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Direct electrochemistry and electrocatalysis of hemoglobin on undoped nanocrystalline diamond modified glassy carbon electrode

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Abstract

Direct electrochemistry of hemoglobin (Hb) was observed at glassy carbon electrode (GCE) modified with undoped nanocrystalline diamond (UND) and Hb multilayer films via layer-by-layer assembly. UV–VIS absorbance spectroscopy, electrochemical impedance spectroscopy and cyclic voltammograms were employed to characterize the film. The results showed that the UND had the effect of enhancing the electron transfer between Hb and the electrode surface. Hb in the multilayer films maintained its bioactivity and structure. It also exhibited a good catalytic activity towards the reduction of H_2O_2. The reciprocal of catalytic current showed a linear dependence on the reciprocal of H_2O_2 concentration ranging from 0.5 μM to 0.25 mM with a detection limit of 0.4 μM. The apparent Michaelis–Menten constant was estimated to be 0.019 mM.

Keywords: Undoped nanocrystalline diamond; Hemoglobin; Layer-by-layer assembly; Direct electrochemistry; Biosensor

1. Introduction

Diamond-based materials, because of their unique properties such as biocompatibility, chemical inertness, chemical stability, wide potential window and optical transparency properties, have attracted increasing attentions in biosensor and biochip applications [1–3]. Due to the lower electrical conductivity of undoped diamond, most works focus on conducting boron-doped diamond thin-film electrodes [4–10] Recently, nanostructuring materials to create nanodimensional shapes have received intense interests due to the promise of discovering new properties and applications. Hamers and coworkers employed undoped nanocrystalline diamond (UND) thin films for DNA hybridization via photochemical modification of diamond surfaces, and showed that both as semiconductors, nanocrystalline diamond has superior properties over the crystalline silicon for the DNA hybridization [11–13]. Garrido et al. [14,15] reported that hydrogen-terminated nanocrystalline diamond films were modified by using a photochemical process to generate a surface layer of amino groups, to which proteins were covalently attached to nanocrystalline diamond thin films. The immobilized proteins at the surface are still fully functional and active. Loh et al. [16] reported a simple re-growth strategy to construct a thin, UCD layer with high graphitic content for making an active biosensing electrode. These successful examples using UND thin film for biosensing application are profiting from the microwave plasma chemical vapor deposition method by using special instruments for fabricating thin diamond film, which inevitably limited its application in common laboratories.

We previously reported a glucose biosensor based on electrochemical pretreatment of undoped nanocrystalline diamond modified gold electrode for the selective detection of glucose [17]. In that work, UND particles modified layers were used instead of UND thin films via vapor deposition. Here, we further investigate the application of UND particles in the direct electron transfer of proteins. Layer-by-layer (LBL) assembly method was used to construct multilayer films of UND and hemoglobin on glassy carbon electrode (GCE). Studies showed that UND particles not only offer a friendly platform to assemble protein molecules, but also enhance the electron

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transfer process between protein molecules and electrode. The study may help to provide another way to explore the application of undoped nanocrystalline diamond.

2. Materials and methods

2.1. Reagents

Chitosan (92.5% deacetylation) was purchased from Sigma and its solution of 0.2 wt.% could be formed by dissolving chitosan in 0.05 M HCl and then stirring for 3 h at room temperature. The indiscerptible material was filtered before the pH of the solution being adjusted to about 5.0 by 1.0 M NaOH solution. Undoped nanocrystalline diamond (UND) was purchased from ShenZhen JINGANGYUAN New Material Development Co., Ltd. The size of the UND particles is about 3.2 nm and its conductivity is 1.3×10^{-8} S/cm. Human hemoglobin (Hb, MW 66,000) and Hemin (Mr 651.96) were purchased from Sigma and used as received without further purification. Solutions of 2.5 mg/mL Hb (solvent: pH 6.0 phosphate buffer solution) and hemin (solvent: pH 9.0 phosphate buffer solution) were stored at 4 °C. Hydrogen peroxide (H_2O_2, 30% (w/w)) was purchased from Siopharm Chemical Reagent Co., Ltd and its dilute solution was freshly prepared daily. Phosphate buffer solution (PBS, 25 mM) was prepared by mixing the storage solution of K_2HPO_4 and KH_2PO_4. Its pH was adjusted by 1.0 M H_3PO_4 or 1.0 M NaOH. Other reagents were of analytical grade and used as received. All solutions were prepared with twice-distilled water.

