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Comparative Immobilization of Antibodies on Modified Screen-Printed Graphite Electrodes

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Abstract—Immobilization of polyclonal antibodies was studied on native screen-printed graphite electrodes (SPEs) and variously modified electrodes. SPEs coated with didodecylammonium bromide (DDAB, a synthetic membranelike substance) films with gold nanoparticles gave the maximum electrochemical response. DDAB and gold nanoparticle films strongly changed the surface morphology, and the electrochemical signal became more intense and stable. This immobilization method increased the concentration of immobilized antibodies while their activity was retained. The detection limit of the enzymatic label (horseradish peroxidase) was 0.02 ng/L of sample.

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Biosensor engineering has recently become an actively studied field. The creation of highly selective immunosensors is an important line of activity in this field, because antibodies are capable of high-specific recognition of biologically active compounds. This capability makes it possible to analyze real multicomponent samples, such as blood serum, urea [1], and foodstuffs [2, 3]. Electrochemical analyses take a special place among the analytical methods used as detection systems in biosensors. Electrochemical biosensors have been developed most actively; they do not require complicated recording devices, can be used in field settings, and can be implanted into a human body for continuous monitoring of various biologically active compounds.

As a rule, the biological component in biosensors is in an immobilized state; therefore, the analytical parameters of biosensors depend on whether the properties of biomolecules are retained upon immobilization.

This work studies various methods for immobilizing specific antibodies on screen-printed graphite electrodes (SPEs). These electrodes have several strengths: a large working surface, simple design and operation, good precision, miniature size, low manufacturing cost, and high standardization. The immunological activity of immobilized antibodies was determined in their complexing with an antigen labeled with an electrochemically active label (horseradish peroxidase; HP). The model antigen used was chloramphenicol (CAP), a wide-spectrum antimicrobial antibiotic. It is widely used in veterinary science and animal husbandry against a number of infectious diseases. As a result, there is a real danger that remnant amounts of this antibiotic will appear in foodstuffs of animal origin; therefore, the development of a rapid and sensitive method for CAP determination in foodstuffs is a challenge.

MATERIALS AND METHODS

The chemicals used were didodecyldimethylammonium bromide (DDAB), $HAuCl_4 \cdot 3H_2O$, sodium borohydrides, cathechol (Sigma-Aldrich), horseradish peroxidase (RZ 3.3, Biozyme), hydrogen peroxide, ferrocyanide, acids, alkalis, casein, buffer solution components (Khimmed), and polyclonal chloramphenicol antibodies from rabbit (NVO Immunotekh). Three-probe SPEs with a graphite indicator electrode, graphite auxiliary electrodes, and a silver/silver chloride reference electrode (Elkom) were used. Chloramphenicol–horseradish peroxidase conjugate (CAP–HP) was prepared using the standard activated ester method [4]. Water deionized with Milli-Q System (Millipore) was used to prepare buffer solutions.

Spectral studies were carried out on a Shimadzu UV 1602 spectrophotometer; electrochemical measurements, on an Autolab (Eco Chemie) potentiostat equipped with GPES software. Electrochemical experiments were carried out in 0.1 M potassium phosphate buffer (pH 7.4); the indicator electrode diameter was 2 mm. Cyclovoltammograms (CVs) were recorded at scan rates of 10 to 100 mV/s. The parameters used in square-wave voltammetry (SWV) were as follows: the initial potential, 200 mV; final potential, 700 mV (for reduction processes); potential step, 5 mV; ampli-

