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Review

The application of boron-doped diamond electrodes in amperometric biosensors

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ABSTRACT

Boron-doped diamond (BDD) electrodes outperform conventional electrodes in terms of high stability, chemical inertness, wide potential window and low background current. Combining the superior properties of BDD electrodes with the merits of biosensors, such as specificity, sensitivity, and fast response, amperometric biosensors based on BDD electrodes have attracted the interests of many researchers. In this review, the latest advances of BDD electrodes with different surfaces including hydrogen-terminated, oxygen-terminated, metal nanoparticles-modified, amine-terminated, and carboxyl-terminated thin films, and microelectrodes, for the construction of various biosensors or the direct detection of biomolecules were demonstrated. The future trends of BDD electrodes in biosensing were also discussed.

Contents

1. Introduction

Owing to their specificity and sensitivity, biosensors have been increasingly developed for many applications in environmental monitoring and control, food and drug analysis, detection of biological metabolites, etc. [1,2]. Biosensors can also meet the need for continuous, real-time, and in vivo monitoring to replace the intermittent analytical techniques used in industrial and clinical chemistry [3]. Thereinto, amperometric biosensors, which combine the advantages of the electrochemical techniques with the high substrate specificity of the enzymes, have emerged as the most promising alternative for the monitoring of biologically related species in the past few years. The development and the performance of amperometric biosensors mainly depend on the physico-chemical characteristics of the materials employed for the construction of the transducer and the methods used for the enzyme immobilization. Many research groups have been studied the employment of carbon-based materials including, carbon paste, porous carbon, glassy carbon (GC), carbon nanotubes, and carbon nanofibers as electrochemical transducers in the field of biosensors

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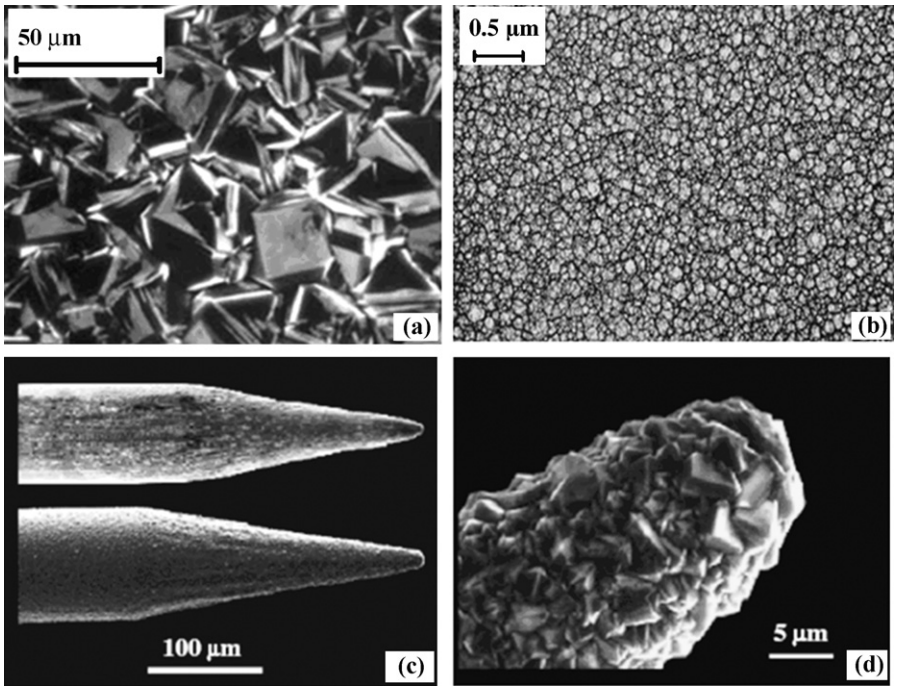

Fig. 1. SEM images of (a) a polycrystalline BDD thin film, (b) a nanocrystalline BDD thin film, (c) an electrochemically sharpened Pt wire (top) and a Pt wire covered with a polycrystalline BDD film (bottom), and (d) the tip of a 76 μm Pt wire covered with a BDD film.

[4–7]. The often-cited advantages of carbon electrodes include simple preparation methods, large positive potential ranges, and suitability for chemical modification. Despite their advantages, traditional carbon electrodes still suffer drawbacks, for example, electrode fouling. This problem limits their long-term stability and leads to frequent polishing or disposal of the electrode after a few uses.

Boron-doped diamond (BDD) thin films are novel carbon materials and are gaining big interests. BDD thin films were prepared by microwave plasma-assisted chemical vapor deposition (MPCVD) and hot-filament CVD (HFCVD). Methanol, B_2O_3 and hydrogen were the most commonly employed as the carbon source, boron source and carrier gas, respectively. The highly doped BDD films with metallic conductivity ($[B] > 3 \times 10^{20}\ cm^{-3}$) were used as electrodes. Nowadays, the development of diamond growth by CVD has enabled the preparation of the BDD electrodes with different surface structures on various substrates. According to the crystal size, the BDD electrodes could be divided into polycrystalline (grain size in μm range) films and nanocrystalline films (grain size below 100 nm). Several groups, such as, Fujishima, Swain, Panizza, Tryk, Marken and Compton groups, have been studied the electrochemical properties of the BDD electrodes and their application in electrosynthesis, water treatment, and electroanalysis of inorganic and organic compounds [8–12]. The results have demonstrated that the BDD electrodes possess many outstanding properties as described below.

