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Biosensors and Bioelectronics 19 (2003) 177–183
BIOSENSORS & BIOELECTRONICS
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Reagentless glucose biosensor based on direct electron transfer of glucose oxidase immobilized on colloidal gold modified carbon paste electrode

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Received 18 July 2002; received in revised form 7 April 2003; accepted 24 April 2003

Abstract

The direct electrochemistry of glucose oxidase (GOD) adsorbed on a colloidal gold modified carbon paste electrode was investigated. The adsorbed GOD displayed a pair of redox peaks with a formal potential of $-(449\pm1)$ mV in 0.1 M pH 5.0 phosphate buffer solution. The response showed a surface-controlled electrode process with an electron transfer rate constant of (38.9 ± 5.3)/s determined in the scan rate range from 10 to 100 mV/s. GOD adsorbed on gold colloid nanoparticles maintained its bioactivity and stability. The immobilized GOD could electrocatalyze the reduction of dissolved oxygen and resulted in a great increase of the reduction peak current. Upon the addition of glucose, the reduction peak current decreased, which could be used for glucose detection with a high sensitivity (8.4 μA/mM), a linear range from 0.04 to 0.28 mM and a detection limit of 0.01 mM at a signal-to-noise ratio of 3σ. The sensor could exclude the interference of commonly coexisted uric and ascorbic acid.

Keywords: Biosensors; Carbon paste electrode; Glucose oxidase; Electrocatalysis; Colloidal gold; Glucose

1. Introduction

The determination of glucose concentration is very important in clinical, biological and chemical samples, as well as food processing and fermentation (Yoshimura and Hozumi, 1996; Mizutani et al., 1997; Xu and Chen, 2000). Many techniques such as fluorescence (Chen et al., 1999), electrochemistry (Haruyama and Aizawa, 1998; Sugawara et al., 2000), and flow injection (Shi and Crouch, 1999) have been developed for this purpose. Among the electrochemical techniques, the amperometric biosensors based on electron transfer between an electrode and immobilized glucose oxidase (GOD), which can catalyze the oxidation of glucose, are especially promising. This technique is simple and convenient and can be realized in two different ways. The first is an indirect method by using mediators to shuttle the electrons (Cass et al., 1984; Ikeda et al., 1985; Losada and Armada, 1997; Kulys, 1999; Reiter et al., 2001). The mediators used in these sensors include ferrocene derivatives (Cass et al., 1984; Amine et al., 1993; Murthy and Sharma, 1998), quinones (Ikeda et al., 1985) and poly-2-aminoaniline polymer (Losada and Armada, 1997) etc. Another way is based on direct electron transfer between GOD and electrode, producing mediatorless glucose sensors (Pandey et al., 1999; Wang et al., 1994). However, the sensitivity and application of these unmediated sensors have been limited due to the lack of a simple approach to immobilize and stabilize enzyme and the difficulty of direct electron transfer between redox enzyme and electrode, which results from the thick insulating protein layer surrounding the face of the active center. Thus, it is necessary to search a way to develop a new glucose sensor based on the direct electrochemistry of GOD.

The direct electron transfer of immobilized GOD, with regard to the GOD(FAD) to GOD($FADH_2$) conversion, where FAD is flavin adenine dinucleotide, has been achieved on platinum or sputtered platinum (Lu et al., 1994; Os van et al., 1996), gold (De Taxis Du

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doi:10.1016/S0956-5663(03)00172-6

Poet et al., 1990), glassy carbon (Narasimhan et al., 1986), carbon paste (CP) (Savitri and Mitra, 1998) and graphite surface (Ianniello et al., 1982) by amperometric and cyclic voltammetric techniques. Wang et al. (1994) reported a glucose biosensor by mixing GOD and rhodium-dispersed CP to produce an intimate contact between enzyme and carbon particles and achieve the direct electron transfer. The metal colloidal films have been used to construct the interface for direct electron transfer of redox-active proteins and retaining their bioactivity (Horisberger, 1983). Colloidal gold is an extensively used metal colloid, which has been used for study of direct electrochemistry of proteins (Zhao et al., 1992; Brown et al., 1996; Xiao et al., 2000; Gu et al., 2001; Ju et al., 2002; Liu and Ju, 2002). It provides an environment similar to that of redox proteins in native systems and gives the protein molecules more freedom in orientation, thus reducing the insulating property of the protein shell for the direct electron transfer and facilitating the electron transfer through the conducting tunnels of colloidal gold. In this work we combines the advantageous features of colloidal gold and CP technology to develop a reproducible glucose biosensor based on the direct electron transfer of GOD.

