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Recent advances in Third Generation Biosensors based on Au and Pt Nanostructured Electrodes

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ABSTRACT

This review presents recent advances in Au and Pt nanostructured-based electrochemical biosensors belonging to the third generation. We will list different routes to modify electrodes with Au and Pt nanoparticles and nanoporous films in more detail. Also, the advantages of such nanostructured electrodes on the electron transfer properties of hemoglobin, laccase, cytochrome c and peroxidase will be summarized. Future perspectives in this emerging field will be discussed in the conclusion part.

Contents

1. Introduction

Nowadays, third generation biosensors, based on the *direct electron transfer* (DET) between redox proteins and the electrode, are gaining increasing attention [1]. Some advantages are the simpler design as compared to biosensors using redox mediators [2], the independence from O_2 content in solution [3] and the reduction of interfering species [4]. Enzymes exhibit a relatively fast electron-transfer process in homogenous systems, conversely when they are confined onto the electrode surface the rate of electron transfer is lowered or missing. This is due to the deep burying of their electroactive center and the enzyme denaturation onto flat metal surfaces [5].

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Au and Pt nanostructured surfaces with both *nanoparticles* (NPs) [6] and nanoporous layers [7] can overcome these issues. They have shown excellent electrochemical and conductivity properties that speed up the rate of electron transfer [8]. Thanks to their high surface-to-volume ratio, the enzyme loading remains high, and a strong retention of the protein lifetime and native-like structure has been widely proven [6]. NPs and nanopores have the same size of enzymes resulting in more freedom in the redox protein orientation. This property minimizes the distance between the electrochemically active center of enzymes and the electrode surface [6].

Taking into account the before-mentioned considerations, the objective of this review is to discuss selectively the latest advances on the use of Au and Pt nanostructured electrodes for enhancing the electron transfer between the enzymes and the electrode. The principal approaches employed in the surface modifications with NPs and nanoporous coatings made of Au and Pt will be described. Then, the electrochemical characteristics of electron transfer of the four enzymes (hemoglobin, laccase, cytochrome c and horseradish peroxidase) at such modified electrodes will be illustrated.

2. Au and Pt nanoparticle-based electrodes

In the last decade, Au and Pt NPs have been widely employed in catalysis because of two main advantages: a high surface to volume ratio and an enhanced catalytic effect (selectivity, electroactivity) as compared to bulk material counterparts. In the following subsections, we summarize the most important physical and chemical methods to form a homogeneous layer of NPs fully covering the electrodes.

2.1. Electrodeposition

Electrodeposition is a fast and well-controlled technique for confining NPs onto electrodes. It includes the application of a controlled voltage or current (fixed or variable). The nucleation of nanostructures on the electrode substrate during electrodeposition is strongly influenced by the electrode surface characteristics. Initially, all the nuclei form instantaneously on the electrode substrate, and then grow and increase in number with the electrodeposition time. The cell contains three electrodes: reference, working and counter electrode [9]. Li and coworkers [10] studied the DET between *hemoglobin* (Hb) and Au NPs synthetized by this method. They obtained differently shaped (flower-like, spherical, and convex polyhedron) and sized (hundreds of nm) Au NPs by a one-step electrodeposition only changing the applied voltages. A possible way to have a better control and a narrow distribution of the NP size is the deposition *via* a variable voltage as reported in ref. [11].

2.2. Self-assembly monolayer (SAM) and Layer-by-layer (LbL)

An interesting approach to immobilize Au and Pt NPs on electrode surfaces is to assemble them *via* thiol/amines bonds onto a variety of substrates (Fig. 1a). For instance, Feng et al. [6] synthesized chitosan-stabilized Au NPs and immobilized them on cysteine-modified Au electrodes to fabricate some heme protein-based biosensors. Other researchers [12] immobilized HRP on Au NPs/*poly(diallyl dimethyl ammonium chloride)* (PDDA) self-assembled on a *glassy carbon electrode* (GCE) thanks to the aggregation of the two molecules with opposite charge (positively charged PDDA and negatively charged Au-NPs). More complex Au and Pt NP architectures have been obtained by *layer-by-layer* (LbL) self-assembly of NPs and other materials on electrodes. Liu [13] reported a modification of Nafion-coated Au electrodes with cysteine. The colloid Au NPs and HRP were deposited multiple times. Another approach used by Zhang [14] and Yuan [15] was to deposit negatively charged MWCNTs on GCE before successive depositions of Hb and Au NPs.

2.3. Polymer and dendrimer-encapsulation

The most widely used methods for developing electrochemical sensors including Au and Pt NPs is to embed them in polymers and/or dendrimers (Fig. 1b). Chitosan is a well-known polymer used for its excellent film-forming ability with good adhesion, water permeability, chemical stability and biocompatibility. Chitosan is a polysaccharide polymer with a high content of ammine residues that make it ideal for functionalization with NPs [16,17]. Dendrimers are nanometer-scaled star-shaped macromolecules with branches exposing surface functional groups. Rahman and coworkers [18] used dendrimers to capture Au NPs among branches and then the carboxylic groups of the branches were used to bind covalently a laccase *via* amide bonds with the amine groups of the enzyme. However, the small conductivity of chitosan and dendrimers can limit their application in biosensors. Conductive polymers are a valid alternative to chitosan and dendrimers as matrix to entrap NPs because of their higher electrical conductivity [19].