(i) Wide electrochemical potential window in aqueous electrolyte solutions: the characteristic property, which allows the BDD electrodes to be used to detect molecules oxidization at high potentials, is ascribed to the extremely low catalytic activity of BDD electrodes for both hydrogen and oxygen generation; (ii) low and stable capacitive background current: the low electrostatic capacity minimizes the time to stabilize the background current during the amperometry, indicating the BDD electrodes superior to other electrode materials, with enhancements in the sensitivity for the low concentration detection; (iii) high response reproducibility and long-term response stability: the properties of the insensitivity to the presence of oxygen dissolved in aqueous solution and the high resistance to deactivation by fouling due to low and weak adsorption of polar molecules, make BDD film a stable electrode for electroanalysis; (iv) morphological and microstructural stability at extreme anodic and cathodic potentials and current densities because of the high stability of sp^3-bonded carbon; and (v) good biocompatibility due to the carbon materials, etc. Therefore, these prominent properties make BDD thin film an ideal electrode substrate for biosensors.

So far, the applications of the construction of various biosensors or the direct detection of biomolecules by the use of BDD electrodes, which have been widely reported, have not yet been summarized. As we know, electrochemical reactions proceed at the interface between electrolyte solutions and electrodes surfaces, so the surface structures and properties of BDD electrodes are important for electrochemical detection. Thus, the present paper summarizes the biosensors based on BDD electrodes materials with different surfaces, including hydrogen-terminated, oxygen-terminated, metal nanoparticles-modified, amine-terminated, and carboxyl-terminated thin films, and BDD microelectrodes.

2. Detection at hydrogen-terminated BDD electrodes

The surface of an as-deposited polycrystalline BDD electrode prepared by CVD using hydrogen gas as carrier gas is recognized to be hydrogen-terminated and its scanning electron microscopy (SEM) images is shown in Fig. 1a [9]. A clean and homogeneous hydrogen-terminated BDD surface could be obtained by treatment of oxygen-terminated BDD surfaces with hydrogen-plasma or heating at high temperatures (800–1000 °C) under hydrogen atmosphere. The hydrogen-terminated BDD electrodes have high stability and sensitivity for analysis of a number of biological species and the performances for the detection of several selected compounds are summarized.

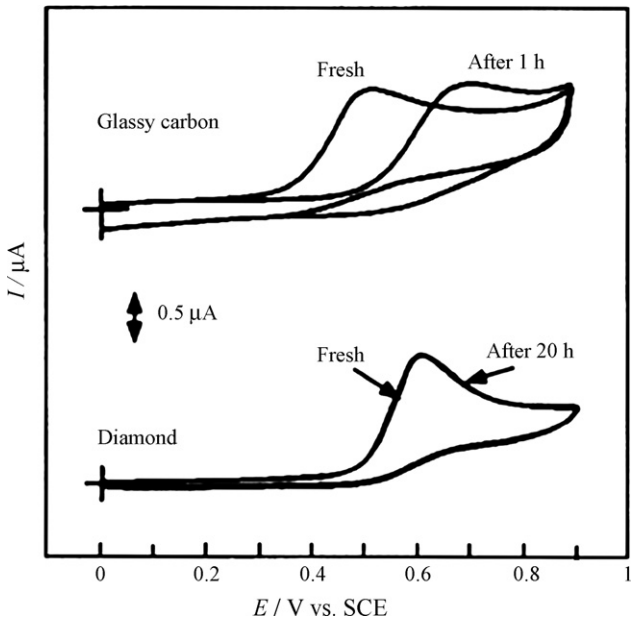

Fig. 2. CVs for 50 μM NADH at GC and as-deposited BDD electrodes in 0.1 M PBS (pH 7.0). The scan rate was 20 mV s^{-1}.

2.1. Detection of nicotinamide adenine dinucleotide

The electrochemical detection of nicotinamide adenine dinucleotide (NADH) is of great interest because it is a cofactor in a large number of dehydrogenase-based biosensors. A major problem with the bare GC and other electrodes is the deactivation rapidly due to the irreversible adsorption of oxidation products on the surfaces. Another problem with the unmodified and modified electrodes is the high background current due to the influence of oxygen present in the solution. Fujishima and his coworkers investigated electrochemical oxidation of NADH at as-deposited BDD electrodes [13,14]. The as-deposited BDD electrodes exhibited highly reproducible and stable response of cyclic voltammograms (CVs) for NADH oxidation, unlike GC electrodes, at which a significant shift of ~200 mV in the peak potential was observed within one hour, as shown in Fig. 2. In addition, due to the inertness toward the adsorption of reactants and products, the oxidation peak at the BDD electrode was more positive than that at fresh GC electrode. A high sensitivity with a detection limit of 10 nM was also obtained. An as-deposited BDD electrode incorporating an NADH mediated dehydrogenase-based ethanol biosensor also revealed good performances, indicating the feasibility of use of BDD electrodes in NADH-based biosensors.

