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Covalent immobilization of oligonucleotides on *p*-aminophenyl-modified carbon screen-printed electrodes for viral DNA sensing

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DNA-sensing platforms were prepared by covalently attaching oligonucleotide capture probes onto *p*-aminophenyl-functionalized carbon surfaces and applied to the determination of an amplified herpes virus DNA sequence in an electrochemical hybridization assay.

Electrochemical nucleic acids sensors have gained considerable interest in the field of genetic diagnostic since they allow the convenient and inexpensive sequence-specific DNA detection of a PCR-preamplified product through its hybridization with a single-stranded oligonucleotide capture probe bound onto the electrode surface. The immobilization of capture probes on electrode surfaces is thus a crucial step to obtain reliable DNA hybridization platforms.[1] A covalent attachment on gold electrodes is usually achieved by self-assembling a high density of thiol-functionalized oligonucleotides[2] while many strategies have been developed to improve the selectivity and the sensitivity of the hybridization tests on carbon electrode surfaces.[3–9] Adsorption is one of the simplest techniques to immobilize nucleic acids on carbon surfaces because it does not require reagents or special oligonucleotide modifications.[3–5] However, the resulting multiple-point linkage usually leads to poor hybridization efficiency. Moreover, the release of the nucleic acids from the surface during the hybridization is a potential problem. The covalent attachment of an oligonucleotide monolayer on a chemically-functionalized carbon surface appears more advantageous because it not only overcomes the drawbacks mentioned above but also allows the use of more stringent washing conditions to reduce the background and therefore to increase the sensitivity of the assay. Consequently, the modification of carbon electrode surfaces is an important objective to further provide high oligonucleotide probes surface coverage with an accurate orientation. Chemical and electrochemical oxidation processes resulting in the generation of oxygenated groups on the carbon surface that can be further coupled with amine-terminated-nucleic acids have been proposed to build DNA-sensing surfaces[6,7] but these corrosive methods often lead to a high capacitive current in electrochemical applications. Alternatively, DNA-sensing carbon surfaces can be prepared by electrodepositing a redox polymer that can subsequently covalently bind an amine-terminated oligonucleotide.[8,9]

In this paper, we report a non-corrosive method to covalently attach unmodified oligonucleotides on the surface of carbon screen-printed electrodes (SPEs).[10] Our strategy relies on the extension of a procedure which has been used for the covalent attachment of nucleic acids to the surface of cellulose or glass functionalized with diazonium groups.[11] During the coupling reaction, the aromatic rings of adenine, guanine and cytosine that all contain an amine electron-releasing group undergo an electrophilic attack by *in situ* generated diazonium ions. Here, nucleic acids were attached on chemically-modified SPEs made according the overall process schematically depicted in Figure 1a. Briefly, in the first two steps, we took advantage of a versatile non-corrosive approach based on the electrochemical reduction of diazonium salts to covalently grafting *p*-aminophenyl groups on the surface of SPEs (step (ii)) from an ice-cold aqueous acidic solution of a freshly prepared *p*-nitroaniline diazonium salt (step (i)).[12] As shown in Figure 1b, the cyclic voltammetry recorded at a SPE on which was deposited a microvolume of a *p*-nitroaniline diazonium salt solution exhibits a characteristic broad irreversible cathodic peak located at ~ +0.40 V *vs.* Ag/AgBr (A) which disappears after two cycles because of the generation of highly reactive radicals and their rapid grafting on carbon surfaces.[12] This first one-electron peak is immediately followed by a second irreversible six-electron wave at ~ −0.30 V *vs.* Ag/AgBr (B) which corresponds to the reduction of the nitro group into an amino group, and which therefore allows the grafting of the aromatic moiety and the reduction of the nitro group into an amino group to be achieved at the same time by setting the potential of the SPE at the beginning of the second wave (usually $E = -0.45$ V *vs.* Ag/AgBr).[12] Another way to generating *p*-aminophenyl functions on the surfaces of a second series of SPEs was also investigated. This procedure involved the grafting of *p*-nitrophenyl moieties by applying a potential of + 0.25 V *vs.* Ag/AgBr and their subsequent chemical reduction into aniline groups with a sodium dithionite solution.[11a] Because diazonium salts are unstable at room temperature, the aniline-functionalized SPEs were converted into diazobenzyl ones by treatment with a 30 µL droplet of a solution containing HCl and $NaNO_2$ (step (iii)) just before reacting with single-stranded nucleic acids (step (iv)). The covalent binding of a 25-mer synthetic oligonucleotide probe (P_1) was initially studied by epifluorescence microscopy using a 5′-Cy3-labeled oligonucleotide solution (P_1-Cy3). The images resulting from the *in situ* diazotized surfaces showed non-uniform fluorescent spots with a surface coverage of ~5% whereas at undiazotized SPEs (bare and aniline-modified SPEs) the absence of fluorescent signals indicated no non-specific bindings (NSB) of P_1-Cy3. Similar low P_1-Cy3 coverages were obtained for both series of grafted SPEs. Such a low coverage probably results from the multi-step procedure and the lack of stability of the diazotized species at room temperature. The one-step reductive electrochemical process was selected for the further studies.