2.4. Sol-gel

An attractive method to prepare Au and Pt NP-based electrodes consists in preparing a dispersion of NPs, mixing it with a sol and finally casting the mixture on the electrode surfaces. Generally, the sol-gel is based on processes of controlled hydrolysis, condensation or polycondensation of compounds, usually alkoxides $M(OR)_x$ (e.g., M = Si [20,21]), in aqueous or organic media (e.g., alcohol). These reactions form colloidal suspensions of particles (sol). Then, the particles interconnect producing a porous and rigid structure (gel). Sol-gel methods have been widely used to fabricate biosensors because of the easy manufacturability under ambient conditions, the chemical and terminal stability, the high porosity and the negligible swelling of the resulting network when exposed to aqueous environments. However, one should take into account the scarce conductivity that limits its application for biosensing. Some authors have used the sol-gel technology entrapping NPs with self-assembly. Di [20] and Jia [21] used (3 -mercaptopropyl)-trimethoxysilane (MPS) sol-gel solution to form three-dimensional silica gel entrapping Au NPs chemisorbed onto the -SH groups of the sol-gel network (Fig. 1c). The resulting sol-gel contained -SH groups and formed a self-assembly on Au electrode. Finally, the enzyme was immobilized on Au NPs by simple adsorption. Coupling sol-gel-NPs and self-assembly technologies has some advantages. First, thiol-containing silica gel forms a three-dimensional network that can be assembled onto a Au electrode and provide many anchoring points for NPs. Second, self-assembly of sol-gel nanocomposite on Au electrode creates the necessary conditions to realize conduction-pathways from the enzymes or proteins to the electrode surface. Third, a high loading of enzymes is possible on Au NPs entrapped in sol-gel network with good stability over time.

2.5. Dispersion in ion liquids (ILs)

Ionic liquids (ILs) are salts containing poorly coordinated ions that end-up in a liquid state generally below 100°C and sometimes even at room temperature. One component is a nitrogen-containing cation with a delocalized charge and the other is an inorganic or organic anion. The large difference between the size of the cation and the small anion prevents the formation of a stable crystal lattice. As a consequence, the ions remain disorganized, which results in a liquid state at room temperature. Low toxicity and biocompatibility, non-flammability, excellent chemical and thermal stability, high ionic conductivity and a wide electrochemical window usage are some

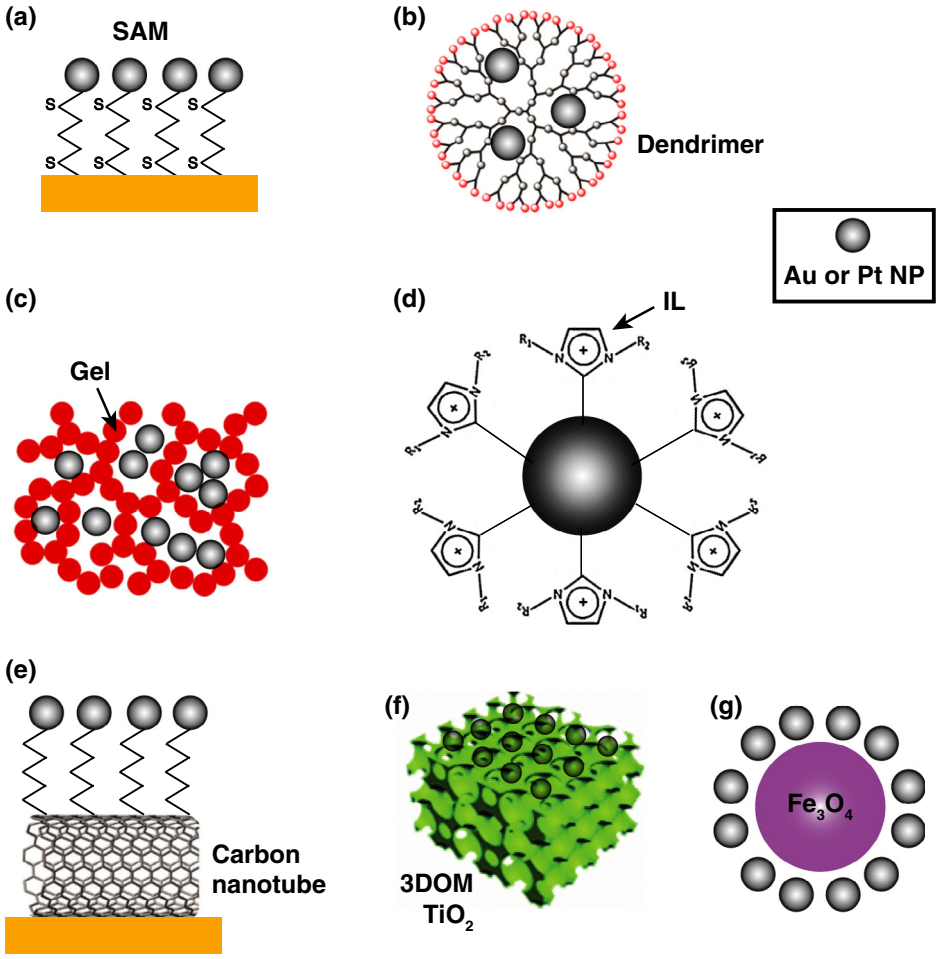

Fig. 1. Fabrication strategies of Au and Pt NP-based electrodes: (a) SAM, (b) embedded in dendrites, (c) sol-gel, (d) dispersion in ILs, immobilization on (e) carbon nanotubes, on (f) 3DOM TiO_2 and on (g) Fe_3O_4 magnetic particles.

