Chapter 11

Methods for the Preparation of Electrochemical Composite Biosensors Based on Gold Nanoparticles

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Summary

Methods for the construction of electrochemical composite biosensors using gold nanoparticles and Teflon as nonconducting-binding material are described in detail. The advantages of the incorporation of gold nanoparticles to the composite electrode matrices are highlighted, giving rise to bioelectrodes with improved analytical performance in terms of stability and sensitivity with respect to other biosensor designs. Three different biosensors have been considered: a tyrosinase biosensor in which the enzyme and gold nanoparticles are incorporated into graphite–Teflon composite electrode matrices by simple physical inclusion, a progesterone immunusensor in which the antibody is directly attached to the electrode surface and amperometric transduction is carried out at a colloidal gold–graphite–Teflon–tyrosinase composite biosensor, and a mediator-less glucose oxidase biosensor constructed by bulk incorporation of the enzyme into colloidal gold-multiwall carbon nanotubes–Teflon composite electrodes.

Key words: Electrochemical biosensors, Gold nanoparticles, Enzyme biosensors, Immunosensors, Composite electrodes, Carbon nanotubes.

1. Introduction

Nowadays, the construction of electrochemical biosensors based on the use of gold nanoparticles constitutes an intensive research area because of the unique advantages that this nanomaterial lends to biosensing devices. So, gold nanoparticles provide a stable surface for immobilization of biomolecules with no loss of their biological activity. Moreover, they facilitate direct electron transfer between redox proteins and electrode materials, and constitute useful interfaces for the electrocatalysis of redox processes of molecules such as H_2O_2 or NADH involved in many biochemical reactions *(1, 2)*.

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Also, the incorporation of nanomaterials into composite electrodes has been demonstrated to be an extremely useful strategy to construct electrochemical biosensors with an improved analytical performance. These biosensors exhibit both the characteristics of the involved nanomaterials and the advantages derived from the use of composite electrodes, such as their regeneration ability and the great versatility that they offer as a consequence of the feasible incorporation of different substances into the bulk of the electrode matrix by simple physical inclusion. Although there are several antecedents in the literature in which the advantageous features of colloidal gold and carbon paste electrode fabrication have been combined *(3)*, this chapter is focussed on composite electrode matrices constructed using Teflon as nonconducting-binding material. Details on the preparation of several electrochemical biosensors following this methodology as well as on their analytical performance and applicability are given below.

2. Materials

2.1. Composite Electrode Matrices

1. Graphite powder (99.997% purity; Goodfellow, Cambridge Ltd., Huntingdon UK).
2. Teflon (poly(tetrafluorethylene) powder (> 40 µm, Aldrich, Saint Louis, Missouri, USA).
3. Multi-wall carbon nanotubes (MWCNTs, 30 ± 15 nm ø), with a 95% purity (NanoLab, Brighton, MA).

2.2. Colloidal Gold

1. $HAuClO_4$ $3H_2O$ (>49% as Au, Sigma, Saint Louis, Missouri, USA). A 1% (w/w) solution was made with Milli-Q water.
2. Sodium citrate (99.0% purity, Sigma). A 1% (w/v) solution was prepared in Milli-Q water.
3. Water is obtained from a Milli-Q purification system (Millipore, Bedford, NA, USA).

2.3. Tyrosinase Biosensor

1. Tyrosinase, Tyr (from mushroom, EC.1.14.18.1, activity of 2,590 units per mg of solid) (Sigma). The enzyme was used as received.
2. Graphite and Teflon powder are the same described in **Subheading 2.1**.
3. Catechol (Sigma, 99%), phenol (Sigma, 99%) 3,4-dimethylphenol (Aldrich, 98%), 4-chloro-3-methylphenol (Aldrich, 99%), 4-chlorophenol (Aldrich, ≥99%), 4-chloro-2-methylphenol (Aldrich, 99%), 3-methylphenol (Aldrich, 99%), and