2.2. Apparatus

UV–VIS absorbance spectra were performed on a UV–VIS-NIR Spectrophotometer (UV-3600 SHIMADZU). Electrochemical measurements were performed on an AutolabPG-STAT-30 digital potentiostat/galvanstat, (Eco Chemie BV, Utrecht, Netherlands), during the experiment, a conventional three-electrode system was used: the modified GCE was used as a working electrode, a platinum electrode was used as an auxiliary electrode and a saturated calomel electrode (SCE) was used as a reference electrode. Electrochemical impedance spectroscopy (EIS) was performed in 0.10 M KNO_3 solution containing 5.0 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1:1), using an alternating current voltage of 5.0 mV and the impedance measurements were done in frequency range of 10^{-1}–10^{6} Hz at its open circuit potential. All electrochemical experiments were done in an unstirred electrochemical cell at room temperature and oxygen in the solution was removed by importing a high-purity nitrogen stream. The solutions were kept in a nitrogen atmosphere through the measurements.

2.3. Preparation of $\{UND/Hb\}_n$ film modified electrodes

Glassy carbon electrodes with diameter of 3.0 mm were polished carefully with 0.5 and 0.3 μm α-Al_2O_3 powders, washed with distilled water after each step, and then sonicated in water and acetone, respectively. The cleaned GCE was firstly formed a positively charged film in the surface by electrodepositing in 0.2 wt.% chitosan solution at –2.0 V for 5 min. Then the electrode modified with chitosan was dipped into UND aqueous solution and Hb solution (2.5 mg/ mL) for 20 min in turns. After each step, the electrode was washed with distilled water. The modified electrode was stored at 4 °C when it was not in use.

ITO glass slides were cleaned by ethanol, acetone and twice-distilled water in an ultrasonic bath respectively, and then dried in air. Using the above method, the ITO glass slides were prepared for UV–VIS spectroscopy experiments.

3. Results and discussion

3.1. Layer-by-layer assembly of the $\{UND/Hb\}_n$ modified electrode

Our previous work validated that the UND particles have several functional groups of –OH, −C=O, –COOH, –C–O–C and –CN and are negatively charged [17]. Hb with isoelectric point of 6.8 [18] is positive charged in pH=6.0 PBS solutions. Chang et al. [19] reported that carboxylated/oxidized diamond nanoparticles (nominal size 100 nm) exhibit exceptionally high affinity for proteins through both hydrophilic and hydrophobic forces. Hence, the layer-by-layer method can be successfully employed to construct the films of $\{UND/Hb\}_n$ in this experiment via the driving force of hydrophilic and hydrophobic interactions.

3.2. UV–VIS spectroscopic characteristization

UV–VIS absorbance spectroscopy can provide the conformational structure change of heme proteins. The possible denaturation of heme proteins can be speculated from the shape and position of the Soret absorption band [20,21]. Fig. 1 shows the UV–VIS spectra of Hb in PBS solution (curve b) and modified on ITO glass slides (curves c, d and e). The Soret absorption band of Hb in solution could be observed at about 406 nm (curve b). When Hb and UND films were anchored on the ITO glass slides modified with chitosan, an obvious Soret

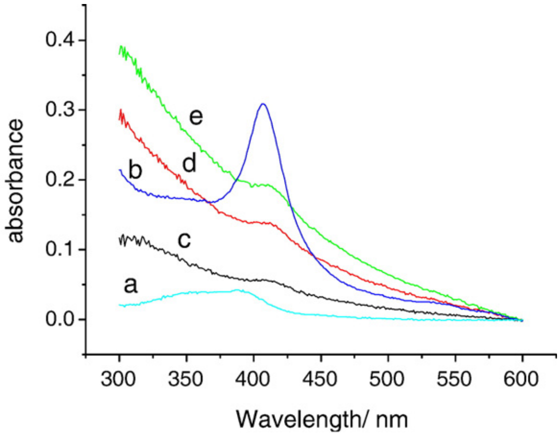

Fig. 1. UV–VIS spectra of modified ITO glass slides with the number of UND and Hb films:c-1, d-2, e-3; a and b are the UV–VIS spectra of the Hemin and Hb in solution, respectively.

