protein cytochrome *c* was investigated. Although several works have reported on the electrochemical characterization of cytochrome *c* on boron-doped nanocrystalline diamond electrodes by measuring the electron transfer between the protein in solution and the electrode,[42,43] evidence of the electroactivity of cytochrome *c* immobilized on diamond surfaces is very scarce.[21]

From the perspective of realizing hybrid systems based on proteins immobilized on inorganic electrodes, it is crucial to show that the modified electrode offers a suitable environment for the protein to retain its functionality, even when it is covalently bound. As shown in Figure 3a, this is not the case for cytochrome *c* on H-terminated diamond (dotted line), where a large cathodic current is observed in the cyclic voltammogram. This cathodic current is attributed to the catalytic reduction of oxygen at the electrode surface induced by the denaturation of the cytochrome *c*, i.e., from the exposition of the protein heme center to the electrode surface due to the alteration of the tertiary structure. Denaturation of the proteins on hydrophobic surfaces, like the H-terminated diamond, has been already reported in the literature.[22] Passivation of the surface with 6-aminocaproic acid, a short alkyl chain known to be reactive with H-terminated diamond surfaces,[44,45] results in a reduction of the catalytic effect of the denatured cytochrome *c* (dashed curve). However, the cathodic current corresponding to the catalytic oxygen reduction can be completely prevented when the electrode surface is intrinsically hydrophilic (like for the case of OH-terminated diamond). The cyclic voltammogram of cytochrome *c* immobilized on an OH-terminated diamond electrode passivated with 6-phosphonohexanoic acid (solid curve in Figure 3a) does not show signs of protein denaturation. On the contrary, it is evidence of the absence of any undesired side redox reaction and a very low background current in the potential range of interest. Figure 3b shows a cyclic voltammogram of cytochrome *c* immobilized on OH-terminated diamond in a narrower voltage range. The two redox peaks, symmetrically positioned around the formal potential of cytochrome *c*, show the direct charge transfer between the protein and the electrode. Together with the absence of the catalytic reduction of oxygen at low negative potentials, the redox activity of cytochrome *c* evidences that the protein maintains its functional conformation and it is able to exchange charges with the electrode in a reversible way. Integration of the current density peak in the cyclic voltammogram, after background subtraction and normal-

ization for the scan rate, results in a total amount of transferred charge of 0.1 μC cm^{-2}, which corresponds to about 0.007 molecules of cytochrome c/nm^2. The maximum density of molecules, assuming a hexagonal packing and a diameter of 3.4 nm for cytochrome c, is 0.1 molecules/nm^2. The resulting coverage is around 7% of the maximum theoretical value.

CONCLUSION

6-Phosphonohexanoic acid has been covalently bound onto OH-terminated diamond surfaces and has been demonstrated to be a valuable anchor system for covalent immobilization of redox species and small proteins on diamond electrodes. XPS analysis performed before and after annealing assesses the covalent nature of the bond between the phosphonic groups and the diamond surface. In addition, an insight on the functionalization mechanism is gained from the evidence that the covalent immobilization does not take place on H-terminated diamond surface, suggesting a reaction mechanism similar to the one observed between phosphonic acid and other OH-terminated semiconductors.[28] The redox molecule amino-methylferrocene was covalently grafted to the linker molecule, enabling a complete electrochemical characterization of the modified diamond electrode. This study evidenced a fast charge transfer and a satisfactory coverage of the active species immobilized on the surface, paving a new way to functionalize diamond electrodes. Finally, the protein cytochrome c was successfully immobilized on an organophoshonate-modified diamond surface without evidence of protein denaturation, as shown by cyclic voltammetry. The evidence of direct charge transfer between the protein and the modified diamond surface qualifies this new functionalization method for any application in which a suitable interface between an inorganic electrode and a biological unit is needed.

ASSOCIATED CONTENT

Supporting Information

ATR-FTIR spectra of diamond substrates before and after functionalization, additional XPS spectra, and cyclic voltammogram of the diamond electrode before functionalization with AmMeFc and with the species in solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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