2.2. Detection of biogenic amines

The analysis of biogenic amines, which are a group of naturally occurring amines derived by enzymatic decarboxylation of the natural amino acids, is essential for the early diagnosis of neurotransmission defects. Sarada et al. [15] investigated the electrochemistry determination of histamine and serotonin in 0.1 M phosphate buffer solution (PBS, pH 7.2) at as-deposited BDD electrodes. As shown in Fig. 3, well-defined CVs for histamine were observed at the BDD electrodes and the oxidation peak was more positive than that at the GC electrode as described above. In contrast, the oxidation peak current at the BDD electrode was more obvious and higher than that at the GC electrode under the condition of the similar electrode area. Importantly, the voltammetric signal-to-noise (S/N) ratios obtained at BDD electrodes were 1 order of magnitude higher than those obtained from GC electrodes. The comparison of the voltammograms for BDD and GC electrodes indicated the superior behavior of BDD electrode in terms of surface inertness to adsorption and response sensitivity. By use of the flow injection analysis technique, the BDD electrodes gave a good response with a linear response range of 0.5–100 and 0.01–50 μM and a detection limit of 0.5 and 0.01 μM for histamine and serotonin, respectively.

2.3. Detection of glucose

Sensitive, selective, reliable and fast monitoring of glucose has become a major concern throughout the world, especially for the control and treatment of diabetes. There are two major types, i.e., enzymatic and nonenzymatic sensors, for the electrochemical detection of glucose. Although the glucose oxidation utilizing glucose oxidase (GOx) has high selectivity, the glucose biosensors based on GOx suffer from difficulties in instability. Therefore, the electrochemical determination of glucose without employing an enzyme has attracted increasing interest due to its stability, simplicity, reproducibility and being free from oxygen limitation. Metal electrodes including gold, platinum, copper and nickel are known for showing the electrocatalytic oxidation of glucose. However, in real biological matrices, a major problem for direct electrochemical detection of glucose is the coexistence of many interfering compounds, for example, uric acid (UA) and ascorbic acid (AA). These compounds can be oxidized at a close potential to that of glucose, which results in the overlap of voltammetric response at above metal electrodes.

Lee and Park [16] reported that bare BDD electrodes annealed with a hydrogen flame can be used to detect glucose directly without any modification with enzymes or metallic catalysts. Fig. 4a showed a series of CVs for the oxidation of glucose at the hydrogen-terminated BDD electrodes and the oxidation peak current (650 mV vs. Ag/AgCl) kept on increasing with the increasing concentration of glucose. An anodic peak was also observed during the reverse scan, which indicated that glucose was strongly adsorbed on the electrode surface, and was continuously oxidized during the reverse scan. The formation of fouling from the oxidized glucose on the electrode prevented further oxidation of glucose. However, this passive film could be easily removed by simply rinsing the electrode with deionized water. Thus, the hydrogen-terminated BDD electrode gave a linear response range of 0.5–10 mM for glucose, which well encompasses the physiological range of 3–8 mM. Importantly, the selective detection of glucose in the presence of AA (Fig. 4b) or UA (not shown here), could be obtained at the hydrogen-terminated BDD electrode. Therefore, the superior performance of the BDD electrode may bring it as a future glucose detector in the medical field.

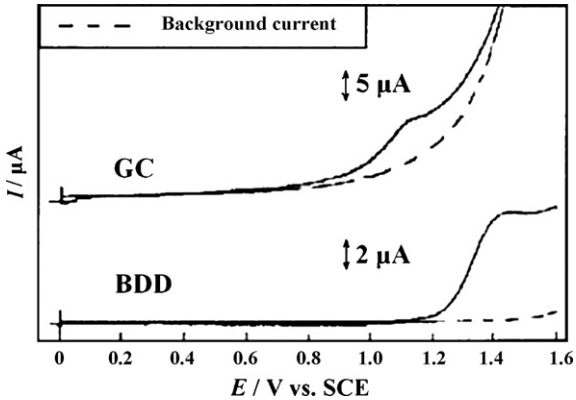

Fig. 3. Linear sweep voltammograms for 100 μM histamine in 0.1 PBS (pH 7) at GC (0.196 cm^2) and BDD (0.189 cm^2) electrodes. The scan rate was 100 mV s^{-1}.

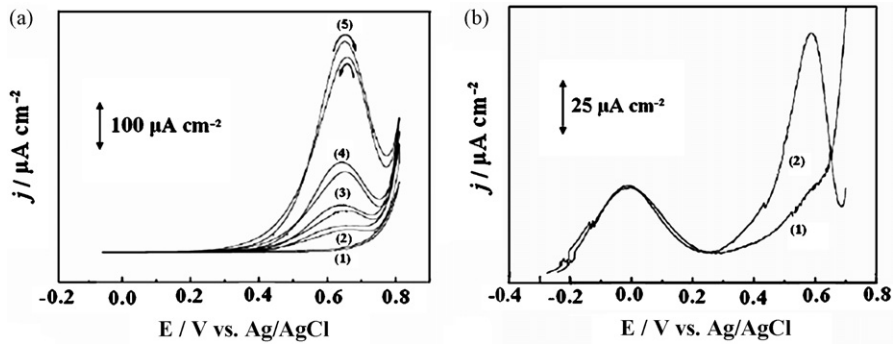

Fig. 4. (a) CVs of (1) 0, (2) 0.5, (3) 1.0, (4) 2.0, and (5) 5.0 mM glucose in 1.0 M NaOH at hydrogen-terminated BDD electrodes. The scan rate was 20 mV s^{-1}; (b) square wave voltammograms of (1) 1.0 mM AA and (2) also containing 5.0 mM glucose in 1.0 NaOH at hydrogen-terminated BDD electrodes. The pulse height was 25 mV, and the frequency was 10 Hz cm^{-2}.