properties that make ILs interesting binders to modify biosensors. Both Au [22] and Pt [23,24] NPs have been synthetized by decomposition of an organometallic metal precursor in ILs. Studies [25,26] demonstrated that NPs were stable and catalytically active in ILs (Fig. 1d). The high sensitivity of biosensors based on NPs and ILs was probably due to the high conductivity of the IL, the electron transfer properties of the metal NPs and the excellent immobilization of enzymes on such a matrix. Evidence of the interaction between ILs and NP surface was demonstrated (IL protective layer surrounds the NPs) while NPs were keeping their catalytic properties [25,26]. The larger the NP size is, the better the catalytic activity is due to the presence of highly uncoordinated atoms.

2.6. Combination with other nanomaterials

To further enhance conductivity, surface area of the electrode, Au and Pt NPs are often coupled with other nanostructures. Carbon nanomaterials including fullerene [27] graphene [28], carbon nanofibers [29] and carbon nanotubes [14] are excellent supporting materials of NPs (Fig. 1e) due to their high surface area. Additionally, electrochemical active sites of Au and Pt NPs seem to be less susceptible to poisoning by various compounds present in the solution if they are immobilized on carbon nanomaterials rather than on bare electrodes. Carbon nanomaterials also favor an easy immobilization of the enzyme by adsorption.

Three-dimensionally ordered macroporous (3DOM) materials have also received extensive attention as materials to modify electrodes thanks to their high stability and the increase of up to two orders of magnitude of the real surface area. In particular, the excellent biocompatibility, environmental safety and good electrical conductivity make 3DOM TiO_2 [30] a very attractive surface for Au NPs incorporation and an improved DET from the enzyme to the electrode (Fig. 1f).

Liu [31] and Yu [32] have reported the immobilization of Au NPs in Fe_3O_4 magnetic particles (Fig. 1g). The NP shell on Fe_3O_4 particles was synthetized by reduction of Au precursor. Briefly, the solution containing both Fe_3O_4 magnetic particles and Au salt was heated to boiling point and vigorously stirred for some time to allow the reduction of the Au precursor. Au-Fe_3O_4 nanocomposites were precipitated by using a magnet. The presence of Au NPs improves biocompatibility, conductivity, chemical stability and dispersion capability of magnetic particles providing also multiple sites to attach thiolated molecules. In general, protective layers are added to prevent the aggregation of Fe_3O_4/Au NPs complexes and to provide further exposed groups for functionalization.

Other hybrid materials (e.g., Ag and Pt NPs [22], Au NPs and $CaCO_3$ microspheres [33]) are further model materials with excellent properties of electron transfer.

3. Au and Pt nanoporous-based electrodes

Within this section we sum up the principal approaches to realize nanoporous layers of Pt and Au used as enzymatic support materials for direct electrochemical sensing. These electrodes can be

manufactured by: dealloying (3.1), LbL (3.2), hydrothermal methods (3.3) and electrodeposition (3.4), the latter in combination or not with solid or dynamic templates.

3.1. Dealloying

In spite of the large number of works concerning the study of DET at bare and NP-modified electrodes, little investigation has been focused on nanoporous metals. An efficient approach to generate nanoporous-based electrodes is the dealloying from binary or ternary alloys. An annealing step is required for homogenization or formation of the alloy. Finally, dealloying is carried out to selectively dissolve the unwanted metal either chemically [34] or electrochemically [35]. After the dealloying, the more noble metal component is left behind with a porous structure. Salaj-Kosla et al. [7] and Qui et al. [36] have studied for the first time the electron transfer from laccase adsorbed on nanoporous Au. Here, dealloying of sputtered Au/Ag alloy in concentrated nitric acid produced the modified electrode (Fig. 2a). An enlargement of the pore size was obtained by thermal annealing and the effect of the pore size on laccase immobilization was investigated [36].

3.2. Layer-by-layer

Zhou and coworkers [37] studied the DET of *cytochrome c* (cyt c) at nanoporous Au film fabricated by LbL and SAM techniques from colloidal Au/Ag multilayer films (Fig. 2b). Briefly, electrodes were immersed in a colloidal Au solution first and then in an alcoholic solution of 1,5-pentanedi-thiol to produce thiols for the following adsorption of Ag colloids. Composite multilayer Au/Ag coatings were fabricated. The dissolution of Ag NPs produced the nanoporous Au. Cyt c was finally immobilized by physical adsorption.

3.3. Hydrothermal technique

Hydrothermal process represents an alternative deposition technique of Au and Pt nanoporous films performed at high pressure and high temperature. After removal of the oxide layer and a roughening of Ti plates in HCl solution at 85°C, the plate is transferred to an autoclave containing Pt or Au precursors and a formaldehyde solution as reducing agent. After cooling down the temperature to 25°C, an annealing at 250°C under Argon completes the nanofabrication [38].