4-methylphenol (Aldrich, 99%). 1.0×10^{-2} M solutions of cathecol, phenol, 4-chlorophenol, and 4-chloro-2-methylphenol are prepared in 0.1 M phosphate buffer of pH 7.4 (prepared from $NaH_2PO_4\,2H_2O$ and Na_2HPO_4, ≥99%, Scharlab, Barcelona, Spain). 1.0×10^{-2} M solutions of 3,4-dimethylphenol, 4-chloro-3-methylphenol, 3-methylphenol, and 4-methylphenol are prepared in methanol (Scharlab, gradient HPLC grade, 99.99%). More diluted standards were prepared by suitable dilution with the 0.1 M phosphate buffer solution.

2.4. Progesterone Immunosensor

1. Monoclonal anti-progesterone (anti-Prog) (clone 2H4 rat cell culture supernatant, Sigma). A 0.01 mg/mL solution is prepared in Tris (tris(hydroxymethyl)aminomethane) (Sigma, 99%) buffer solution of pH 7.0.
2. Progesterone labeled with alkaline phosphatase (AP-Prog) (Ridgeway Science Ltd., Alvington, Gloucestershire, UK) was prepared by reaction of 9 mg of alkaline phosphatase with 0.5 mg progesterone glucoronide in a 2 mL final volume.
3. Unlabeled progesterone (Prog) (Aldrich, 98%): A stock 1 mM solution of progesterone is prepared in methanol (Scharlab, gradient HPLC grade, 99.99%) and stored at 4°C. More diluted solutions are prepared daily by dilution with 0.1 M Tris buffer solution of pH 7.0.
4. Tyrosinase, Tyr (from mushroom, EC.1.14.18.1, activity of 2,590 units per mg of solid) (Sigma).
5. Phenyl phosphate disodium salt dihydrate (Sigma, 98%): A 0.01 M phenyl phosphate solution is prepared in 0.1 M Tris buffer of pH 7.0.
6. Phenol (Merck, 99%) 0.01 M phenol solutions are prepared daily in 0.1 M Tris (pH 7.0) buffer solution.
7. Bovine serum albumin (BSA) (97%) and magnesium chloride (99%) are all from Merck.
8. Graphite and Teflon powder are the same as described in **Subheading 2.1**.

2.5. Glucose Oxidase Biosensor Based on Colloidal Gold–Carbon Nanotubes

1. Glucose oxidase, GOx (Sigma, 151,000 U/g solid).
2. Teflon powder is the same mentioned in **Subheading 2.1**.
3. Glucose (>99%, Panreac, Barcelona, Spain). Stock 0.5 M solutions are prepared in 0.05 M phosphate buffer, pH 7.4, and let to stand overnight before using to allow equilibration of anomers. More diluted standards were prepared by suitable dilution with the same phosphate buffer solution.

2.6. Apparatus

1. Electrochemical measurements with the tyrosinase biosensor and the progesterone immunosensor are carried out with a

PGSTAT 12 potentiostat from Autolab (EcoChemie B.V., Utrecht, The Netherlands). The electrochemical software was the general-purpose electrochemical system (GPES).

2. Amperometric measurements in stirred solutions with MWCNTs-colloidal gold composite biosensors were carried out using a PalmSens Electrochemical Sensor Interface (Palm Instruments BV, Houten, The Netherlands) controlled by a Pocket PC Software.
3. A three-electrode cell (a BAS VC-2 10-mL glass electrochemical cell) consisting of a platinum wire counter electrode (BAS MW-1032), a BAS MF-2063 Ag/AgCl/3M KCl reference electrode, and the corresponding composite electrode as the working electrode, was used. All experiments were performed at room temperature.

3. Methods

3.1. Preparation of Colloidal Gold Dispersions

1. 2.5 mL of the 1% sodium citrate solution are added to 100 mL of a boiling aqueous solution containing 1 mL 1% (w/w) $HAuCl_4$.
2. The mixture is allowed to boil for 15 min.
3. Heating is stopped and the mixture is stirred by hand with a glass rod for 15 min.
4. Colloidal gold suspension is left to reach room temperature and stored in dark glass bottles at 4°C until use. The diameter of gold nanoparticles is 16 ± 2 nm.