GOD adsorbed on the surface of colloidal gold mixed in CP exhibits a fast electron transfer with electrode. Its reduced form, GOD($FADH_2$), can electrocatalyze the reduction of dissolved oxygen according to following equations (De Taxis Du Poet et al., 1990; Garjonyte and Malinauskas, 2000).

$$GOD(FAD) + 2e^- + 2H^+ \rightleftharpoons GOD(FADH_2) \quad (1)$$

$$GOD(FADH_2) + O_2 \rightarrow GOD(FAD) + H_2O_2 \quad (2)$$

In the presence of glucose the electrocatalytic reaction is restrained due to the enzyme-catalyzed reaction between the oxidized form of GOD, GOD(FAD), and glucose,

$$\text{Glucose} + GOD(FAD) \rightarrow \text{gluconolactone} + GOD(FADH_2) \quad (3)$$

which results in a decrease of electrocatalytic response. Based on the decrease, a new method for glucose determination is proposed. This method is different from the usual glucose amperometric sensors on which the determination is realized by monitoring either the consumption of oxygen (Shinohara et al., 1988) or the production of hydrogen peroxide (Mu et al., 1991; Garjonyte and Malinauskas, 2000). The latter requires a high anodic potential, thus results in an interference of cooxidizable substances such as ascorbic acid and acetaminophen. The proposed method can efficiently exclude the interference of commonly coexisted uric and ascorbic acid.

2. Experimental

2.1. Materials

GOD (EC 1.1.3.4, 35.3 units/mg) and β-D(+)-glucose was purchased from Sigma and used as received. Serum standard sample was provided by Zhongsheng Biology Engineering Co. (Beijing) and prepared according to the instructions. $AuC1_3HC1{\cdot}4H_2O$ (Au% > 48%) was obtained from Aldrich. Carbon graphite powder (< 325 mesh, Johnson Matthey) and paraffin oil (from Fluka) were used for the preparation of CP. All other chemicals were of analytical grade and used without further purification. Colloidal gold was prepared according to the literature (Ju et al., 2002). 0.1 M phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH. All solutions were made up with twice-distilled water.

2.2. Electrode preparation

The CP was prepared by thoroughly mixing carbon graphite powder and paraffin oil (1 mg: 0.36 μl). Prior to use, the graphite powder was treated at 700 °C for 30 s in a muffle furnace and then cooled to room temperature in a desiccator in the presence of activated silica gel. The colloidal gold modified CP was prepared according to the following procedure. Hundred milligram of pretreated graphite powder was mixed thoroughly with 300 μl 24-nm colloidal gold solution. After evaporation of water in a desiccator for 3 h, 36 μl paraffin oil was added to the mixture. A portion of the resulting pastes were put into plastic syringe tubes with the inner diameter of 0.51 ± 0.01 mm to form carbon paste electrode (CPE) and colloidal gold modified carbon paste electrode (Au/CPE). Electrical contact to the pastes was established by inserting a copper wire down the plastic syringe tube and into the back of mixture. The GOD/Au/CPE and GOD/CPE were prepared by dropping 10 μl 3 mg/ml GOD in 0.1 M pH 5.0 PBS to the Au/CPE and CPE, respectively, after the electrode tips were smoothed manually with clean paper and then at a plane glass surface to produce a flat surface. All electrodes were stored in PBS at 4 °C.