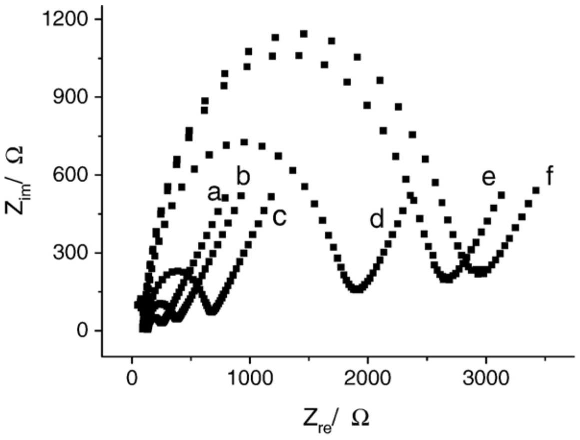

Fig. 2. Electrochemical impedance spectroscopy of bare GCE (curve a), and modified GCE with Chit (curve b), Chit/UND (curve c), Chit/UND/Hb (curve d), Chit/{UND/Hb}$_2$ (curve e), Chit/{UND/Hb}$_3$ (curve f) in 0.10 M KNO_3 solution containing. 5.0 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1:1).

band appeared at about 412 nm (Fig. 1 c, d and e). And the intensity of absorbency enhanced if the number of {UND/Hb}$_n$ film increased, from which Hb is testified to be adsorbed on UND film successfully. For comparison, we tested the UV–VIS spectrum of hemin, a free heme group, the Soret band was observed at 390 nm in solution (curve a). The slightly red shift (not blue shift) of Soret band for Hb on UND modified ITO glass slides suggests that the heme prosthetic group of Hb does not split out from Hb polypeptide matrix [22].

3.3. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy is a useful tool to compare the impedance of electrodes, with which the interface properties of surface modified electrode can be studied [23]. The curve of EIS present as Nyquist plot consists two parts: one part is the semicircle part corresponding to the electron transfer limited process and locating at the higher frequency. The electron transfer resistance (R_{ct}) can be gained by measuring its diameter; the other is the linear part affording the information about the diffusion process in solution and locating at the lower frequency. Fig. 2 denotes the EIS of bare GCE (curve a), and modified GCE of each step (curves b to f) in 0.10 M KNO_3 solution containing 5.0 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1:1). Compared with the bare GCE, the semicircle of the chitosan modified GCE increased dramatically, which indicates that chitosan film was an obstacle making the electron transfer of interface more difficult. When UND nanoparticles were anchored on the chitosan film, the electron transfer resistance further increased. This could be ascribed to the weak conductivity of UND. The evident change of Nyquist plot between the modified GCE with Chit/UND (curve c) and Chit/UND/Hb (curve d) indicates that Hb was successfully adsorbed. With the number of bilayer of {UND/Hb}$_n$ increases, the diameter of semicircle increases according, indicating {UND/Hb}$_n$ films were successfully immobilized on the GCE.

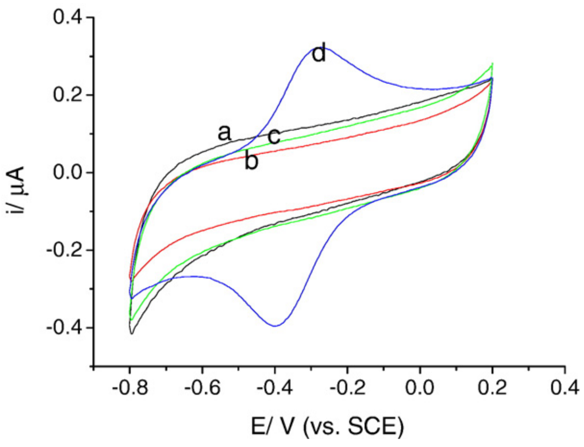

Fig. 3. Cyclic voltammograms(CVs) of GCE(curve a) and modified GCE with Chit(curve b), Chit/UND(curve c), Chit/UND/Hb (curve d) in pH=7 PBS solution: scan rate:100 mV/s.