3.2. Preparation of the Au_{coll}–Graphite–Teflon Electrodes

A scheme of the fabrication procedure of composite colloidal gold–graphite–Teflon electrode matrices is depicted in **Fig. 1**.

1. Graphite (150 mg) and colloidal gold (900 µL) suspension prepared as described above are thoroughly mixed using a magnetic stirrer (Selecta, Barcelona, Spain) for 2 h in a dark glass flask.
2. Water is evaporated under air current at room temperature.
3. Teflon powder (450 mg) is added and thoroughly mixed by hand with a glass rod until a homogeneous mixture is obtained.
4. The mixture is pressed into pellets by means of a Carver pellet press (Perkin Elmer, Norwalk, CT, USA) at 10,000 kg/cm for 10 min. Pellets are 1.3 cm diameter and around 0.4 cm thick.
5. From each main pellet, several (five or six) 3.0 mm diameter cylindrical portions are bored with a drill. Each portion constitutes a different composite electrode.

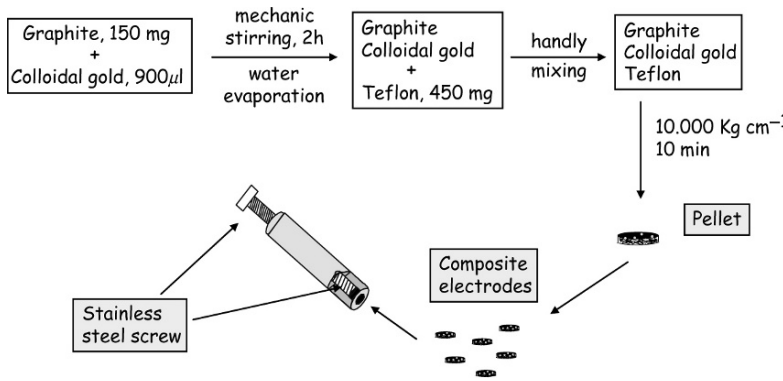

Fig. 1. The procedure of fabrication of composite Au_{coll}–graphite–Teflon electrodes.

6. Each electrode is press-fitted into a Teflon holder.
7. The electrical contact was made through a stainless steel screw.

3.3. Tyrosinase Biosensor Based on a Composite Au_{coll}–Graphite–Teflon Electrode

A tyrosinase bioelectrode with improved stability and sensitivity with respect to other biosensor designs is constructed by incorporation of the enzyme and gold nanoparticles into graphite–Teflon composite electrode matrices by simple physical inclusion. Amperometry at –0.10 V vs. Ag/AgCl for different alkyl and chlorophenols allows the achievement of detection limits of 3 nM for catechol and approximately 20 nM for the rest of phenolic compounds. This high sensitivity is attributed to the presence of colloidal gold in the composite matrix, which gives rise to enhanced kinetics of both the enzyme reaction with tyrosinase and the electrochemical reduction of the corresponding *o*-quinones at the electrode surface. Moreover, the simple renewability of the electrode surface by polishing, which is an inherent characteristic of the rigid composite bioelectrodes, coupled with the gold nanoparticles ability to attach proteins retaining their biological activity permit the fabrication of reusable and reproducible biosensors exhibiting a lifetime of at least 39 days without apparent loss of the immobilized enzyme activity *(4)*. The composite biosensor was applied to the estimation of the phenolic compounds content in water samples of different origin.

3.3.1. Preparation of the Tyr–Au_{coll}–Graphite–Teflon Biosensor

1. **Steps 1 and 2** of **Subheading 3.2** are carried out.
2. 34.75 mg tyrosinase and 400 µL of the 0.1 M phosphate buffer solution of pH 7.4 are incorporated to the mixture by stirring during 2 h with a magnetic stirrer (Selecta) in an ice bath.
3. The resulting homogeneous mixture is dried by passing an Ar stream.
4. Teflon (415.25 mg) is added and thoroughly hand mixed using a glass rod until a homogeneous mixture is obtained.