3.4. Electrodeposition

Electrodeposition is a practical and versatile approach to selectively nanostructure conductive substrates. *Template electrodeposition* is the most used technique to produce nanoporous Au and Pt films (Fig. 2c). It requires three steps: (i) fabrication of a template with the desired porosity on conductive electrodes (ii) electrochemical filling of pores and (iii) template removal. Wang et al. [39] have recently studied the adsorption and DET from Hb into a nanoporous Au film synthesized electrochemically by an inverted *colloidal* crystal template technique. Firstly, SiO_2 colloidal spheres were self-assembled on an Au electrode. Then, Au was electrodeposited into

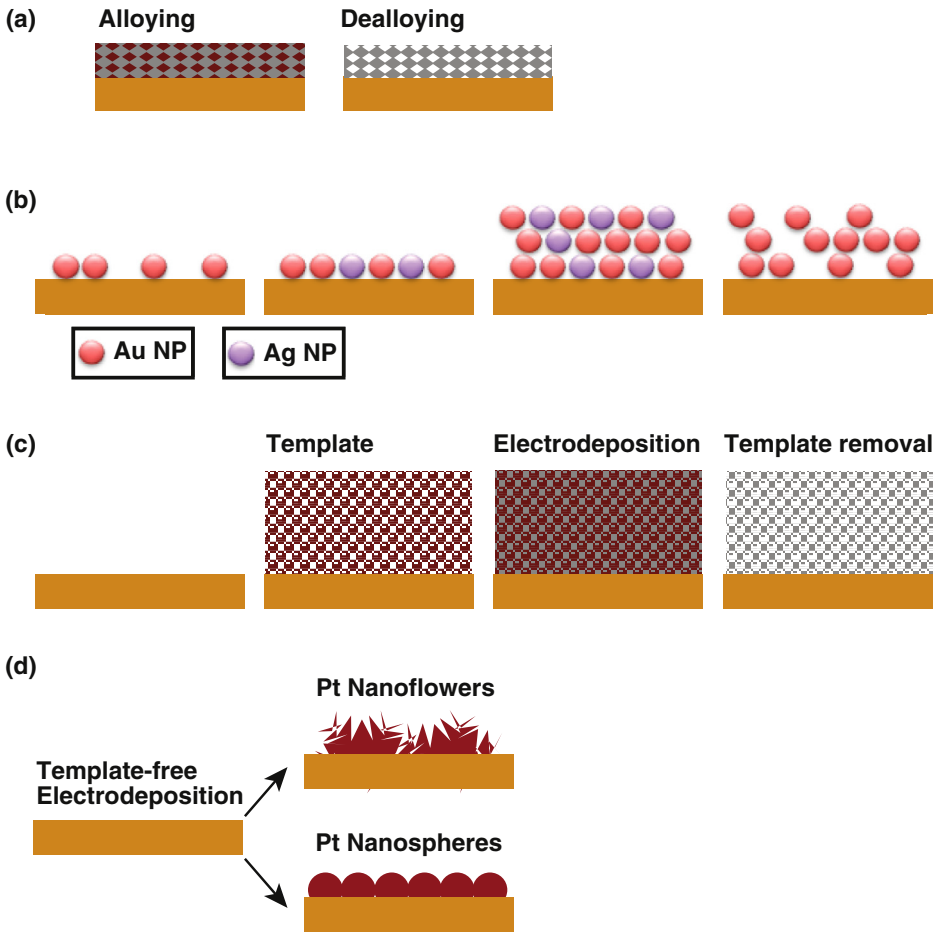

Fig. 2. Methods to produce Au and Pt nanoporous electrodes. (a) Alloying-dealloying. (b) LbL. (c) Template electrodeposition. (d) Template-free electrodeposition (c).

the interspaces of the silica-crystals and finally removed by a chemical etching in fluorhydric acid. Other mono- and multilayers (3D and 2D) closely packed colloidal crystals can be used as templates on electrode surfaces by immersion or evaporation methods (typically, polymers as polystyrene [40]). Polymeric spheres are etched by using solvents [15].

Other types of solid templates are hard and soft templates. The most used *hard* template is the porous anodic aluminum oxide membrane containing 1D vertical structures (to form nanotubes or nanowires) [41]. Another method to synthetize nanostructured electrodes is the *soft* templating. A variety of both biological and artificial structures (micelles, reverse micelles in organic solvents, liposomes, vesicles, microemulsions, biomacromolecules and viruses) could be self-assembled onto the electrode surface and can be used as templates [42]. After the electrodeposition of metals, the template removal is obtained by rinsing with water. The main advantage of the soft templating synthesis is the possibility to change sizes of the pores and the coating thickness.

A more intriguing strategy to fabricate Au and Pt nanoporous coatings is based on *dynamic templates*. Chen [43] fabricated a three-dimensional Au layer prepared by hydrogen bubbles as dynamic templates. At negative applied voltage (−1.0 V), a large amount of hydrogen bubbles evolves in the solution containing acid and a gold salt precursor. The bubbles acted as templates to fabricate three-dimensional coatings. Then, Hb was self-assembled on the produced layer to improve the DET between the enzyme and the electrode. In our recent work, we have used hydrogen bubbles as dynamic templates to modify electrodes with Au-Pt coatings starting from solutions containing NH_4^+ as source of hydrogen bubbles and salts of the two metals.

We have recently introduced *template-free electrodeposition* protocols from H_2SO_4-containing solutions [44] to produce differently shaped-Pt nanostructured layers. During the deposition, the acid anions adsorb on specific Pt planes promoting their growth. Spherical-like structures resulted by applying a low voltage while Pt nanoflowers were produced at more negative applied voltage and/or by prolonging the growth time (Fig. 2d).