2.3. Electrochemical measurements

Electrochemical measurements were performed with a BAS-100B electrochemical analyzer connected a PA-i preamplifier (Bioanalytical Systems Inc., USA). A three-electrode system comprising a prepared working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference was employed for all electrochemical experiments. All ex-

periments were performed at room temperature (18 ± 2 °C) in 0.1 M PBS as the supporting electrolyte. The experiments for glucose determinations and electrocatalytical studies were carried out in air-saturated solutions, other experiments were performed in the solutions deoxygenated by bubbling highly pure nitrogen for 15 min and maintained under nitrogen atmosphere during measurements. The geometric area of the CPE was determined by the slope of plot of the anodic peak current of 1.0 mM K_3 [Fe(CN)$_6$] in 0.1 M KCl vs. the square root of scan rate to be 1.9×10^{-3} cm^2.

3. Results and discussion

3.1. Optimization for preparation of the enzyme electrode

The immobilization of enzyme on the surface of the colloidal gold modified carbon paste electrode can be achieved with different methods. In previous work (Ju et al., 2002; Liu and Ju, 2002), we have immobilized cytochrome *c* by polishing the Au/CPE in the enzyme solution on a glass surface and horseradish peroxidase by entrapping it into the blend of colloidal gold solution and carbon paste. These methods were very effective for preparation of the cytochrome *c* and horseradish peroxidase modified carbon paste electrodes.

After Au/CPE was polished in 3 mg/ml GOD solution on a glass surface or immersed in 3 mg/ml GOD solution at 4 °C for 8 h, and then rinsed thoroughly with water, the electrode showed a poor response of GOD in 0.1 M pH 5.0 PBS. Thus, the adsorption of GOD at Au/CPE was rather difficult due to the different structure of GOD from cytochrome *c*.

As shown in Fig. 1, a couple of stable and well-defined redox peaks for the direct electron transfer of GOD could be observed upon the cyclic sweep of the GOD/Au/CPE electrode in 0.1 M pH 5.0 PBS (curve d). The redox peak potentials were −404 and −498 mV at 50 mV/s. No peak was observable at both CPE and Au/CPE (Fig. 1 curves a and b). The presence of gold colloid resulted in a slight decrease in the background current. When GOD was immobilized on the surface of carbon paste electrode without the presence of gold colloid, the cyclic voltammogram showed a small response of GOD (Fig. 1 curve c), indicating that carbon particles were favorable to the direct electron transfer of GOD. However, the response was three times smaller than that of GOD/Au/CPE. Thus, colloidal gold played an important role in improving the GOD adsorption and facilitating the electron exchange between the GOD and carbon particles.

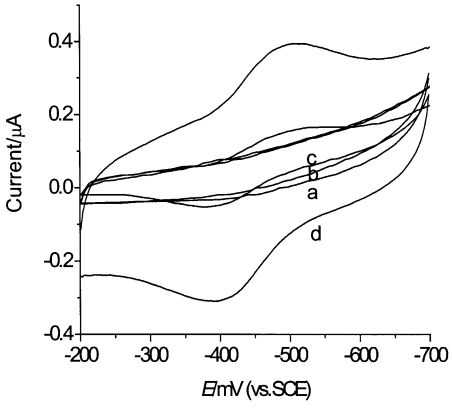

Fig. 1. Cyclic voltammograms of CPE (a) Au/CPE (b) GOD/CPE (c) and GOD/Au/CPE (d) in 0.1 M pH 5.0 PBS at 50 mV/s.

3.2. Electrochemistry of GOD adsorbed on colloidal gold modified electrode

FAD, a part of the GOD molecule, is known to undergo redox reaction where two protons and two electrons are released or taken up (Janik and Elving, 1968; Scheller et al., 1979; Ianniello et al., 1982; Savitri and Mitra, 1998). In GOD, FAD is deeply seated in a cavity and therefore not easily accessible for conduction of electrons to the electrode surface. According to the conclusion of Ianniello et al. (1982), the electrochemistry response of GOD immobilized on the heterogeneous surface is due to the redox of FAD.

At scan rates lower than 500 mV/s the anodic peak potential of the voltammogram of GOD/Au/CPE shifted to a more positive value with an increasing scan rate, while the cathodic peak potential shifted in a negative direction (Fig. 2). The redox peak currents were proportional to the scan rate in the range less than 100 mV/s (inset A in Fig. 2), indicative of a typical of the surface-controlled quasi-reversible process. From the integration of reduction peaks of GOD/Au/CPE at scan rates less than 100 mV/s, the surface coverage of GOD was calculated with the geometric area of the electrode to be $(9.8 \pm 0.6) \times 10^{-12}$ mol/cm^2.