The final Teflon percentage is of 70% and provides suitable mechanic and conducting characteristics to the composite material.

5. **Steps 4–7** of **Subheading 3.2** are accomplished.

3.3.2. Monitoring of Phenolic Compounds in Water Samples

1. Aliquots of 200 µL of undiluted samples are directly added to the electrochemical cell containing 10 mL of 0.1 M phosphate buffer solution of pH 7.4.
2. Amperometric measurements under constant stirring conditions are performed with the Tyr–Au_{coll}–graphite–Teflon biosensor by applying a potential of –0.10 V and allowing the steady-state current to be reached.
3. The analysis of samples was carried out by applying the standard additions method by injecting appropriate volumes of 1.0×10^{-4} M phenol stock solutions prepared in the above-mentioned phosphate buffer.
4. The content of phenolic compounds is expressed as a concentration of phenol.

3.4. Progesterone Immunosensor Using a Tyrosinase–Au_{coll}–Graphite–Teflon Biosensor as Amperometric Transducer

The synergic combination of the advantages provided by gold nanoparticle nanostructured biosensors and composite electrode matrices into which gold nanoparticles are incorporated by simple physical inclusion allows the development of a progesterone immunusensor in which the antibody is directly attached to the electrode surface, and amperometric transduction is carried out at a colloidal gold–graphite–Teflon–tyrosinase composite biosensor (see procedure below) *(5)*.

The immunosensor functioning is schematized in **Fig. 2**, and it is based on a sequential competitive assay between progesterone (Prog) and the antigen-labeled with the enzyme alkaline phosphatase (AP-Prog) for the binding sites of the immobilized antibodies (anti-Prog). Antibodies are preferentially linked to gold nanoparticles at the electrode surface as a consequence of the gold nanoparticles ability to adsorb proteins retaining their biological activity. Phenyl phosphate is employed as the alkaline phosphatase-substrate, generating phenol which is catalytically oxidized by tyrosinase to the *o*-quinone. This quinone is electrochemically reduced at –0.10 V at the tyrosinase–colloidal gold composite electrode.

As it has been commented in **Subheading 3.3**, the presence of gold nanoparticles in the composite electrode matrix allows a high sensitivity for the detection of phenol. The calibration graph for progesterone showed a linear range between 0 and 40 ng/mL, with a detection limit of 0.43 ng/mL. Furthermore, the immunosensor lifetime is of 14 days without need of a regeneration procedure. The practical usefulness of the immunosensor was demonstrated by determining progesterone in spiked milk samples