In a word, hydrogen-terminated BDD shows advantages for electrochemical oxidation of a broad range of biological compounds [13–21], such as NADH, biogenic amines, glucose, L-cysteine, oxalic acid, glucose, and DNA, etc. Some of the analytical characteristics of above and other compounds are summarized in Table 1. Its wide electrochemical potential window allows the detection of biomolecules oxidizing at high potentials, and its resistance to the adsorption of chemical species on the surface allows the stable electrochemical detection of above molecules. Thus, in most of these studies, BDD electrodes were found to outperform GC electrodes in terms of stability and sensitivity. Additionally, at the oxygen-terminated BDD electrodes obtained by the treatment of electrochemical oxidation, the oxidation reaction of the above negative biomolecules is very less because of the electrostatic repulsion between the carbon–oxygen dipoles of the electrode surfaces and the negative charged compounds [19,20]. However, at the hydrogen-terminated surface the positive dipolar field created attracts the above biomolecules, facilitating the electrochemical reaction. Therefore, the control of surface termination is important for the electrochemical detection of some negative charged molecules by the use of BDD electrodes.

3. Detection at oxygen-terminated BDD electrodes

A hydrogen-terminated BDD electrodes can be altered to oxgen-terminated by a variety of methods, e.g. anodic treatment at highly positive potentials, oxygen plasma treatment, high treatment temperatures (300–1000 °C) under O_2 atmosphere, boiling in strong acid, and oxidation by strong oxidant. Electrochemical oxidation is convenient and popular for the use of electrochemistry, because the treatment can be performed in the same system as the electrochemical measurements. After treatment, a certain amount of C–OH and C=O groups confirmed by X-ray photoelectron spectroscopy measurement were introduced on BDD surface [22]. Compounds with positive charge maybe easily oxidized at oxygen-terminated BDD electrode in comparison with that at as-deposited BDD electrode since electrostatic attraction force from carbon–oxygen functionalities. While, as discussed above, negative charge compounds were more clearly obtained at as-deposited BDD electrode than those at oxygen-terminated BDD electrode due to the electrostatic repulsion at the oxidized surface existed. Thus, oxygen-terminated BDD electrodes could show selectivity (either enhancement or suppression) for detection of some biomolecules due to the carbon–oxygen dipoles of surface.

3.1. Selective detection of dopamine in the presence of ascorbic acid

Dopamine (DA) is an important neurotransmitter in mamalian central nervous system. A loss of DA-containing neurons may result in serious disease such as Parkinson's disease. The electrochemical detection of DA at carbon-based electrodes has received intense

Table 1
Some analytical characteristics for the detection of compounds at the BDD electrodes.

Electrode	Analyte	Linear range (μM)	Sensitivity (nA/μM)	Limit of detection (μM)	Repeatability RSD[a] (%)	Useful lifetime	Re.[b]
Hydrogen-terminated	NADH	0.01–0.5	~50	0.01	–	3 months	[14]
Hydrogen-terminated	Histamine	0.5–100	4	0.5	5 (15 injections)	–	[15]
Hydrogen-terminated	Glucose	500–1×10^4	~3.8	–	–	–	[16]
Hydrogen-terminated	Polyamines	1–1000	0.247–2.58	1	9.7[c] (50 injections)	–	[17]
Hydrogen-terminated	L-Cysteine	0.1–100	12	0.021	3.5 (five runs)	1 month	[18]
Hydrogen-terminated	Oxalic acid	0.05–10	110.31	~0.0005	2.05 (10 injections)	3 months	[19]
Hydrogen-terminated	DNA	0.1–8[d]	–	0.0037–0.01[d]	–	–	[20]
Hydrogen-terminated	Purines	50–250	0.0633[e]	2.1[e]	4.4[e] (8 injections)	–	[21]
Oxygen-terminated	Dopamine	1–70; 0.1–1	~70	0.05	–	3 months	[26]
Oxygen-terminated	Uric acid	0.05–1	12.2	0.015	1.15 (20 measurements)	>3 months	[28]
Oxygen-terminated	Glutathione	1–250	–	0.0014	< 3 (3 days)	–	[29]
Oxygen-terminated	Purines, pyrimidines	0.1–10	–	0.03–0.16	2.63–4.89 (1 month)	–	[30]
AuNPs-modified	Dopamine	0.01–10	–	0.001	~1 (10 measurements)	–	[31]
Tyrosinase-modified	*p*-Cresol	1–200	76.4	0.1	1.9 (10 measurements)	5 weeks	[36]
Cyt *c*-modified	H_2O_2	1–450	18.6	0.7	2.1 (10 measurements)	6 weeks	[41]
Microelectrode	Catecholamines	0.06–1	–	0.051	4.5 (5 measurements)	–	[49]

[a] Relative standard deviation.
[b] References.
[c] Cadaverine.
[d] μg mL^{-1}.
[e] Xanthine.

attention in part due to the possible future development of practical in vivo sensors. Major problem of electrochemical detection DA is the overlapping of oxidation waves between DA and interfering compounds (e.g. AA) at the above bare electrodes. Recently, some nano-materials, e.g. carbon nanotubes [23], gold nanoparticles [24], and polymer thin film [25] modified GC electrodes were used to carry out the selective detection of DA in the presence of AA. However, there are difficulties remaining to be solved, such as complicated preparation methods and long-term stability.