We have investigated the direct electrochemistry of a laccase at Pt nanospheres, Pt nanoflowers and Au-Pt nanostructured films.

4. Direct electron transfer in biosensors

Enzymes are typically proteins that speed up the rate of reactions involving the transformation of molecules in some products. Electrochemical biosensors, containing an enzyme immobilized onto the transducer, have been developed to specifically sense only one molecule. Indeed, each enzyme contains active sites that are specific for binding and reacting with a certain molecule. In the third generation biosensors, this reaction causes the response signal without involving any mediator because the integrated enzyme directly exchanges electrons with the electrode.

Third generation biosensors can sense small (e.g., H_2O_2 [38] and O_2 [7]) as well as big molecules (e.g., luteolin [22]) for a variety of applications (e.g., medicine [24], food analysis [18]).

In direct electrochemistry, nanostructures used as binding materials of enzymes, serve as channels to carry the charge from the enzyme to the electrode. Examples of enzymes used to fabricate such devices are hemoglobin, laccase, cytochrome c and horseradish peroxidase.

4.1. Hemoglobin (Hb)

Hemoglobin (Hb), an important respiratory protein in red cells with a molecular weight of approximately 64500 Da, consists of four polypeptide chains, each one with an electroactive iron heme group [45]. The function of Hb is to deliver oxygen to cells. Investigation of the DET between electrode surface and Hb is crucial for fundamental studies [14,46] as well as for further developments in biosensing [47,48]. However, Hb displays poor redox kinetics at conventional electrodes due to the sluggish electron transfer. The electroactive center of Hb is buried inside the non-conductive peptide chains, resulting in inaccessibility of its electroactive center to the electrode surface. Additionally, the slow electron transfer may be caused by the unfavorable orientation of Hb molecules on the electrode, which increases the distance between Hb heme center and surface of immobilization. Finally, the adsorption of impurities could block the electron communication between heme and electrode and could cause denaturation of the enzyme too. Many works focused on the study of the DET between Hb and electrodes modified with Au and Pt nanostructures (Table 1).

Direct electrochemistry of Hb immobilized on nanoporous Au has been studied. Nanoporous Au network produced under a hydrothermal condition by Kafi [38] effectively sensed successive increases of H_2O_2. The linearity ranged between 0.05 μM and 0.2 mM with a detection limit of 0.02 μM (S/N = 3). The *apparent Michaelis–Menten constant* (K_M^{app}) was 0.261 mM. Nanoporous Au fabricated by template electrodeposition [39] showed a high *electron transfer rate* (k_s) equal to 0.95 s^{-1} of the adsorbed Hb on the nanostructured film.

A wider linear range (from 0.015 mM to 0.48 mM), a higher detection limit of 0.0074 mM (S/N = 3) for H_2O_2 determination was obtained by Feng [47] as compared to the work of Kafi [38]. In Feng's work [47], the efficient electron transfer of Hb was realized on Au-Zr phosphate NP-modified GCE due to the synergistic effect between

Table 1
Direct electrochemistry of Hb on electrodes modified with Au or Pt nanostructures

Electrodes	Linear range	Detection limit (μM)	Response time	Potential	Analyte	k_s (s^{-1})	K_M^{app} (mM)	Ref.
Au nanoporous	0.05 μM to 0.2 mM	0.02	–	–0.3 V *vs* Ag|AgCl	H_2O_2	–	0.261	[38]
Au-Zr NPs	0.015 mM to 0.48 mM	7.4	–	–0.25 V *vs* SCE	H_2O_2	–	0.65	[47]
3D Au-SAM 3-mercaptopropylphosphonic acid	0.078 to 91 μM	0.025	<1 s	–0.3 V *vs* SCE	H_2O_2	15.8 ± 2.0	–	[43]
Graphene-Pt NPs	0.01 mM to 1 mM	1	–	–0.26 V *vs* SCE	H_2O_2	0.14	0.54	[28]
Chitosan-Au NPs	0.74 to 13 mM	6.4	8 s	–0.25 V *vs* SCE	H_2O_2	–	1.4	[6]
Chitosan-Au NPs	0.14 μM to 6.6 mM	0.045	10 s	–	H_2O_2	–	0.12	[15]
Chitosan-Au NPs	0.44 to 44 μM	0.028	–	–0.4 V *vs* SCE	H_2O_2	–	0.021	[16]
3DOM TiO_2-Au NPs	5.0 μM to 1.0 mM	0.6	–	–0.52 V *vs* SCE	H_2O_2	1.12	–	[30]
ZnO-Chitosan-Au NPs	0.194 μM to 1.73 mM	0.097	<4 s	–0.28 V *vs* SCE	H_2O_2	–	0.075	[46]
Fe_3O_4-Au NPs	3.4 μM to 4.0 mM	0.67	–	–	H_2O_2	–	2.3	[32]
Graphene-Au NPs	0.72 μM to 7.92 μM	0.012	<3 s	–0.82 V *vs* Ag|AgCl	Nitrite	4.8 ± 5	–	[49]
Graphene- ZnO-Au NPs	6.0 μM to 1.13 mM	0.80	<2 s	–0.3 V vs SCE	H_2O_2	1.3	0.17	[50]
Carbon nanotubes-Au NPs	0.21 μM to 3 mM	0.08	<5 s	–0.3 V *vs* SCE	H_2O_2	–	0.26	[8]
Carbon nanotubes-Au NPs	3.6 μM to 3.0 mM	0.96	–	–0.1 V *vs* SCE	Nitrite	–	0.62	[14]

the two NPs. A couple of stable and well-defined redox peaks of Hb was observed at about +134 and +35 mV *vs* SCE in pH 6.0 buffers. The K_M^{app} was 0.65 mM.