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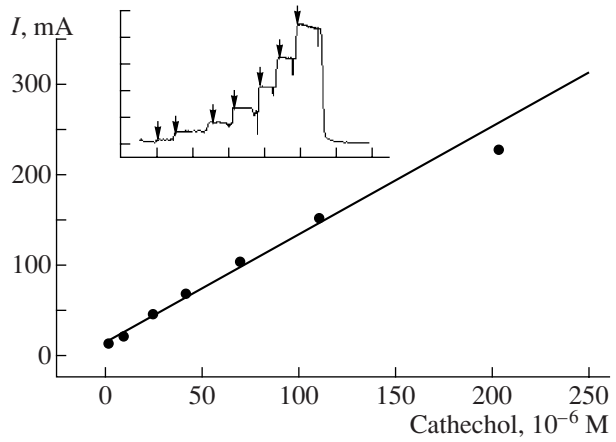

Fig. 1. Electrochemical current vs. cathechol concentration in a flow-through cell.

tude, 20 mV; and frequency, 10 to 100 Hz. The parameters used in differential pulse voltammetry (DPV) were as follows: the pulse amplitude, 25 mV; initial potential, 200 mV; final potential, 700 mV; potential step, 1 mV; and pulse duration, 50 ms. All measurements were carried out at room temperature.

Modification of SPEs by gold nanoparticles and DDAB. A DDAB-stabilized colloidal gold solution was synthesized as follows: to 1 mL of 0.1 M DDAB solution in chloroform, 0.5 mL of 10 mM $HAuCl_4 \cdot 3H_2O$ solution in water was added with stirring. Next, 0.2 mL of a fresh 0.4 M $NaBH_4$ solution in water was slowly added with vigorous stirring. After 2 h, a colored organic layer was separated and washed with an equal volume of water. The DDAB-stabilized colloidal gold solution in chloroform was characterized using absorption spectroscopy: $\lambda_{max} = 520$ nm [5, 6]. The gold nanoparticle concentration in 0.1 M DDAB in chloroform was calculated according to the reaction stoichiometry (5 mM).

Anodic oxidation of SPEs lasted 5 min in an electrochemical cell containing acetate buffer (the potential was 5 mV).

Antibody immobilization on SPEs. 2 μL of 5 mM colloidal gold solution in 0.1 M DDAB in chloroform was spread over the surface of the indicator electrode. After chloroform vaporized (which took 10 min), 2 μL of an antibody solution was applied. The electrodes were left for 12 h at 4°C in a moist cell to keep them from completely drying. Then, the electrodes were rinsed with PBS and PBST solutions in a flow-through system for 5 min; the flow rate was 1 mL/min. To inhibit nonspecific binding, the electrode was incubated for 30 min in 0.5% casein at 37°C. A CAP–HP solution in PBS was passed in order to study antibody complexing with the antigen. After rinsing, the electrode was placed into the electrochemical cell, and the electrochemical activity of HP on the electrode surface was determined while passing the substrate (0.5 mM hydrogen peroxide) and mediator (0.1 mM cathechol). The peak electrochemical current was used as the analytical signal.

SEM Characterization of electrode surfaces. Electrode surfaces were characterized using a Stereoscan-240 (Cambridge Instruments) scanning electron microscope.

RESULTS AND DISCUSSION

The need to develop highly sensitive analyses for monitoring various compounds in real objects entails the need to create analytical devices with high sensitivity, precision, and operational stability. Important problems in immunosensor engineering are the discovery of immobilization techniques that would provide the necessary concentration of a biorecognition element on the electrode surface and the retention of the ability of antibodies to complex with antigens.

We used several approaches to immobilize specific antibodies on graphite electrode surfaces: physisorption on both untreated electrodes and electrodes after anodic oxidation, as well as immobilization on electrodes modified by a membranelike synthetic substance and gold nanoparticles. Scanning electron microscopy was used to characterize electrode surfaces [7].