Fujishima's group researched the electrochemical detection of DA in the presence of AA at BDD electrodes [26,27]. At as-deposited BDD (hydrogen-terminated) electrode, the anodic peak potential ($E_{p,a}$) of DA and AA was 0.76 and 0.80 V (vs. saturated calomel electrode, SCE) in 0.1 M $HClO_4$, respectively. After electrochemical treatment of BDD (oxygen-terminated) electrode, the $E_{p,a}$ of DA and AA was 0.80 and 1.3 V (vs. SCE), respectively. CV for a solution containing both DA and AA in 0.1 M $HClO_4$ at the oxygen-terminated BDD electrode exhibited two well-defined anodic peaks. Therefore, DA can be selectively determined in the presence of AA using the oxygen-terminated BDD electrode, and as a result, a low detection limit of 50 nM (S/N = 3) and long-term stability (3 months) was obtained. Possible explanation of the selective detection for DA is that the oxidized BDD surface acquires surface dipoles as a result of introducing C=O functional groups, which then electrostatically repel the oxygen-containing group on AA with strong dipoles. That is to say, the AA oxidation is impeded owing to the high potential required and the spatial locations separates from oxidized BDD surface. For the protonated DA, the interaction between the ammonium group of DA is relatively strong with both hydrogen- and oxygen-terminated BDD electrode, so that the equilibrium distances and the electron transfer rates are not greatly different. For the same reason, excellent selectivity and sensitivity for the detection of UA in the presence of high concentrations of AA was obtained at oxygen-terminated BDD electrodes [28].

3.2. Simultaneous detection of oxidized and reduced glutathione

Glutathione (GSH) is among the most important antioxidant in cells involved in enzymatical reduction of hydroperoxides and nonenzymatical retainment of vitamin E and C in reduced and functional forms. In the presence of oxidants, GSH is oxidized to glutathione disulfide (GSSG), which is either reduced enzymatically by glutathione reductase or excreted from cells into extracellular fluids. Thus, the GSSG/GSH ration serves as an indicator of oxidative stress. The early techniques for the measurement of GSH and GSSG were based on an enzymatic recycling and high performance liquid chromatography method, which suffer from low sensitivity and complicated pretreatment. Electrochemical method is simple and sensitive, but the electrochemical detection of GSSG, which is a positively charged molecule, is difficult due to the high oxidation potentials.

Terashima et al. [29] studied the amperometric detection of GSSG and GSH at anodically oxidized BDD electrodes. Fig. 5 showed a comparison of CVs for GSSG obtained at oxidized BDD, GC, and Pt electrodes in acidic medium (pH 2). At the Pt electrode, a small oxidation current was observed in the potential as the peak of surface oxide formation, and the peak current overlapped with oxygen evolution when increasing potential. In case of GC electrodes, surface fouling due to the adsorption of reaction products was observed during oxidation of GSSG, and the background current caused an increase because of the relatively high potential. For comparison, the response of GSSG at oxidized BDD electrodes showed two well-defined peaks with diffusion controlled limiting currents. However, the GSSG oxidation was not so sensitive at as-deposited BDD electrodes. These were because, at the oxidized BDD electrode, the GSSG oxidation was ascribed to the electrostatical attraction between the positively charged GSSG molecule in acidic solution and a negative dipolar field formed by oxygen functional groups of electrode surface. To obtain the simultaneous quantification of GSSG and GSH, liquid chromatography coupled with the oxidized BDD electrodes as electrochemical detection was used. The detection limits for GSH and GSSG were 1.4 and 1.9 nmol (S/N = 3), respectively. The variation in day-to-day response during 3 days were less than 3%. The high stable amperometric response of the oxidized BDD electrode could be ascribed to the high electrochemical stability and the surface reactivation of oxidized electrodes as a detector. Moreover, high performance liquid chromatography system using oxygen-terminated BDD electrochemical detector has been also demonstrated to be a promising approach for the simultaneous detection of purine and pyrimidiane bases [30].

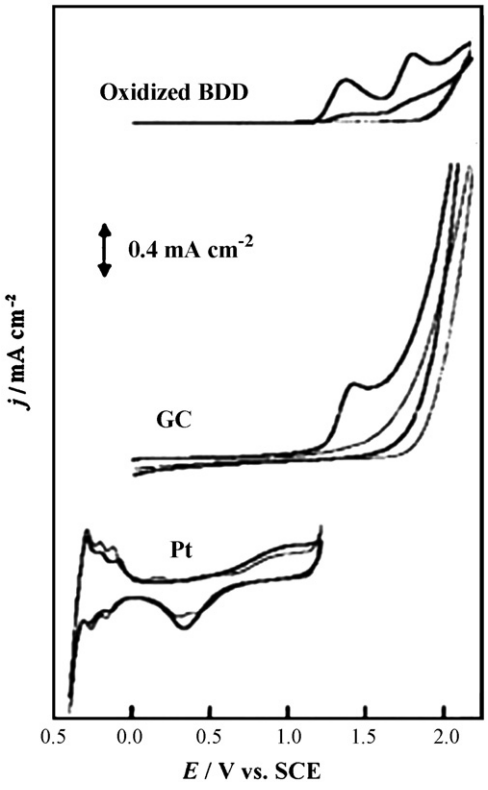

Fig. 5. CVs for 1 mM GSSG in Britton–Robinson buffer solution (pH 2) at oxidized BDD, GC, and Pt electrodes. The scan rate was 100 mV s^{-1}. Thin lines represent background current.