Other authors immobilized multilayered Hb molecules on three-dimensional Au film electrode modified with SAMs of 3-mercaptopropylphosphonic acid [43]. DET of the multilayered Hb was observed with high thermal and electrochemical stability. The inner Hb molecules directly transfer electrons to the electrode surface and the other Hb proteins communicate electrons *via* protein–protein exchange. The k_s was computed and resulted equal to (15.8 ± 2.0) s^{-1}. This electrochemical sensor showed fast response (less than 1 s), wide linear range (0.078 to 91 μM) and particularly low detection limit (0.025 μM).

Feng et al. [28] found that Hb retained its natural configuration after the immobilization onto graphene–Pt nanocomposite,. The k_s resulted equal to 0.14 s^{-1}. The immobilized Hb showed a pair of stable and well-defined peaks in cyclic voltammetry at +0.26 V *vs* Ag|AgCl. The modified electrode exhibited a higher value of upper limit of linearity (1 mM) as compared to the before-mentioned research works, no interferences and good reproducibility. Graphene increased the enzyme loading, the rate of electron transfer and improved the Hb thermal stability [28].

Not only graphene [28,49,50] but also carbon nanotubes have been used as nanomaterials to immobilize Au NPs and study the DET of Hb [14,46]. The best results were obtained by Chen et al. [8]. They proposed a biosensor based on carbon nanotubes and Au NPs that displayed a broader linear range (0.21 μM–3 mM) and a lower detection limit (0.08 μM, S/N = 3) as compared to the H_2O_2 biosensors only based on carbon nanotubes. The K_M^{app} value was estimated to be 0.26 mM. The proposed biosensor displayed a response time of less than 5 s and good stability and reproducibility.

Another hybrid coating developed by Duan et al. was proven to effectively enhance electron transfer between Hb and electrode [46]. Hb, ZnO NPs, chitosan and Au NPs were immobilized on GCE. Biocompatible ZnO-chitosan created a suitable microenvironment to keep Hb activity (K_M^{app} = 0.075 mM). This biosensor had a fast response time (less than 4 s) and excellent linear range from 0.194 μM to 1.73 mM with detection limit of 0.097 μM (S/N = 3).

Yu et al. adsorbed Hb on an hybrid NP-film made of Fe_3O_4 and Au [32]. Stable and well-defined redox peaks were observed at about –350 mV and –130 mV *vs* SCE in pH 6.0 buffer. The modified electrode was used for H_2O_2 detection with a linear range from 3.4 μM to 4.0 mM and 0.67 μM of detection limit (S/N = 3). The K_M^{app} was 2.3 mM.

Electrodeposited flower-like, spherical, and convex polyhedron Au NPs on *boron-doped diamond* (BDD) surface were fabricated by Li [10] and used to study the DET of Hb. Different NPs were synthetized only by changing electrodeposition potential and concentration of $HAuCl_4$. The electrochemical properties of these differently shaped Au NPs were quite distinct from each other. Hb immobilized on flower-like Au NPs showed k_s higher (0.34 s^{-1}) than that related to spherical and convex polyhedron Au NPs (0.16 s^{-1}, and 0.13 s^{-1}, respectively).

Feng et al. [6] used chitosan-stabilized Au NPs to incorporate Hb. The proteins retained their secondary structures. This film also enhanced the DET between enzyme molecules and the underlying surface. The electrode displayed a linear range from 0.74 to 13 mM and a limit of detection of 6.4 μM. The K_M^{app} was determined to be approximately 1.4 mM. Chitosan and Au NPs were also used by Yang [15] and Jia [16,17] and they obtained lower values of limit of detection (0.045 and 0.028 μM, respectively) and of K_M^{app} (0.12 and 0.021 mM, respectively) as compared to those in ref. [6].

Finally, 3DOM TiO_2 Au NPs was an excellent composite material to promote the DET of Hb [30]. In fact, this biosensor showed a k_s of 1.12 s^{-1}, which is higher than that reported by other authors [10,28].

4.2. Laccase

Laccase is a multicopper oxidase that catalyzes the oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as of some inorganic ions coupled to the reduction of molecular dioxygen to water. The enzyme contains four metal ions classified into three types (e.g., T1, T2, T3) according to their spectral characteristics. The most relevant characteristic of laccase is the redox potentials of the T1 Cu sites. Laccases are classified into three groups according to the redox potential of the T1 site and their primary structures, namely low, middle, and high redox potential laccases [51,52].

Zapp [23], Franzoi [22] and Brondani [23,24] immobilized laccase in a new IL phase based on Au or Pt NPs. The biosensors were used for the determination of different compounds showing excellent linearity and detection limits.

Another work [18] reported the immobilization of laccase on Au NPs encapsulated-dendrimers. To immobilize dendrimer-encapsulated AuNPs onto the electrode substrate, a functionalized conducting polymer layer with two amine groups was used. Laccase was then covalently immobilized to carboxylic groups of dendrimers. Two redox peaks of the Cu center of the laccase were observed at –0.03 and +0.13 *vs* Ag|AgCl and the electron-transfer rate constant was 1.28 s^{-1}. The biosensor was linear in the concentrations range from 0.1 to 10 μM with a detection limit of (0.05 ± 0.003) μM towards catechin.