Because the hydrophobicity of the aromatic groups grafted on the DNA-sensing platform should dramatically increase nucleic acids NSB in an hybridization test,[13] carboxylate groups were

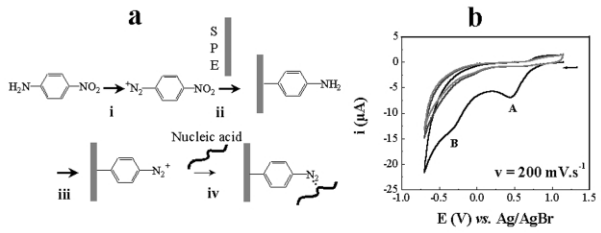

Fig. 1 a) Main steps involved in making diazotized carbon SPEs for the subsequent covalent immobilization of single-stranded nucleic acids. (i) *p*-nitroaniline (25 mM in 1 M HCl), $NaNO_2$ (0.11 M), ice-cold, 5 min; (ii) 30 µL droplet, −0.45 V *vs.* Ag/AgBr, 1 min, 4 °C; (iii) $NaNO_2$ (0.2 M, 30 µL), ice-cold, 30 min; (iv) oligonucleotide (10 µL, 60 mg.L^{-1}), 4 °C; 2 h. b) Repetitive cyclic voltammetry at an ice-cold SPE on which was pipetted a 30 µL droplet of an acidic solution of a freshly prepared *p*-nitroaniline diazonium salt.

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introduced in the *p*-aminophenyl monolayers by electrodepositing solutions made by mixing at several molar ratios the diazonium salt of the *p*-nitroaniline with that of the *p*-aminohippuric acid.[14] When the molar ratio *p*-aminohippuric acid/*p*-nitroaniline of the solution-phase was raised from 0 to 0.4, the surface coverage of P_1-Cy3 remained unchanged whereas the observed fluorescent response dramatically decreased for higher molar ratio. No other ways to prevent NSB have been investigated yet since the grafting of a carefully chosen amount of free carboxylate groups (i.e. which did not limit the covalent attachment of P_1) on the carbon surface appeared to be efficient enough to reduce the NSB during the hybridization assay described below.