In brief, the oxygen-terminated BDD electrodes have outstanding features in a much wider potentials window, and higher surface stability from fouling compared to hydrogen-terminated BDD electrodes. Importantly, the oxygen-terminated BDD electrodes are able to achieve selective detection of certain compounds under certain conditions. The significant advantages including very ease of preparation, very high stability, high sensitivity and good selectivity, make oxygen-terminated BDD electrodes an interesting candidate for the study of the direct detection of biological molecules.

4. Detection at metal nanoparticles-modified BDD electrodes

Assembly of ordered metal nanoparticles, in particular gold nanoparticles (AuNPs) have a wide range of applications in electronics, catalysis, and analysis. Metal nanoparticles could be immobilized on BDD surface by vacuum vapor deposition, electrochemical deposition, sputtering or layer-by-layer self-assembly. Considering the outstanding properties of BDD electrodes, assem-

bly of ordered metal are expected to have a wide range of applications in the field of biocatalysis.

To determine DA with high sensitivity and selectivity, the modification of AuNPs on BDD electrode was studied. Weng et al. [31] reported that BDD electrode was modified with gold clusters by electro-deposition and the electrochemical performance of DA and AA was investigated on the Au/BDD electrode. AuNPs with a size distribution between 20 and 400 nm were deposited on the BDD surface after deposition. The AuNPs-modified BDD electrode showed a higher activity for DA oxidation than AA; the oxidation peak of DA shifted to a less-positive potential (0.11 V) than that of AA (0.26 V), and a much higher peak current could be observed for DA oxidation than that of AA. As a result, the AuNPs-modified BDD electrode could selectively determine DA in the presence of a large excess of AA with detection limit of 0.1 μM, but it could not resist fouling. After the further modification of carboxylic groups on the AuNPs, the carboxyl-terminated electrode had a better antifouling effect and higher sensitivity for DA detection due to the electrostatic attraction. The minimal detection limit of 1.0 nM and linear range of 10 nM to 10 μM could be achieved. The detection limit was lower than that of direct detection of DA at oxygen-terminated BDD electrodes discussed in section of 3.1, indicating that the AuNPs introduced on the surface are indeed a useful catalyst to the DA oxidation.

In addition, complex nanoparticles of negatively charged AuNPs and polymer spheres were modified on BDD electrodes and the modified BDD electrodes were used to study the electrochemical behaviors of DA and AA [32]. The modified BDD electrode exhibit high electrocatalytic activities toward the DA oxidation, while the AA oxidation showed almost no response and the unoxidized AA would not cause any side reactions due to electrostatic repulsion negatively charged AuNPs and AA. The results displayed an effective detection of DA in the presence of AA at the modified BDD electrode with detection limit of DA of 0.8 μM.

In brief, the modification of metal nanoparticles on the BDD electrode makes it a candidate to prepare highly active electrode for the catalytic oxidation (or reduction) of biomolecules. Especially, the immobilization of charged nanoparticles on BDD electrode could determinate special biomolecules in biological system with high selectivity and high sensitivity.

5. Biosensors based on immobilizing tyrosinase at amine-terminated BDD electrodes

Due to the inert nature of BDD surfaces, the immobilization of biomolecules on BDD surfaces for the fabrication of amperometric biosensors requires surface activation procedures to provide the reactive groups, such as amino and carboxylic groups. Generally, amine-terminated BDD electrodes could be achieved by modification several methods, e.g. (i) etching a hydrogen-terminated BDD surface by NH_3 plasma in specific reactor, (ii) chemical modification of an oxidized BDD surface with (3-aminopropyl) triethoxysilane [33], (iii) photochemical reaction of amino molecules containing a vinyl group with a hydrogen-terminated BDD surface by free radical mechanism [34], and (iv) diazonium functionalization of 4-nitrobenzendiazonium tetrafluoroborate with hydrogen-terminated BDD electrodes by combined chemical and electrochemical processes [35]. Thus, a layer of amine groups introduced on the BDD surface could serve as binding sites for attachment of biomolecules.

On the other hand, tyrosinase, also known as polyphenol oxidase, can catalyse two reactions: ortho-hydroxylation of phenols to catechol and the further oxidation of catechols to *ortho*-quinones, both in the presence of molecular oxygen. Then the quinone generated could be reduced by the electrodes, reforming the original phenols, thus forming a bio-electrocatalytic amplification cycle. Moreover, the polymerization of phenols and interference from oxidizable species could be prevented because of the low potential for reduction of *o*-quinones on tyrosinase-based sensors. The key aspects in the construction of this kind of biosensors is the choice of method and substrate for immobilizing the tyrosinase on the electrode surfaces. Tyrosinase-based biosensors reported have employed conventional electrode materials as substrates, such as GC, carbon-paste, gold, and other materials. But these enzyme electrodes suffer from complication in manipulation, desorption of enzyme from electrode materials, and/or weak in retaining the bioactivity of tyrosinase. Thus, biosensors based on negatively charged tyrosinase (p*I* 4.5) immobilized on the positively charged amino groups of BDD electrodes in neutral buffer solution are of considerable interest due to the outstanding properties of BDD films and the reliability of covalent immobilization.