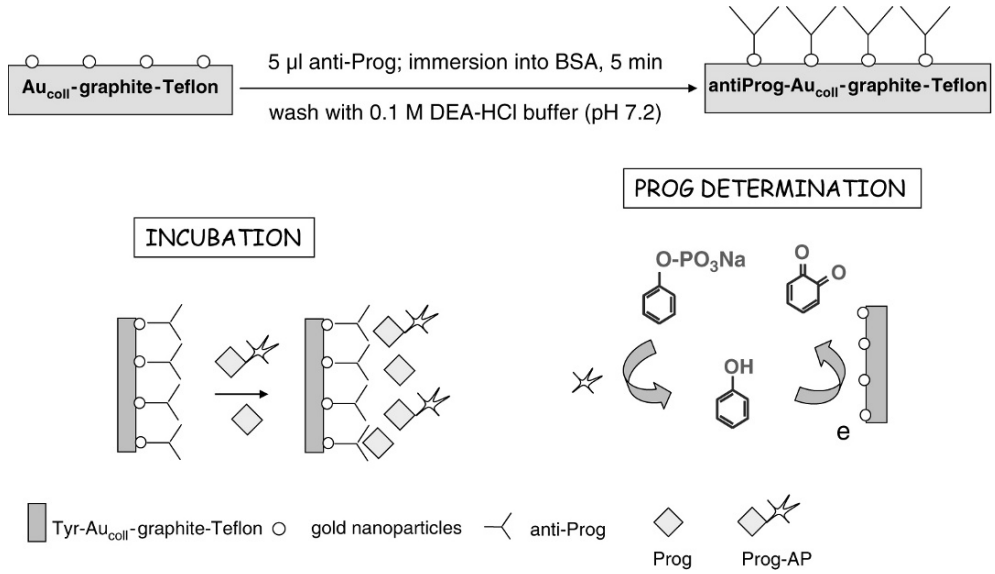

Fig. 2. The progesterone immunosensor using a colloidal gold–graphite–Teflon–tyrosinase composite biosensor as amperometric transducer.

at the 5.0 and 1.5 ng/mL concentration levels. Using the very simple experimental procedure described below, mean recoveries (n = 7) of 98 ± 3% and 99 ± 3%, respectively, were obtained.

3.4.1. Preparation of the Anti-Prog–Tyr–Graphite–Teflon Immunosensor

1. A Tyr-Au$_{coll}$-graphite-Teflon electrode is constructed as described in **Subheading 3.3**.
2. A 5.2-µL aliquot of the 0.01 mg/mL anti-Prog solution in Tris is deposited on the surface of the Tyr–Au$_{coll}$–graphite–Teflon composite biosensor and allowed to dry at air under room temperature.
3. The bioelectrode is immersed for 10 min into a 2% (w/w) BSA solution in 0.1 M Tris buffer of pH 7.0.
4. The biosensor is washed carefully with 0.1 M Tris buffer solution of pH 7.0.
5. The resulting anti-Prog-(Tyr-Au$_{coll}$-graphite–Teflon) immunosensor is incubated in 10 mL of the analyte Prog solution for 30 min.
6. The immunosensor is subsequently incubated in 10 mL of the AP-Prog solution mentioned in the **item 2** of **Subheading 2.4** during 40 min.

3.4.2. Determination of Progesterone with the Anti-Prog–Tyr–Au$_{coll}$–Graphite–Teflon Immunosensor

1. The immunosensor is immersed into the electrochemical cell containing 10 mL of the 0.1 M Tris buffer of pH 7.0 and 1 mM $MgCl_2$.
2. 20 µL of the 0.01 M phenyl phosphate solution are added to the electrochemical cell.

3. Amperometric measurements under constant stirring conditions of the *o*-quinone reduction current were carried out at −0.10 V vs. Ag/AgCl and allowing the steady-state current to be reached.

3.4.3. Determination of Progesterone in Milk with the Anti-Prog–Tyr–Au_{coll}–Graphite–Teflon Immunosensor

1. The immunosensor is immersed in the spiked untreated milk sample for 30 min.
2. Subsequently, it is incubated in the AP-Prog solution during 40 min.
3. Amperometry at −0.10 V is used to monitor the affinity reaction.
4. A calibration graph obtained with spiked milk that does not contain detectable endogenous progesterone is employed for progesterone quantification.

Glucose Oxidase Biosensor Based on Colloidal Gold–Carbon Nanotubes

Hybrid nanoparticles/nanotubes materials have shown to possess interesting properties, which can be profited for the development of electrochemical biosensors. In particular, gold nanoparticles–CNTs hybrids constitute biocompatible materials with important electroanalytical features because of the coupling of the above-mentioned capabilities of gold nanoparticles with the electrocatalytic ability of carbon nanotubes toward the electrooxidation of several molecules of biochemical interest. The bulk incorporation of glucose oxidase into colloidal gold–carbon nanotubes composite electrodes using Teflon as the binder allows the construction of a mediator-less glucose oxidase biosensor, GOx–Au_{coll}–MWCNTs–Teflon, which exhibits improved analytical performance with respect to previous biosensor designs using carbon nanotubes. The biosensor is applied for the rapid determination of glucose in beverages recommended for sport practice *(6)*.