Salaj-Kosla et al. [7] described the electrocatalytic reduction of O_2 by a *Trametes hirsuta* laccase immobilized on unmodified nanoporous Au electrodes. The potential of approximately +650 mV for the reduction of O_2 (O_2 saturated solution) resulted close to the redox potential of the T1 site of the laccase.

In a very recent work, we have used coatings nanostructured with Pt nanoflowers, Pt nanospheres and Au-Pt nanoferns produced by a one-step electrodeposition to study the DET of a laccase. Laccase from *Trametes versicolor* was immobilized onto the modified electrodes employing 0.5% Nafion as physical entrapping agent. The efficient immobilizing procedure, without loss of biochemical properties, was confirmed by determining the electron transfer rate constant. The value of the constant was 0.08, 0.24 and 28 s^{-1} for Pt nanospheres, Pt nanoflowers and Au-Pt nanoferns, respectively. The combination of Au and Pt enhances the direct electron transfer because of the cooperative activity of the two metals.

Table 2 lists recent works on the determination of compounds *via* laccase-based Au and Pt nanostructured biosensors.

4.3. Cytochrome c (cyt c)

Cytochrome c (cyt c) is a kind of basic redox protein embedded in inner membranes of mitochondrion. It carries out electron transport by the redox reaction of iron ion in its iron-porphyrin prosthetic group.

Among various redox proteins, the study of the DET between cyt c and the electrode has gained increasing attention due to its biological function [53,54]. However, it is very difficult for cyt c to exhibit a voltammetric response at a bare electrode because of the extremely slow electron transfer kinetics at the electrode/solution interface, the scarce time-stability and the slow response time on a metal surface.

Some authors [54] have successfully fabricated macroporous Au films by combining hydrogen bubbles as templates and galvanic replacement processes. The large area of Au film modified with 11-mercaptoundecanoic acid was used to immobilize covalently cyt c. The k_s was 1.73 s^{-1}.

Nanoporous Au film produced by LbL assembling of Au and Ag NPs following Ag etching showed a higher k_s of 3.9 s^{-1} [37]. By sensing

Table 2
Direct electrochemistry of laccase on electrodes modified with Au or Pt nanostructures

Electrodes	Linear range (μM)	Detection limit (μM)	Response time	Potential	Analyte	k_s (s^{-1})	Ref.
Polyethyleneimine-Au NPs	0.36 to 11	0.03		+0.25 V	Catechol	0.4	[19]
	0.8 to 17.4	0.03		+0.25 V	Guaiacol		
	1.7 to 19.6	0.14		+0.18 V	Pyrogallol		
	2.9 to 22.0	0.21		+0.12 V *vs* Ag/AgCl	Hydroquinone		
Au NPs-dendrimers	0.1 to 10	0.05 ± 0.003	<10 s	+0.13 V *vs* Ag|AgCl	Catechin	1.28	[18]
ILs-Au NPs	100 to 5800	28 ± 2		+0.4 V *vs* Ag|AgCl	Luteolin	–	[22]
ILs-Pt NPs	0.97 to 72	0,235	–	+0.2 V *vs* Ag|AgCl	Methomyl	–	[23]
ILs-Pt NPs	1 to 213	0.293		–0.2 V *vs* Ag|AgCl	Adrenaline	–	[24]

changes of H_2O_2, this biosensor had a linear range between 10 μM-12 mM, a detection limit of 6.3 μM and a fast response time (8 s).

Xiang et al. [55] developed a robust and effective composite film based on AuNPs/room temperature ILs/MWCNT/GCE by means of a LbL self-assembly technique. Cyt c was immobilized on the electrode by electrostatic adsorption. A pair of well-defined quasi-reversible redox peaks was obtained in 0.10 M pH 7.0 phosphate buffer solution. An enhanced DET between cyt c and the underlying electrode was demonstrated with k_s of (0.78 ± 0.04) s^{-1}. The biosensor exhibited a wide linear range (50 μM - 1.5 mM) and a low detection limit (3 μM) towards the reduction of H_2O_2.

4.4. Horseradish peroxidase (HRP)

Horseradish peroxidase (HRP) is a heme-containing glycoprotein with a molecular mass of approximately 42000 Da. Efficient electron transfer between HRP and electrodes has been reported for many years [56]. Although HRP can directly catalyze the electrochemical reduction of H_2O_2, direct electrochemistry of HRP is rarely observed, due to their deeply located redox centers. Moreover, the direct immobilization of HRP monolayer onto metal electrode often resulted in denaturation and loss of enzyme activity [57].

Di et al. [20], immobilized Au NPs and HRP simultaneously in silica sol-gel network on Au electrode surface in the presence of cysteine. Direct electron transfer of HRP was observed with k_s of 7.8 s^{-1}. The linear range was from 1.6 μM to 3.2 mM and the detection limit was 0.5 μM. The K_M^{app} was found to be 1.1 mM.

Zhao et al. [17] stabilized Au NPs by chitosan hybridized with clay nanoplates *via* electrostatic interaction. HRP was entrapped between two layers of clay/Au chitosan. The HRP showed a pair of quasi-reversible redox peaks at –0.195 V *vs* Ag|AgCl in 0.1 M PBS (pH 7.0) and the biosensor displayed a linear range towards H_2O_2 sensing from 39 μM to 3.1 mM. The detection limit was 9.0 μM and k_s was (2.95 ± 0.2) s^{-1}. The obtained K_M^{app} was 23.15 mM.