3.4. Electrochemical behaviors

The technique of cyclic voltammetry can be used to track the electrochemical properties of the modified electrodes. Fig. 3

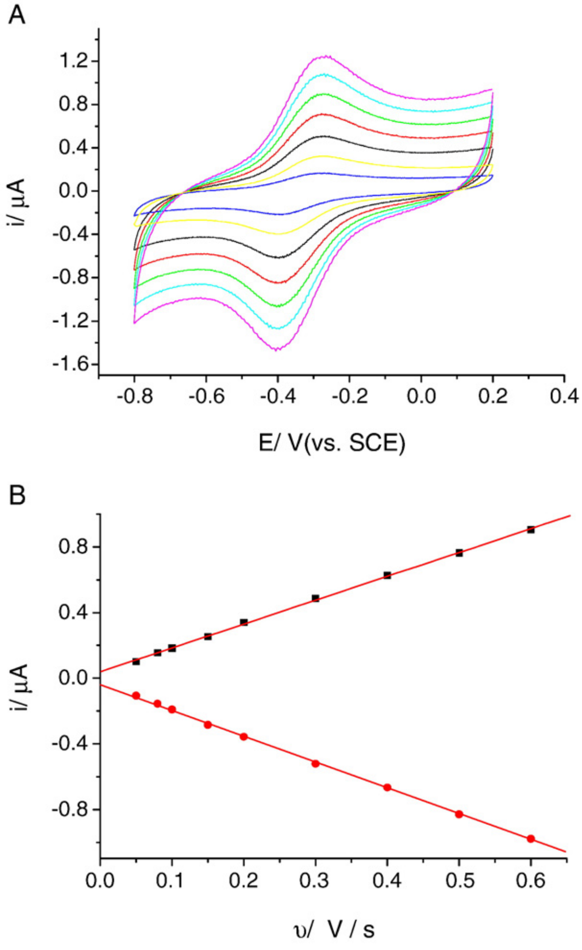

Fig. 4. A: CVs of GCE modified with Chit/{UND/Hb}$_3$ in pH=7.0 PBS solution with different scan rate:50, 100, 200, 300, 400, 500, 600 mV/s (from inner to outer). B: Plot of peak currents versus scan rate.

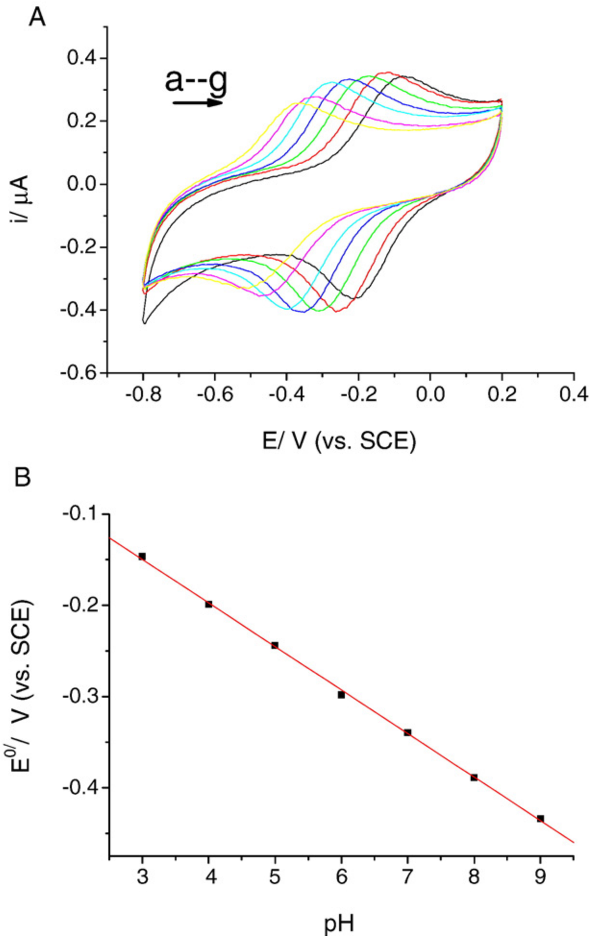

Fig. 5. A: CVs of GCE modified with Chit/{UND/Hb}$_3$ in PBS solution with different pH: 9.0, 8.0, 7.0, 6.0, 5.0, 4.0, 3.0 (from a to g). B: Plot of the formal potential versus pH of PBS solution. Scan rate: 100 mV/s.