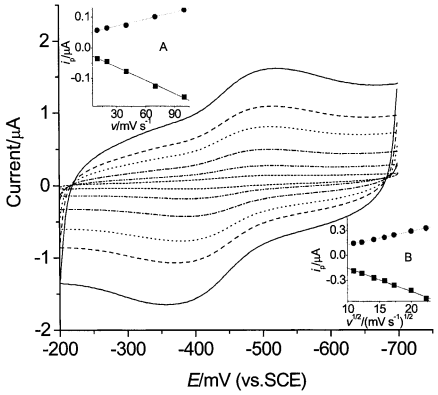

Fig. 2. Cyclic voltammograms of GOD/Au/CPE in pH 5.0 PBS at 10, 40, 100, 200, 300 and 500 mV/s (from inner to outer). (Inset) A: Plots of peak currents vs. *v*. B: Plots of peak currents vs. $v^{1/2}$.

When the scan rate was larger than 100 mV/s, the redox peak currents were proportional to the square root of scan rate, $v^{1/2}$ (inset B of Fig. 2). According to the dependence of redox peak potentials of immobilized GOD on solution pH (Fig. 3), proton participated in the redox processes of GOD. Thus, a proton gradient was produced during the electrochemical reaction at high scan rates. The involvement of the proton gradient resulted in an electron transfer process with diffusion-controlled behavior (Brown and Anson, 1978). Considering the ratio near unity for the cathodic to anodic peak currents, the electrode reaction was a typical of diffusion-controlled quasi-reversible process. The diffusion of protons from the solution to the electrode surface was the rate-limited step at the scan rates more than 100 mV/s.

The formal potential of FAD/$FADH_2$ redox couple calculated from the average of anodic and cathodic peak potentials at pH 5.0 in the scan rate range was -449 ± 1 mV, which was -544 mV at pH 7.0 and near the standard electrode potential of -0.46 V (vs. SCE) for FAD/$FADH_2$ at pH 7.0 (25 °C) (Tinoco et al., 1978), suggesting that most GOD molecules preserved their native structure after the adsorption process (Ju et al., 2002; Liu and Ju, 2002).

In the scan rate range less than 100 mV/s, the kinetics of the direct electron transfer was analyzed using the model of Laviron (1979). The plot of cathodic peak potentials vs. the logarithm of scan rates gave a charge transfer coefficient α of 0.68. The peak-to-peak separation was 70, 75, 82, 88 and 95 mV at 10, 20, 40, 70 and 100 mV/s, respectively. Considering the values of alpha and the peak-to-peak separation less than 100 mV, the electron transfer rate constant k_s was estimated to be (38.9 ± 5.3)/s according to the formula $k_s = mnFv/RT$, where m is a parameter related to the peak-to-peak separation.

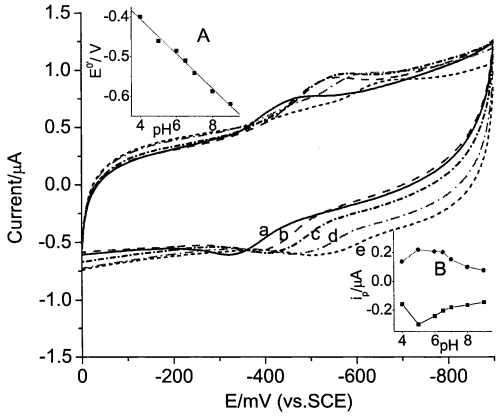

Fig. 3. Cyclic voltammograms of GOD/Au/CPE in 0.1 M pH 4.0 (a), 5.0 (b), 6.0 (c), 7.0 (d) and 9.0 (e) PBSs at 50 mV/s (Inset) A: Plot of peak potential vs. pH. B: Plots of peak currents vs. pH.

