Biotinylation of Screen-Printed Carbon Electrodes through the Electrochemical Reduction of the Diazonium Salt of *p*-Aminobenzoyl Biocytin

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Many strategies have been developed for the immobilization of bioactive molecules on electrode surfaces to improve the selectivity and sensitivity of electrochemical measurements. Immobilization may be accomplished by physical adsorption or chemical attachment of monolayers on electrode surfaces or by entrapment within polymer or gel coatings.[1] The diversity of biomolecules that may be used for the construction of biosensors (i.e., enzymes, antibodies, peptides, DNA ...), as well as the growing interest for the development of multianalyte sensors (e.g., arrays of immunosensors),[2] implies the need for a spatial distribution of biomolecules on surfaces through a simple, rapid, and versatile immobilization strategy. The linkage of any biocomponents via the biotin/avidin/biotin coupling is an attractive universal immobilization approach.[3] The complex allows the construction of a molecular sandwich[3] and the assemblage of ordered protein multilayers[4] owing to the strong binding of four biotin molecules to one avidin unit (dissociation constant of 10^{-15} M). Moreover, many biotinylated molecules or avidin conjugates are commercially available, allowing a large variety of biosensors to be rapidly fabricated.

Several approaches for biotin immobilization on electrode surfaces have been investigated.[5−9] Photopatterning is one of the techniques used to achieve a spatially distributed monolayer of biotin on a carbon electrode, but it requires sophisticated equipment.[6] Electrodeposition, an alternative method, is less expensive, since it allows the amount of deposited biotin to be accurately controlled in a one-step process and to be locally confined on any shape of electrode.[7,8] Moreover, this a noncorrosive method favorable to low voltammetric detection limit, because the capacitive current recorded in linear scan or cyclic voltammetry (LSV or CV) remains unchanged or becomes even lower after electrodeposition.[10] Biotin functionalized by a phenol[7a] or a pyrrole group[7b,c] has been recently electropolymerized, leading to a biotinylated polymer coating. However, a large amount of biotin is entrapped within the polymer film and, therefore, it becomes inaccessible for binding, i.e., a low biotin coverage is achieved.[7c] Moreover, electrodeposited polymer films produce an additional diffusion barrier slowing down response times and reducing sensitivities, and they lead to significant nonspecific binding (NSB).[7a,b] The covalent attachment of a monolayer of biotin on gold[9] or glassy carbon electrode[5,6,8] (GCE) surfaces appears more advantageous. For example, a high density of thiol-functionalized biotins with a low NSB has been self-assembled on a gold surface,[9a] and rapid amperometric response times have been achieved at an enzyme-modified carbon-fiber electrode using the biotin/avidin/biotin coupling.[5a,b] An electrochemical approach based on the electrooxidation of an amine-containing biotin has also been proposed for the grafting of a biotin monolayer on a GCE,[8] but the modified electrode was not further investigated for the assemblage of proteins.

We describe, in this paper, a noncorrosive cathodic approach for the electrodeposition of a biotin monolayer on the surface of disposable carbon-based screen-printed electrodes (SPEs)[11] fabricated with a conductive carbon ink of graphite particles embedded in a polystyrene binder (3:2 ratio).[12] The SPEs possess a very low double-layer capacitance (1.6 μF cm^{-2}, i.e., 20 times lower than that at GCE), leading to low detection limits in LSV or CV, and they show a good electrochemical response in a wide potential range (from −1.5 to +1.5 V vs Ag/AgCl in Tris buffer, pH 9.0).[13] For the biotin electrodeposition, we took advantage of a novel route devised for modifying carbon surfaces in acetonitrile[14a−c] and, more interestingly, in acidic water[14d] (the SPEs are soluble in most of the organic solvents). This route, which leads to very stable modified surfaces,[14b,c] exploits the arylation of carbon electrodes via the electrochemical reduction of diazonium salts,[14] and the method has been applied to the functionalization of glassy carbon,[14a−c] highly oriented pyrolitic graphite,[14b] and carbon fibers[14d] by various aryl groups.