shows the cyclic voltammograms (CVs) of bare GCE, the Chit modified GCE, Chit/UND modified GCE and the Chit/UND/Hb modified GCE in pH = 7.0 PBS solution, respectively. A pair of stable redox peak could be observed only at the Chit/UND/Hb modified GCE, and the anodic and cathodic peak potentials were about −293 and −386 mV, respectively. For comparison, we also investigated the electrochemical behavior of Hb on chitosan modified GCE without the UND particles. A small redox peak at the same potentials was observed and the peak currents decreased quickly with the continuous cyclic scanning. It indicated that the UND particles can not only promote the direct electron transfer between Hb and the GCE but also enhance stability of immobilized Hb. The redox peak currents gained a flat roof when there were three bilayers of UND/Hb on the modified GCE, Thus we use {UND/Hb}$_3$ film modified electrode for further experiments.

Fig. 4 shows the CVs of GCE modified with Chit/{UND/Hb}$_3$ in pH = 7.0 PBS solution with different scan rates. With the increasing of the scan rate, the redox currents increase linearly (Fig. 4B), and the redox potentials under each scan rate nearly kept a fixed value. These results indicate that the redox process is a typical surface controlled process [24,25]. The formal potential (E^{θ}) was about −0.34 V which was characteristic of the potential of heme ($Fe^{III/II}$) redox couples and was consistent with the literature [20,26]. The biggest separation of the peak potential was 102 mV ($E_{pc} = -0.393$ V, $E_{pa} = -0.291$ V, scan rate: 600 mV/s), illuminating the redox process is nearly reversible [27]. When the scan rate was returned to 100 mV/s from a higher scan rate, the shape and position of CV was the same as the beginning one, with which Hb can be proved to adsorb firmly. According to $\Gamma = Q/nFA$, (where Γ is the electroactive hemoglobin amount, Q represents the average reacted electric quantity, $F = 96485$, n and A are the number of electrons transferred and the area of the GC electrode, respectively), the amount of electroactive hemoglobin molecules at the {UND/Hb}$_3$/GC electrode was estimated to be 5.86×10^{-11} mol/cm^2 (assuming a one-electron-transfer reaction), which is almost 3.1 times higher than that of the theoretical monolayer coverage (1.89×10^{-11} mol/cm^2) [20].

The CVs are also influenced by the pH of PBS solution (Fig. 5A) The peak potential appeared positive shift with the pH decrease. A linear relationship between the formal potential and the pH was obtained with a slope of −47.7 mV/ pH (Fig. 5B), which was close to the expected value of 58.0 mV/ pH for a single electron transfer process. When the modified GCE was measured in pH = 7.0 solution again after the experiment, the