3.3. Dependence of direct electron transfer of GOD on solution pH

Cyclic voltammogram of GOD/Au/CPE showed a strong dependence on solution pH (Fig. 3). In general, all changes in CV peak potentials and currents with pH were reversible in the pH range of 5.0–7.0, that is, the same CV could be obtained if the electrode was transferred from a solution with a different pH value to its original solution. An increase in solution pH caused a negative shift in both cathodic and anodic peak potentials. Plot of the formal potential vs. pH (from 4 to 9) produced a line with the slope of $-(43.7\pm1.9)$ mV/pH ($R = 0.995$) (inset A in Fig. 3), which was close to the expected value of -58.0 mV/pH, indicating two protons and two electrons attending in the electron transfer process.

Inset B in Fig. 3 shows the effect of solution pH on the peak currents of GOD/Au/CPE. Obviously, the maximum current response occurs at pH 5.0. The irreversible decrease in peak current at pH 4.0 is due to the denaturation of protein, which results from the dissociation of FAD group between pH 4.35 and 3.55 (Kulys and Cenas, 1983). The response of GOD/Au/CPE decreases with an increasing pH from 5.0 to 9.0 is due to the increase of electron transfer distance between the protein active center and colloidal gold nanoparticles (Kulys and Cenas, 1983).

3.4. Electrocatalysis of dissolved oxygen to the electron transfer of adsorbed GOD

In the presence of dissolved oxygen, no peak was observed at Au/CPE (curve c in Fig. 4), though an increase in cathodic current at the potentials more negative than -0.4 V. At GOD/Au/CPE the shape of cyclic voltammogram for the direct electron transfer of GOD changed dramatically with an increase of reduction peak current and decrease of oxidation peak current (curves b and d in Fig. 4). A large current response occurred at the potential of -0.5 V. The

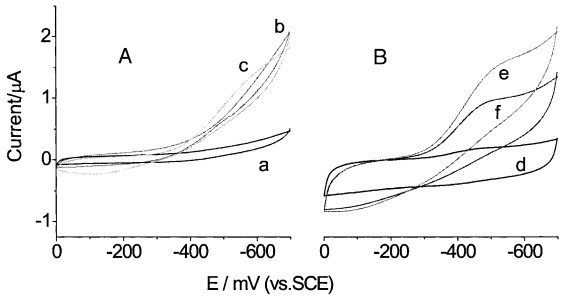

Fig. 4. Cyclic voltammograms of Au/CPE (A) and GOD/Au/CPE (B) in 0.1 M pH 5.0 PBS in the absence (a and d) and presence of dissolved oxygen (b and e) and 0.1 M pH 5.0 PBS+0.08 mM glucose in the presence of dissolved oxygen (c and f) at 50 mV/s.

difference between curve c and curve d was indicative of the electrocatalytic reduction of adsorbed GOD to dissolved oxygen according to Eqs. (1) and (2). In other words, the adsorbed GOD catalyzed the reduction of dissolved oxygen and resulted in a great increase in the reduction peak current.

3.5. Effect of glucose on the electrocatalytic process and its determination

Upon addition of β-D(+)-glucose to air-saturated PBS, the reduction current response of GOD/Au/CPE decreased (curve e in Fig. 4.), while no obvious change was observed at Au/CPE. The decrease increased with an increasing β-D(+)-glucose concentration. As well know, β-D(+)-glucose is the substrate of GOD, its presence will result in a enzyme-catalyzed reaction according to Eq. (3) and decrease the concentration of the oxidized form of GOD on electrode surface. Thus, the addition of glucose restrained the electrocatalytic reaction and led to the decrease of reduction current.

The cyclic voltammograms of GOD/Au/CPE with successive addition of β-D(+)-glucose to air-saturated 0.1 M PBS (pH 5.0) were shown in Fig. 5. With increasing β-D(+)-glucose concentration the reduction current response decreased. Inset A in Fig. 5 shows the plot of peak current decrease vs. glucose concentration. The calibration range of β-D(+)-glucose concentration was from 0.04 to 0.8 mM. The linear response range of the sensor to β-D(+)-glucose concentration was from 0.04 to 0.28 mM with a correlation coefficient of 0.997 and a detection limit of 0.01 mM at a signal-to-noise ratio of 3. The sensitivity of GOD/Au/CPE to β-D(+)-glucose was found to be 8.4 μA/mM. This value is higher than that of 3 μA/mM at Pt/PB/GOD-Pan (Garjonyte and Malinauskas, 2000).