The activity of immobilized antibodies was determined as the electrochemical response after complexing immobilized antibodies with CAP-HP. In this case, immune complexes on the electrode surface can be detected by the appearance of an enzymatic activity of the label (HP), which is directly proportional to the concentration of surface immune complexes. First, the parameters of the electrochemical detection of HP on SPEs were optimized. Using antibodies of a rather large size as the recognition element of biosensors, we failed to implement direct electron transfer from the biocatalyst (peroxidase) to the electrode surface; therefore, we used a mediator (cathechol). The current versus mediator concentration calibration curve was as shown in Fig. 1. There was an optimal mediator concentration providing the maximal signal. In an excess of the mediator, the signal dropped because of the inactivation of immobilized proteins. Similar tendencies were observed for hydrogen peroxide (the peroxidase substrate). Thus, we found the optimal concentrations of the reagents (substrate and mediator) for determining the activity of peroxidase conjugated to chloramphenicol.

Figure 2 displays the SEM images of the surfaces of native electrodes and electrodes with immobilized antibodies. The surface morphology was virtually the same for a native graphite electrode and an electrode modified by anodic oxidation (Figs. 2a, 2b). Antibody physisorption did not markedly alter the surface morphology (Fig. 2c). This is an indication of a small protein surface coverage. Figure 3 presents the results of the electrochemical detection of antibody complexing with CAP–HP on unmodified electrodes and electrodes after

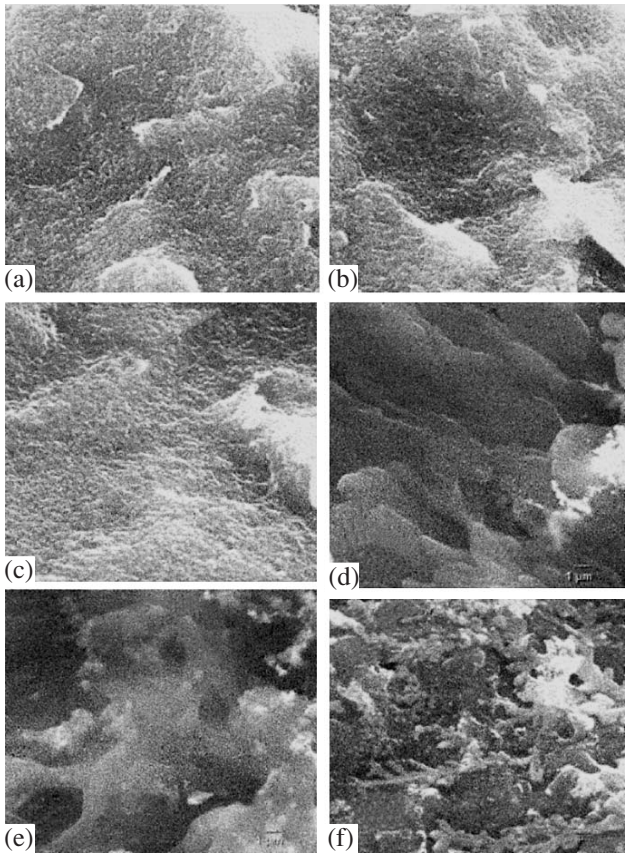

Fig. 2. SEM images of SPE surface structures: (a) SPE, (b) SPE after anodic oxidation, (c) SPE after anodic oxidation with immobilized antibodies, (d) SPE treated with DDAB and colloidal gold, and (e, f) SPE treated with DDAB and colloidal gold (e) with immobilized antibodies and (f) conjugate.

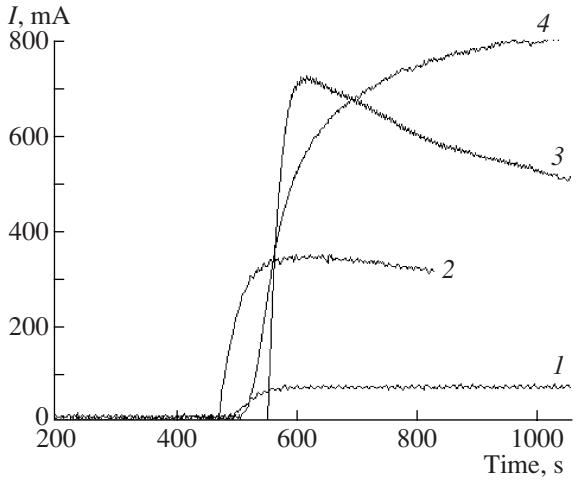

Fig. 3. Electrochemical response of the immunosensor prepared using various methods of antibody immobilization on graphite electrode surfaces: (*1*) SPE, (*2*) SPE with anodic preoxidation, (*3*) DDAB-treated SPE, and (*4*) SPE treated with a solution of DDAB and colloidal gold.

anodic oxidation. Anodic preoxidation of the electrode increases the electrochemical response of the sensor almost threefold.