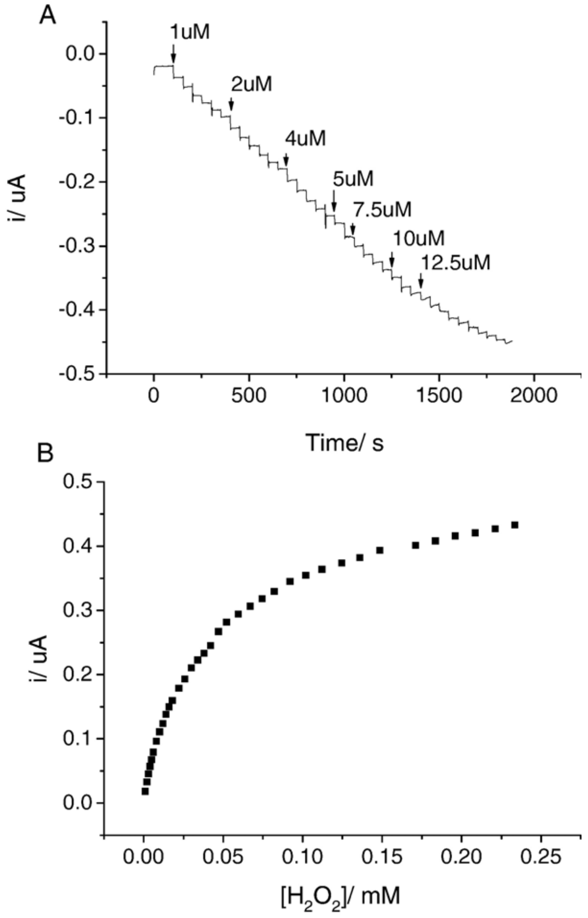

Fig. 6. A: A typical amperometric i–t curve of Chit/{UND/Hb}$_3$ modified GCE at −0.3 V in 2.0 ml PBS solution with pH = 7.0. B: Plot of current versus concentration of H_2O_2.

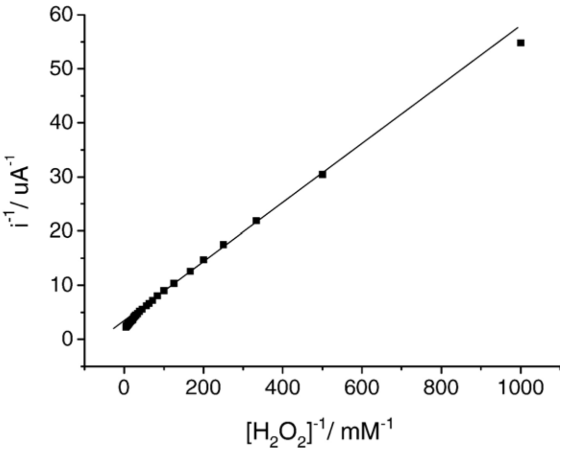

Fig. 7. The linearity relationship between the reciprocals of current and H_2O_2.

shape and position returned to the initialization. This result can also testify the Hb modified electrode had a good stability.

3.5. Electrocatalysis of Chit/{UND/Hb}$_3$ modified GCE to H_2O_2

It is well known that heme proteins can catalyze H_2O_2. In this case, electrocatalytic behaviors of the Chit/{UND/Hb}$_3$ modified GCE were tested and characterized by amperometric measurements. Fig. 6 shows the amperometric i–t curve of the modified GCE at −0.3 V in 2.0 ml PBS solution with successive injection of H_2O_2. The reduction current increased steeply within 6 s to reach a stable value. The reduction current increased linearly with the increase of hydrogen peroxide concentration in the range of 5.0×10^{-7} M–1.8×10^{-5} μM (R=0.996) with the detection limit of 4.0×10^{-7} M. When H_2O_2 concentration was higher than 0.10 mM, a response plateau was observed, showing a typical Michaelis–Menten kinetic mechanism. The apparent Michaelis-Menten constant (K_M) could be calculated from the Lineweaver–Burk equation [28]:

$$1/I_{SS} = 1/I_{max}(1 + K_M/\mathrm{mM}/c) \qquad (1)$$

where I_{ss} (μA) is the steady current, I_{max} (μA) is the current measured when H_2O_2 is saturation in PBS solution and c (mM) is the concentration of H_2O_2. Fig. 7 shows the linearity relationship between the reciprocal of current (I_{max}) and the reciprocal of H_2O_2 concentration (c) in the range of 0.5 μM to 0.25 mM with R=0.998 (N=36). K_M could be estimated to 0.019 mM, much smaller than that of Hb immobilized on gold colloid nanoparticles (0.12 mM) [29] and on carbon nanotube interface (0.675 mM) [27]. Thus, the Hb adsorbed on UND matrix has a higher affinity to H_2O_2.

3.6. Stability of the modified electrode

The electrodes modified with Chit/{UND/Hb}$_3$ were stored in the refrigeratory at 4 °C. Measured by CVs 3 days later, the current had no changes, but it was 0.91 times of the primal value a week later, 0.70 times 2 weeks later. In conclusion, the modified electrodes have a better stability.

4. Conclusion

Undoped nanocrystalline diamond particles and hemoglobin have been successfully modified on glassy carbon electrode surfaces. UND particles not only offer a friendly platform to assemble protein molecules, but also enhance the electron transfer process between protein molecules and electrode. This study provides another way to explore the application of undoped nanocrystalline diamond.

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