Biotin was attached on SPEs as depicted by steps (i) and (ii) in Scheme 1, which are an adaptation of the procedure previously reported.[14d] Indeed, instead of using a recrystallized diazonium salt the diazonation of a biotinylated aniline derivative was carried out in ice-cold acidic water for a short period (step (i)), and then the solution was transferred to an electrochemical cell thermostated at 4 °C, in which the cathodic functionalization of SPE took place (step (ii)). The starting material was *p*-aminobenzoyl biocytin, a commercially available compound currently used as a biotinylating reagent for macromolecules.[3b] Its spacer arm is a hydrophilic lysine residue containing a free carboxylate group,

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(10) This is an advantage over the harsh chemical or electrochemical oxidation methods generally used to generate carboxylic anchoring groups on carbon electrode surfaces, and which lead to high capacitive currents.[5a, b]

(11) The screen-printing technology allows for the mass production of low-cost electrochemical sensors and for a high flexibility in designing the layout. Consequently, SPEs have found successful applications in bioanalytical chemistry.[11a−c] (a) Alvarez-Icaza, M.; Bilitewski, U. *Anal. Chem.* **1993**, *65*, 525A. (b) Hart, J. P.; Wring, S. A. *Trends Anal. Chem.* **1997**, *16*, 89. (c) Hilditch, P. I.; Green, M. J. *Analyst (Cambridge, U.K.)* **1991**, *116*, 1217.

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(13) The high carbon content gives a highly conductive thick film, without compromising its binding and adhesion properties, which allows a sufficiently fast electron-transfer rate (i.e., $\Delta E_p = 63 \pm 4$ mV for 4 μM ferrocenemethanol in PBS, scan rate: 50 mV s^{-1}).[12]

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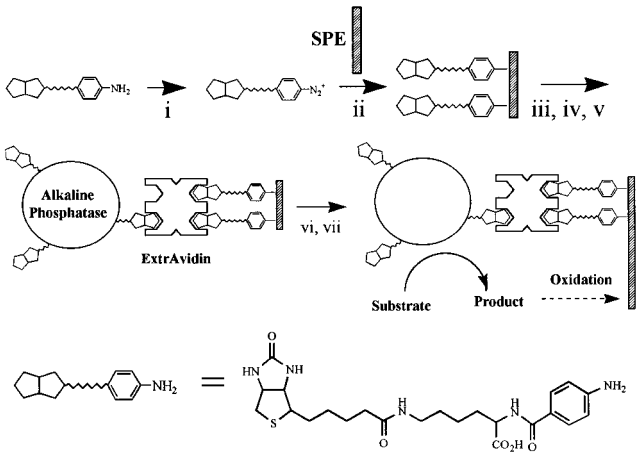
Table 1. Experiments for Controlling the Specific and Nonspecific Bindings

entry	biotinylated electrode	extravidin	biotin	biotinylated AP	AP	$i_{p,a}$ (μA)[a] first run	$i_{p,a}$ (μA)[a] second run[c]	% first run	% second run
1	+	+		+		8.35	10.80	100	100
2	+			+		0.034	0.045	<0.5	<0.5
3	+	+	+[b]	+		0.125	0.033	1.5	<0.5
4	+	+			+	0.172	0.048	2.1	<0.5
5		+		+		0.170		2.0	

[a] Average of two measurements at two different electrodes. [b] 10-fold excess of biotin was premixed with Extravidin. [c] After regeneration of the biotin-modified SPE with a solution of 6 M guanidine hydrochloride.

Scheme 1. Reagents and Conditions[a]



[a] (i) *p*-aminobenzoyl biocytin (1 mg mL^{-1} in 1 N HCl), $NaNO_2$ (40 equiv), ice-cold, 5 min; (ii) 3-fold dilution in 0.1 N HCl (final volume = 4 mL), −0.25 V vs Ag/AgBr, 1 min, 4 °C; (iii) Extravidin (10 μg mL^{-1} in phosphate buffer saline (PBS) containing 0.1% BSA (PBS/BSA), pH 7.4), 35 μL deposited on the biotinylated electrode surface, 30 min, RT; (iv) washing with PBS; (v) biotinylated alkaline phosphatase (10 μg mL^{-1} in PBS/BSA), 35 μL, 30 min, RT; (vi) enzyme reaction: substrate (1 mM in Tris buffer, pH 9.0, containing 1 mM $MgCl_2$), 35 μL, 1 h, RT; (vii) detection: linear sweep voltammetry (50 mV s^{-1}).