Preparation of Glucose Oxidase–Au_{coll}–MWCNTs–Teflon Biosensors

The experimental procedure for preparation of glucose oxidase–Au_{coll}–MWCNTs–Teflon biosensors is schematized in **Fig. 3**. The successive steps are the following:

1. A colloidal gold dispersion is prepared as described in **Subheading 3.1**.
2. Colloidal gold (360 µL) is added to 50 mg of solid MWCNTs in a glass vessel and thoroughly hand mixed for 10 min with a glass rod.
3. Water is evaporated under N_2 current.
4. Glucose oxidase (1 mg) is incorporated to the composite mixture by stirring with a magnetic stirrer (Selecta) for 20 min.
5. Teflon is incorporated to the mixture by adding 50 mg Teflon powder and thoroughly hand mixing until complete homogenization.

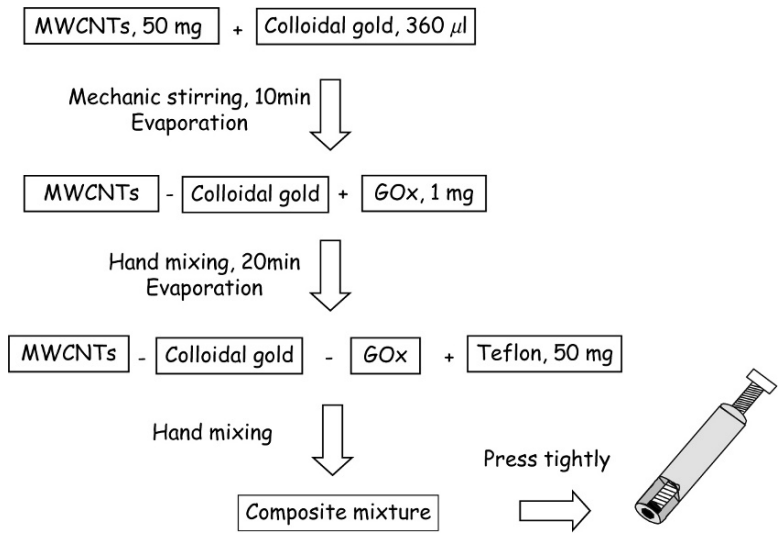

Fig. 3. The procedure for the construction of glucose oxidase–Au_{coll}–MWCNTs–Teflon biosensors.

6. Portions of the resulting mixture are packed into Teflon holders (3 mm inner diameter) and pressed tightly.
7. The electrical contact is made through a stainless steel screw.

3.4.4. Determination of Glucose in Commercial Beverages Recommended for Sport Practice

1. Aliquots of the samples are appropriately diluted with 0.05 M phosphate buffer solution of pH 7.4, and transferred to the electrochemical cell containing the bioelectrode.
2. Amperometry in stirred solutions at +0.5 V vs. Ag/AgCl is employed allowing the steady-state current to be reached.
3. Samples analysis is carried out by applying the standard additions method which involved successive addition of 25 μL of a 2.5×10^{-4} M glucose solution.

4. Notes

1. All glassware used in the preparation of the colloidal gold dispersions must be carefully cleaned in a bath of a freshly prepared 3:1 HNO_3 + HCl solution, and then thoroughly rinsed with Milli-Q water, previously filtered through a 22-μm microporous Nylon membrane filter (Scharlab), and dried in air.
2. Observation of color changes produced after addition of sodium citrate to the $HAuCl_4$ solution in the procedure used for the preparation of colloidal gold is an appropriate way to verify the adequate progress of the reaction. Initially (i.e.,

before sodium citrate addition), the tetracholoroauric acid aqueous solution is yellow. Color changes to purple upon addition of sodium citrate solution, and then it slowly changes to cherry red. This final color indicates that a correct colloidal gold dispersion is obtained.

3. An inherent advantage of the biosensor designs based on the use of rigid composite electrodes is the possibility of surface renewability. This regeneration treatment is performed by simple polishing of the electrode surface for approximately 5 s on a 150 grit SiC paper.

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