To improve the ability of graphite to bind antibodies, the electrode surface was treated with DDAB [8, 9]. Membranelike substance are known to form a stable lyotropic liquid-crystalline film on the electrode surface, this film containing amounts of water sufficient for proteins to retain their structure and activity. The use of such films for immobilization of protein molecules makes it possible to employ milder adsorption parameters and to conserve the native conformation of proteins and, thus, their maximal activity. It was shown earlier that DDAB is an efficient means for improving the electrochemical properties of immobilized proteins. The immobilization of proteins or other macromolecules very frequently causes partial denaturing, which generates an insulation layer and inhibits electron transport [10, 11]. The addition of colloidal gold particles to DDAB solution partially solves this problem; colloidal gold acts as a bridge for electron transport from the protein to the electrode surface [12, 13].

Figures 2c and 2d show the surface structures of DDAB-modified electrodes and the electrodes with antibodies immobilized in DDAB and DDAB + gold nanoparticle films. DDAB markedly alters the surface morphology; addition of gold nanoparticles generates a relatively smooth film, which changes its parameters after the immobilization of antibodies and CAP–HP (Figs. 2e, 2f). In this case, we can speak of a substantial increase in the amount of active immobilized protein on the electrode surface.

The table compiles the maximal electrochemical responses and signal stability data for electrodes with antibodies immobilized by various techniques. The highest signal and high operation stability are characteristic of the electrodes with antibodies immobilized in DDAB + gold nanoparticle films. The detection limit for HP in CAP–HP on the electrode was 0.02 ng/mL

Analytical parameters of SPEs with antibodies immobilized by various methods

Immobilization method/parameter	Adsorption on graphite	Adsorption on graphite pretreated by anodic oxidation	Immobilization in a DDAB film	Immobilization in a DDAB + gold nanoparticle film
I_{max}, nA	100	340	750	790
Residual activity after 1 h, %	98	75	70	95

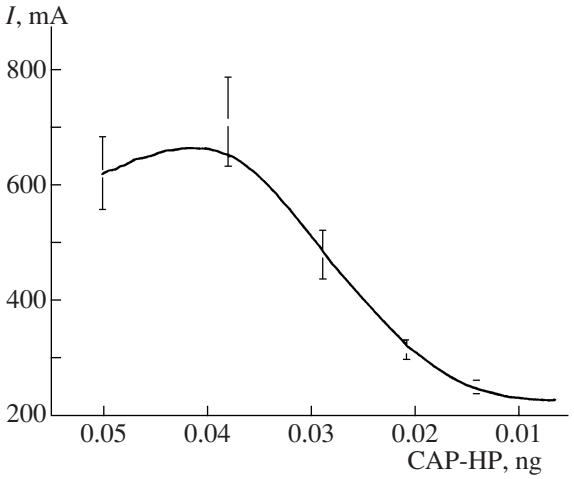

Fig. 4. Calibration curve for the determination of HP in the protein conjugate in a flow-through cell containing cathechol and hydrogen peroxide.

sample. The calibration curve for the determination of HP conjugated to the protein in a flow-through cell containing cathechol and hydrogen peroxide was as shown in Fig. 4.

In summary, the modification of SPEs with DDAB + gold nanoparticle films produces a system sufficiently sensitive for use in engineering highly sensitive immunosensors with peroxidase as a label.

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