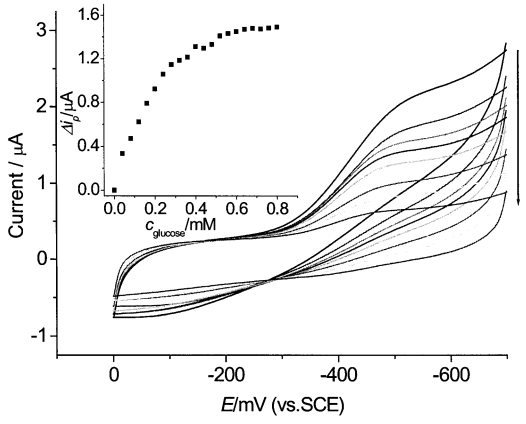

Fig. 5. Cyclic voltammograms of GOD/Au/CPE in 0.1 M pH 5.0 PBS containing dissolved oxygen and 0, 0.04, 0.08, 0.12, 0.16, 0.20, 0.28 and 0.44 mM glucose (from top to bottom) at 50 mV/s. Inset: plot of electrocatalytic current at −0.5 V vs. glucose concentration (A) and linear calibration curve (B).

3.6. Stability of GOD sensor

When the enzyme electrode was stored at 4 °C, it retained 96% of its initial current response for glucose after intermitted use over a 10 day period. Thus, the presence of colloidal gold is very efficient for retaining the enzyme activity of GOD. The sensor could keep a constant current when successively swept for 150 cycles in the presence of glucose, its response then decreased irreversibly due to the dissociation of the immobilized GOD (Kulys and Cenas, 1983). The sensor was reproducible by dropping again the GOD solution to its surface after the electrode tip was gently rubbed on a clean paper and then at a plane glass surface to produce a flat surface. The current response of the renewed surface was examined at a β-D(+)-glucose concentration of 0.20 mM. The relative S.D. was 6.9% for seven successive renewals. Thus the method was rapid, easy and, more importantly, reproducible to remove surface dissociated GOD film. The fabrication reproducibility of six electrodes, made independently, showed an acceptable reproducibility with a relative S.D.s of 7.2% for the current determined at 0.24 mM β-D(+)-glucose concentration.

3.7. Determination of glucose in serum sample

The determination of glucose in serum sample was performed on the sensor utilizing standard addition method. After the current response was determined in 5.0 ml of 0.1 M pH 5.0 PBS containing sample of 40 μl, which depended on the concentration of glucose, four 10 μl 20 mM β-D(+)-glucose solutions were successively added to the system for standard addition determination. All the concentrations of glucose in detection solutions were in the linear response range. The glucose level was determined to be 8.66 mM, close to the value of 8.74 mM obtained by spectrophotometry. The recoveries for the assay of 0.1–0.3 mM glucose were between 97.3 and 103.2% for eight detections. The interference effects were investigated by testing the amperometric response of 0.1 mM glucose in presence of uric acid or ascorbic acid with the gradually increasing concentration. 0.16 mM uric acid or 0.36 mM ascorbic acid caused an increase of 3.3 or 5.1% in reduction current. Therefore, these substances caused hardly any interference to the response of the biosensor.

4. Conclusions

GOD can be effectively immobilized on colloidal gold modified carbon paste electrode to produce a fast direct electron transfer. The immobilized GOD maintains its bioactivity and native structure. Its reduced form can be oxidized by dissolved oxygen to cause an electrocatalytic

reaction, which is restrained by glucose due to the reaction between the oxidized form of GOD and glucose. Based on the decrease of electrocatalytic response, a novel glucose sensor has been developed. The resulting sensor displays a high sensitivity (8.4 μA/mM) and a linear range from 0.04 to 0.28 mM for glucose determination, and can efficiently exclude the interference of commonly coexisted uric and ascorbic acid. Because of its convenient preparation and good properties, this biosensor can be used for glucose determination in serum sample.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (29975013, 20275017), the Science Foundation of Jiangsu (BS2001063), the Doctoral Program for Higher Education from the Education Ministry of China (200028403) and State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry.

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