Another work [33] reported a different hybrid material based on Au NPs, calcium carbonate microspheres ($CaCO_3$) and HRP embedded into a silica sol-gel matrix. The modified electrode exhibited a pair of redox peaks at –0.03 and –0.118 V *vs* SCE. The biosensor exhibited a high upper limit of linearity to 8 mM. The detection limit was 1 μM.

Other authors [58] developed a flowerlike ZnO-Au NPs-Nafion coating to promote the DET of HRP. The enzyme electrode showed a well-defined pair of quasi-reversible redox peaks. The value of peak-to-peak separation was 60 mV at a scan rate of 50 mV/s and k_s was of 1.94 s^{-1}. The modified electrode showed a good stability after successive scans. The biosensor exhibited a linear range from 15 μM to 1.1 mM with a detection limit of 9 μM. The K_M^{app} was calculated to be 1.76 mM.

Bimetallic electrodes have better properties than their monometallic counterparts. Che et al. [59] electrodeposited Au-Pt NPs on a polypyrrole layer. Then, DNA and HRP were self-assembled on the surface of the electrode pre-modified with L-cysteine. The biosensor is linear between 4.9 μM and 4.8 mM with a detection limit of 1.3 μM. The K_M^{app} value was determined to be 0.69 mM.

A list of works concerning the DET of HRP immobilized on Au or Pt nanostructured electrodes is in Table 3.

5. Conclusions

A good electrical contact between enzymes and electrode surfaces is of key importance for high performance third-generation biosensors. Indeed, the active center of redox proteins is surrounded by a thick insulating shell that blocks the electron transfer to the electrode. The excellent conductivity properties and the nanoscale dimensions of metal NPs and nanopores make them powerful for enhancing the DET between the enzyme and the electrode transducer. NPs and nano-protrusions of porous films penetrate the enzyme active center acting as “wires” of electron transit thus connecting protein and electrode.

Table 3
Direct electrochemistry of HRP on electrodes modified with Au or Pt nanostructures

Electrodes	Linear range	Detection limit (μM)	Response time	Potential	Analyte	k_s (s^{-1})	K_M^{app} (mM)	Ref.
Sol-gel-Au NPs	5.0 μM to 10.0 mM	2.0	2.5 s	–0.25 *vs* Ag|AgCl	H_2O_2	–	–	[21]
ZnO-Au NPs	15 μM to 1.1 mM	9	–	–0.32 *vs* Ag|AgCl	H_2O_2	1.94	1.76	[58]
PDDA -Au NPs	0.196 to 0.909 mM	0.99	–	–0.35 *vs* SCE	H_2O_2	–	1.3	[12]
Sol-gel-Au NPs	1.6 μM to 3.2 mM	0.5	5 s	–0.1 *vs* SCE	H_2O_2	7.8	1.1	[20]
$CaCO_3$-Au NPs	40 μM to 8.0 mM	1.0	–	–0.3 *vs* SCE	H_2O_2	–	–	[33]
Nafion-cysteine-Au NPs	1.6 μM to 2.4 mM	0.5	10 s	–0.09 *vs* SCE	H_2O_2	–	–	[13]
DNA-cysteine-Au&Pt NPs-polypyrrole	4.9 μM to 4.8 mM	1.3	–	–0.2 *vs* SCE	H_2O_2	–	0.69	[59]
Pt NPs	0.64 μM to 3.6 mM	0.35	–	–0.3 *vs* SCE	H_2O_2	–	–	[11]
Clay-chitosan-Au NPs	39 μM to 3.1 mM	9.0	–	–0.30 *vs* Ag|AgCl	H_2O_2	2.95 ± 0.20	23.15	[17]
TiO_2 colloids Au NPs	0.41 μM to 0.63 mM	5.6	3 s	–0.3 V *vs* SCE	H_2O_2		0.63	[60]
Carboxymethyl chitosan Au NPs	5 μM to 1.4 mM	0.4	5 s	–0.4 *vs* SCE	H_2O_2		0.57	[61]

This review summarizes recent fabrication protocols of Au and Pt nanostructured electrodes and their attractive sensing properties for third generation biosensors. Au and Pt NP-based electrodes have been proven to improve the electron shuttle. The poor stability and the multiple step preparation procedures of NP-based electrodes have encouraged researchers to explore novel ways of nanostructuration like layers of Au and Pt nanopores. Several recent studies demonstrate that these emerging materials also enhance the direct electrochemistry of redox proteins on electrodes paving the way for dramatic changes in enzyme sensors industry in the future. We have analyzed the performance of Au and Pt nanostructured biosensors based on hemoglobin, laccase, cytochrome c and horseradish peroxidase. The considered biosensors show wide linear ranges (from nano to millimolar concentrations), limit of detection below 1 μM, short response times and high rates of electron transfer. Moreover, the DET of the immobilized enzymes occurs with both high electrochemical and time stability at potentials excluding possible interferences.

The combination of the unique DET properties between the enzymes and the electrodes of Au and Pt nanostructures, along with the manifold advantages of third generation biosensors could significantly impact the biosensing field and has the potential to address challenges in branches like medicine, food and environmental analysis, among others.

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