Notsu et al. [33] reported the immobilization of tyrosinase on a BDD electrode. Firstly, (3-aminopropyl) triethoxysilane was used to modify BDD electrode treated by electrochemical oxidation, ant then a tyrosinase film cross-linked with glutaraldehyde. The low limit with 10^{-6} M for bisphenol-A was achieved at the enzyme electrodes by using a flow injection system. However, the tyrosinase-modified BDD electrode retained its initial activity only for a few days in storage under dry conditions, due to weak bonding of (3-aminopropyl) triethoxysilane with BDD surface.

To improve the stability of tyrosinase-based BDD electrodes, Zhi's group studied the covalent immobilization of tyrosinase onto the amine-terminated BDD electrode [36,37]. The amine active BDD surfaces were obtained by two methods. One was that the hydrogen-terminated BDD surface was treated with allylamine by photochemical reaction. Another was that the hydrogen-terminated BDD surface was treated with 4-nitrobenzenediazonium tetrafluoroborate. The difference of these two methods was that the photochemical reaction linked the amino group to BDD surface via an alkyl chain, while the diazonium method typically used an aromatic ring. Both the two tyrosinase-modified BDD electrodes by the above methods exhibited fast response, high sensitivity and wide linear range for the detection of phenolic compounds. For comparison, the sensitivity of the enzyme electrode by diazonium method was higher than the enzyme electrode by photochemical method, which might be because, the aryl ring of diazonium molecule was more conductive than alkyl chain. That is to say, the aryl ring could be well-suited for the electron transfer between enzyme molecules and the BDD electrodes. The sensitivity of the enzyme electrode by diazonium method was also higher than those of the tyrosinase biosensors reported [38–40], which could be attributed to the high and reliable loading of enzyme by present method. The two developed enzyme electrodes could retain about 90% of its initial activity for the response of phenols after 1 month. The high stability could be ascribed to the strongly covalent bonding of tyrosinase to the BDD electrodes and the high chemical and electrochemical stability of the BDD substrates. To sum up, the amine-functionalized BDD electrodes is an interesting alternative for application in biosensing technology.

6. Biosensors based on immobilizing cytochrome *c* at cayboxyl-terminated BDD electrodes

Direct electron transfer reactions between redox proteins and electrode surfaces has attracted considerable interest. Understanding of these reactions can establish a fundamental and desirable model for studying the natural redox properties of the protein, the interfacial charge transfer process, and the relationship between their structure and biological functions. Moreover, the study of direct electron transfer between proteins and underlying electrodes could also provide a platform for fabricating biosensors. It is known that the heme-containing proteins such as horseradish peroxidase,

cytochrome *c* (Cyt *c*), and myoglobin, have a direct electrochemical behavior for its redox center of $Fe^{III/II}$ at the electrode surface and the ability to electrocatalyze the reduction of H_2O_2. However, direct electrochemistry of these proteins at a bare electrode is difficult because of its extremely slow electron transfer kinetics at the electrode/solution interface and its short-live and transient response on a metal electrode surface. Common electrode materials employed should be modified with mediators by special methods.

On the other hand, compared with the polycrystalline BDD electrodes, nanocrystalline BDD (NBDD) electrodes have a smoother surface while maintaining the intact BDD properties. Fig. 1b shows the SEM images of NBDD electrodes [41]. Furthermore, in addition to boron doping, the sp^2-bonded carbon phase on grain boundaries of NBDD films can provide charge carries and high carrier mobility pathways, which may lead to better reversible properties of electrodes for redox systems [42]. Thus, NBDD electrode is expected to be a more suitable candidate to provide a high activity for biosensors. In fact, surface smoothness of BDD electrodes could affect their amperometric response significantly. It has been demonstrated that mechanical polishing of a polycrystalline BDD electrode to a nanometer-scale finish has shown to result in a well-defined voltammetric response of Cyt *c* in solution [43]. Haymond et al. [44] have also reported that direct electron transfer could occur between Cyt *c* existing in solution and the as-deposited NBDD electrode with quasi-reversible, diffusion-controlled electron transfer kinetics.

Zhi's group reported the functionalization of NBDD films via photochemical reaction with undecylenic acid methyl ester and subsequent removal of the protection ester groups to produce a carboxyl-terminated surface [41]. Then Cyt *c* was successfully immobilized on NBDD electrode by the bonding of negatively charged carboxylic groups and positively charged lysine residue of Cyt *c* (pI 10). The Cyt *c*-modified NBDD electrode showed a pair of quasi-reversible redox peaks with a formal potential (E^0) of 0.061 V (vs. Ag/AgCl) in 0.1 M PBS (pH 7.0) and a high electron transfer constant (k_s) of $5.2 \pm 0.6\ s^{-1}$. Compared to a Cyt *c*-modified polycrystalline BDD electrode, the electron transfer was faster at the Cyt *c*-modified NBDD electrode due to the incorporated sp^2 state carbon as charge transfer mediators on the NBDD surface. It is known that amperometric biosensors based on Cyt *c* immobilized on electrode surfaces could be used to detect H_2O_2 selectively, because Cyt *c* as heme-containing proteins is able to electrocatalyze the reduction of H_2O_2. Investigation of the electrocatalytic activity of the Cyt *c*-modified NBDD electrode toward H_2O_2 exhibited a rapid amperometric response (5 s), a wide linear range (1–450 μM), and a low detection limit (0.7 μM at S/N = 3). In addition, the response stability of Cyt *c*-modified NBDD electrode toward H_2O_2 was nearly equivalent to that of the Cyt *c*-modified polycrystalline BDD electrode but was significantly higher than that of the Cyt *c*-modified GC electrode.