probably releasing avidin away from the hydrophobic environment of the electrode surface and, consequently, avoiding the protein denaturation.[14c] Moreover, the spacer length of the biocytin derivative is well-adapted for an efficient biotin/avidin recognition, since a length of at least eleven atoms is deemed necessary.[3a] The CV curve recorded at a SPE immersed in a solution of the in situ generated diazonium salt presents a broad irreversible cathodic peak, located at −0.1 V vs Ag/AgBr, which tends to disappear when cycling the potential repetitively (Figure 1). This behavior is characteristic of the reduction of an aryl diazonium salt to a highly reactive aryl radical rapidly grafted on the electrode surface.[14] A series of 45 biotin-modified SPEs were prepared by immersing each bare SPE for 1 min under polarization (−0.25 V). The stability of the diazonium salt was monitored by periodically measuring the cathodic peak current, and no significant decrease was observed after preparation of the series (∼90 min). Once biotinylated, the SPEs were reacted with Extravidin, a modified form of avidin with a low NSB. This was followed by incubation with biotinylated alkaline phosphatase, and then an enzyme reaction with the phosphoric acid ester of [(4-hydroxyphenyl)aminocarbonyl]-cobaltocenium, a substrate leading to the sensitive determination of alkaline phosphatase (AP).[12] The phenolic product, enzymatically generated in the droplet (35 μL) deposited on the SPE surface, was quantified by anodic LSV (peak current $i_{p,a}$ located at ∼+0.85 V vs Ag/AgBr). A series of experiments were performed with a first set of biotin-modified SPEs in order to control the specific and nonspecific bindings, and the results are summarized in Table 1 (entries 1−4, first run).

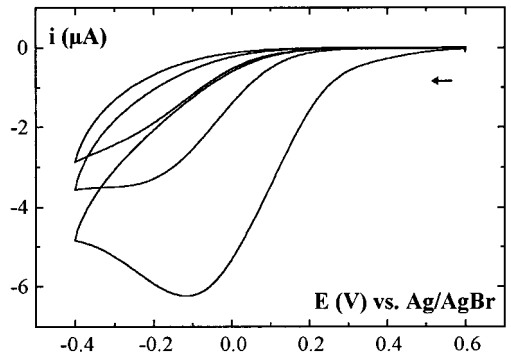


Figure 1. Repetitive cyclic voltammetry at a SPE immersed in a 0.5 mM *p*-aminobenzoyl biocytin in 0.125 N HCl and containing 1.1 equiv of $NaNO_2$ (8-fold dilution of the solution of diazonation). Scan rate: 0.2 V s^{-1}. Temperature: 4 °C.

Entry 1 corresponds to the standard procedure depicted in Scheme 1. In the absence of Extravidin (entry 2), it is clear that biotinylated AP failed to bind. Similar results were obtained when the binding sites of Extravidin are first blocked with biotin (entry 3). The experiment with native AP (entry 4) confirms that there is a low NSB at the Extravidin modified SPE. Finally, when the electrode surface was not biotinylated (entry 5), Extravidin poorly adsorbed on the polystyrene/graphite surface. A second set of biotinylated SPEs were saturated with Extravidin, as done previously, and then regenerated in a denaturant solution of guanidine hydrochloride.[5c] After rinsing with water, the biotinylated SPEs were reused in a second series of experiments (second run in Table 1). Under these conditions, the sensitivity was somewhat increased (entry 1, ∼30% of increase which remained after the third and fourth runs), whereas NSBs became negligible (entries 2−4). The decrease of NSB is suggested to be related to the blockage of holes in the biotin monolayer (the electrodeposition of biotin has not yet been optimized). A relative standard deviation of 12.8% was calculated ($i_{p,a}$ = 10.9 ± 1.4 μA, average of seven measurements at seven biotin-modified SPEs) under the second-run conditions, and the assay of Extravidin gave an estimated detection limit of ∼3 ng mL^{-1} (i.e., 1.4 fmole) with a continuous increasing response up to ∼1 μg L^{-1}.

The convenient electrochemical grafting of disposable SPEs by a monolayer of *p*-benzoyl biocytin and their reusability constitutes a promising versatile platform for the construction of electrochemical biosensor arrays, as well as for the supramolecular assemblage of well-organized mono- or multilayers of biomolecules. Moreover, the carboxylate function of the anchored biocytin may serve to chemically graft a supplementary species such as a redox mediator, and this latter approach might open new opportunities to study factors that govern the electrical communication between the electrode surface and biomolecules.[4b,5d]

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