The DNA-sensing surfaces were thus applied to the determination of a PCR-amplified 406 base-pairs human cytomegalovirus (HCMV) DNA sequences in a sandwich-type hybridization assay, which principle is sketched by the three main steps of Figure 2. Although single hybridization tests of HCMV DNA have been successfully achieved by our group,[5] the sandwich-type format on ready-to-use DNA-sensing surfaces should overcome the time consuming immobilization step of the PCR-amplified target before its analysis and improve the specificity of the assay. In this case, once denatured, 2 µL aliquots of the amplified HCMV DNA were pipetted onto the DNA-sensing platform and simultaneously hybridized with the immobilized capture probe P_1 and 15 µL of a 13 nm colloidal gold labeled detection probe (P_2-Au; 22 pb).[15] The hybridization step (step (1)) was followed by the release of the gold metal atoms anchored in the hybrids by the oxidative gold metal dissolution with an acidic bromide–bromine solution (step (2)) and the resulting Au^{III} ions were quantified by anodic stripping voltammetry at a screen-printed microband electrode (SPMBE) as previously described (step (3)).[5b] To demonstrate the utility and the specificity of the DNA-sensing surfaces in an electrochemical hybridization assay, steps (1) and (3) were deliberately carried out on an SPE (9.6 mm^2) and an SPMBE (8.5×10^{-3} mm^2), respectively.[16] As expected, preliminary experiments clearly demonstrated that the carboxylate function of the hippuric acid grafted from a diazonium salts solution-phase *p*-nitroaniline/*p*-aminohippuric acid molar ratio 70:30 significantly reduced (factor of 15) the NSB of P_2-Au on the DNA-sensing platform. The specificity of the sandwich-type hybridization on mixed-grafted surfaces was further investigated. As evidenced by the results summarized in Table 1, only the DNA-sensing platform made by covalently attaching P_1 led to a significant decrease of the NSB of P_2-Au and allowed the specific detection of the amplified HCMV DNA at 32 nM with an average peak charge Q_p of 0.394 ± 0.160 nC at a signal to noise ratio of 14. The reproducibility and the efficiency of the hybridization step might be improved by a better control of the covalent attachment of P_1, which randomly occurs between one of its A, C, G bases and the grafted diazonium salt.[11b] To favor the immobilization of P_1 by its 5′end, DNA-sensing SPEs are currently prepared with a 5′-terminated poly A, C, or G-spacer

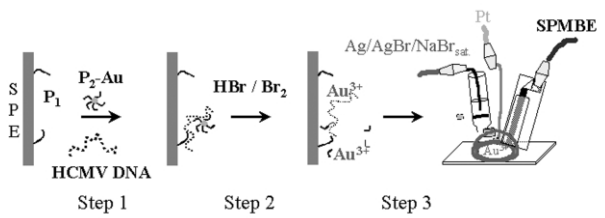

Fig. 2 Schematic representation of the HCMV-amplified DNA electrochemical sandwich-type hybridization assay. (1) One-step hybridization of the denatured HCMV DNA target (2 µL) in the presence of the gold-labeled detection probe (100 ng mL^{-1}; 15 µL), 37 °C, 30 min; (2) Oxidative dissolution of the gold tag in 0.1 M HBr containing 0.1 mM Br_2, 30 µL, 20 min, RT; (**3**) Detection of the released Au^{3+} at a SPMBE: 5 min electrodeposition at −0.3 V *vs.* Ag/AgBr followed by a positive linear sweep voltammetry (50 mV s^{-1}).

Table 1 Experiments for checking the specificity of the HCMV DNA sandwich-type assay on screen-printed surfaces reacted with P_1

SPE	HCMV DNA[c]	Q_p (nC)[d]
Bare	−	0.162
	+	0.286
Anilinated[a]	−	0.179
	+	0.248
Diazotized[b]	−	0.028 ± 0.024 (n = 3)
	+	0.394 ± 0.160 (n = 8)

[a] *p*-Aminophenyl groups were grafted on the surface of the SPEs from a diazonium salt solution-phase *p*-nitroaniline/*p*-aminohippuric acid molar ratio 70:30. [b] Once prepared as described above, the modified-SPEs were diazotized according to step (iii) in Figure 1. [c] The concentration of the amplified HCMV DNA was estimated at 32 nM by agarose gel electrophoresis. [d] Integration of the anodic stripping peak located at ~ +0.95 V *vs.* Ag/AgBr.

P_1. The first tests with P_1 containing a 5′-terminated-12A spacer yielded no significant improvements.

The electrochemical grafting of disposable SPEs by *p*-aminophenyl groups and their subsequent conversion to diazophenyl functions provides a convenient and versatile way to covalently link nucleic acids without prior chemical modification on carbon surfaces. This immobilization process also offers the possibility to produce ready-to-use DNA-sensing platforms since their storage at 4 °C for at least two weeks did not change their efficiency in the sandwich hybridization assay of HCMV-amplified DNA fragments. Work is in progress in our laboratory to achieve a higher capture probe surface coverage with an accurate orientation and to use the DNA-sensing platform for the final electrochemical detection.

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