In a word, the outstanding electrochemical properties, including wide potential window, low background current, and extreme stability, together with its inherent biocompatibility and flat surface, make the conductive NBDD thin films as an interesting candidate for the substrate of a third-generation biosensor.

7. In vivo detection at BDD microelectrodes

The small size of the microelectrodes renders them suitable for use in vivo detection. Carbon fiber microelectrodes have been used widely for biomolecules detection in biological system because of their small size (<30 μm), biocompatible, and temporal response [45]. However, during exposure to physiological environments, surface fouling by biomolecule adsorption makes that the response stability and sensitivity at carbon fiber microelectrodes are low. Besides, for activation of the carbon electrodes, pretreatment could introduce surface roughening, surface oxide formation, and microstructural damage. Recently, BDD microelectrodes have been fabricated by deposited a BDD film on a metal wire substrate [46,47]. SEM images of a microsized Pt wire before and after coating of BDD thin film, and the tip of the Pt wire covered with a BDD film were showed in Fig. 1c and d, respectively. The result of Raman spectra (not shown here) showed that the BDD microelectrode was of good quality under certain deposition condition. The BDD microelectrodes were expected to meet the above listed requirements in biological environments, because of its (i) hard, lubricious and biocompatible nature that enables easy penetration into tissue with minimal damage, (ii) low background current over a wide potential range, (iii) extreme high electrochemical stability ascribed to the hydrophobic sp^3-bonded carbon surface on which weak adsorption of polar biomolecules and contaminants occurs, (iv) chemical inertness. Therefore, BDD microelectrodes are attractive for in vitro electroanalytical measurements.

The catecholamines, such as, norepinephrine, epinephrine, and DA, play an important role in neurotransmission and other physiological process. For clinical and diagnostic reasons, monitoring catecholamine levels in tissue and biological fluids requires highly sensitive and reliable analytical techniques. Swain's group studied amperometric detection for norepinephrine detection in tissue or nervous system using BDD microelectrodes [46–49]. Compared with conventional carbon fiber electrodes, the BDD microelectrodes exhibited improved response performances, including, higher sensitivity, stability, and reproducibility. Specifically, coupled with video microscopy, simultaneously amperometric monitoring with the microelectrode was used to measure norepinephrine released from rat sympathetic nerves innervating meseteric arteries and veins in vitro, and the evoked contractile response. Additionally, by virtue of capillary zone or capillary electrophoresis, the amperometric detection was obtained very high sensitivity with a low detection limit of 51 nM (S/N = 3).

Continuous amperometry with the BDD microelectrode was used to measure serotonin overflow as an oxidation current [50]. With the recording microelectrode positioned about 1 mm above the mucosa, serotonin released from enterochromaffin cells, was elicited by both mechanical and electrical overflow stimulation. Some minor electrode fouling, a common problem with the oxidative detection of serotonin at the carbon fiber microelectrode, was observed for BDD microelectrode but the response was enough stable to record in vitro. The fact that the oxidation current increased in the presence of the serotonin transporter inhibitor indicated that the measured signal was associated with serotonin. Serotonin disposition in the intestinal mucosa of neonatal and adult guinea pigs was also compared using a BDD microelectrode and continuous amperometry [51].

These results could help us to learn about behavioral changes and the onset of neural disease conditions [52]. In view of the remarkable performance of the BDD microelectrode, it is useful for real-time measurement of samples in vivo, providing superior response sensitivity, precision, and stability as compared to a carbon fiber microelectrode.

8. Conclusions

In conclusion, several functional BDD electrodes have been used for determining various bio-analytes over the past decade or so. Because of the excellent properties of BDD substrates, these BDD-based amperometric biosensors exhibited good performances in terms of high sensitivity, selectivity, reproducibility and long-term stability. That is to say, BDD electrodes are interesting candidates for the construction of amperometric biosensors or the direct electrochemical detection of biomolecules.

Despite the impressive progress in the development of BDD-based amperometric biosensors, the promise of the application

of these biosensors in real biological systems has not been fulfilled, and there are still many challenges and obstacles related to the achievement of a highly stable and reliable continuous biomolecules monitoring. Future developments will rely upon the close collaboration of analytical technology, electrochemistry, biological engineering and other relative technologies to ensure effective application and exploitation of BDD electrode materials in amperometric biosensing. There are some areas of interests opened up by these developments. One promising area is in search of reliable modification method of BDD electrode surfaces to further enhance the selectivity and sensitivity for biomolecules detection in complex systems. Another area for future advances is the BDD sensor arrays for detecting several bio-analytes at once. With the development of the electron beam and nanoimprint lithography, there is an opportunities for fabrication of ordered micro- or nanostructure on BDD thin films. Therefore, the prepared BDD microelectrode-arrays could be used for the real-time and in vivo detection of biologically related species in real biological systems.

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