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Hairpin-DNA Probe for Enzyme-Amplified Electrochemical Detection of *Legionella pneumophila*

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An electrochemical genosensor for the detection of nucleic acid sequences specific of *Legionella pneumophila* is reported. An immobilized thiolated hairpin probe is combined with a sandwich-type hybridization assay, using biotin as a tracer in the signaling probe, and streptavidin-alkaline phosphatase as reporter molecule. The activity of the immobilized enzyme was voltammetrically determined by measuring the amount of 1-naphthol generated after 2 min of enzymatic dephosphorylation of 1-naphthyl phosphate. The sensor allows discrimination between *L. pneumophila* and *L. longbeachae* with high sensitivity under identical assay conditions (no changes in stringency). A limit of detection of 340 pM *L. pneumophila* DNA, and a linear relationship between the analytical signal and the logarithm of the target concentration to 2 μM were obtained. Experimental results show the superior sensitivity and selectivity of the hairpin-based assay when compared with analogous sandwich-type assays using linear capture probes.

In recent years, intensive research has been performed to bring down the sensitivity of electrochemical DNA sensors to subnanomolar levels, so that the genoassays are suitable for various applications including clinical diagnosis, environmental control, and forensic analysis.[1,2] Identifying specific nucleic acid sequences of viral or bacterial pathogens, hereditary diseases, or genetic abnormalities requires not only high sensitivity but also excellent selectivity.

Sensitive detection of specific nucleic acids sequences on the basis of the hybridization reaction can be improved by target or signal amplification strategies.[3] The biotin−streptavidin interaction has been established as a representative system for amplifying the signal after hybridization. In this context, alkaline phosphatase−streptavidin conjugates have been used as amplifiers in the electrochemical detection of DNA.[4−10]

The most popular strategies to improve the selectivity of the hybridization assay rely on the control of the experimental conditions of the recognition reaction, i.e., high temperature,[11,12] low ionic strength,[13] high formamide concentration,[14] reaction time,[15] or even the application of an electrostatic field.[16] Alternatively, the advantage of a higher mismatch discrimination of locked nucleic acids (LNAs),[17] peptidic nucleic acids (PNAs),[18] and structured DNA probes such as DNA hairpins[19] has been used to recognize specific sequences.

DNA hairpins have been functionalized at one end with fluorescent[20,21] or electroactive[22−24] labels and then immobilized onto solid substrates to develop useful sensors for "label-free" detection of oligonucleotides. A microtiter assay with enzymatic

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Table 1. DNA Sequences Used in the Development of the Genosensor[a]

name	function	oligonucleotide sequences 5′→3′
Lp	target	AAGTTATCTGTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGG
H	hairpin capture probe	SH-$(CH_2)_6$-CGGCCAGTATTATCTGACCGTCCCATGGCCGT
S	linear capture probe	SH-$(CH_2)_6$-CCAGTATTATCTGACCGTCCCA
I_1	signaling probe	CACAGATAACTT-biotin
I_2	signaling probe	GGTTAAGCCCAG-biotin
I_3	signaling probe	GGTTAAGCCCAGGAATTTCACAGATAACTT-biotin
Ll	interference	AAGTNATCTGTGAAATCCCTRGGCTNAACCTGGGCAGGTCAGATGATACTGG
nH	interference	AAGTTATCTGTGAAATTCCTGGGCTTAACCACCCTGCCAGTCTATTATGACC
nI	interference	TTCAATAGACACTTTAAGGACCCGAATTGGTGGGACGGTCAGATAATACTGG
nHI	interference	GTAGCCGACAGTGCGACTAAACTTAGCTTGACTTGTACAGGACAGCCGAATA
R	interference (random sequence)	ATGTGGAAAATCTCTAGCAGT

[a] Within the interference sequences the bases that mismatch with the target are underlined. R: A or G; N: A, C, G, or T.

labeling and optical detection has also been recently reported.[25] In most of these published studies, short DNA molecules are used as solution targets. However, when the targets are longer than the probes, as is typically the case in biological assays, the secondary structure of the target can lead to significant limitation in hybridization specificity or even in the detection sensitivity.[26] The purpose of this work is the development of a method for the detection of DNA that combines, for the first time, the use of a hairpin oligonucleotide as capture probe, with a sandwich-type hybridization assay that can contribute to the disruption of the secondary structure of the target that otherwise could limit the efficiency of hybridization. The incorporation of an enzyme (alkaline phosphatase) for generating the output electrochemical signal offers the potential for signal amplification, thus improving the sensitivity. The analytical performance of this assay is compared with that of an enzymatic sandwich assay based on the use of linear oligonucleotide probes.

As a target model a 52-mer DNA sequence characteristic for *Legionella pneumophila* was selected. *L. pneumophila* is one of the most common pathogenic species in the world. This bacterium can be found in environmental water sources and cause sporadic as well as epidemic cases of legionellosis, a severe form of acute pneumonia. Although infection with non-pneumophila *Legionella* species (*Legionella longbeachae* and *Legionella micdadei*) can occur, its virulence is much lower. Conventionally, culture-based methods have been used for identification of *Legionella*, but they are time-consuming, a 10-day procedure according to the ISO 11731 norm. Genosensors could be a good alternative for the rapid, sensitive, and specific detection of *Legionella* species in clinical as well as in environmental samples.

EXPERIMENTAL SECTION

Chemicals. The oligonucleotide sequences used in this work were purchased from Sigma-Genosys (London, UK) as desalted products, and their sequences are listed in Table 1.

In order to immobilize the capture probes on the gold electrode they were functionalized at the 5′ end with a thiol group. These sequences were commercially supplied as the respective disulfides. To reduce the S−S bonds and obtain the SH terminal groups, these products were treated with 0.1 M dithiothreitol (DTT, Sigma, Spain) for 16 h at room temperature. The DNA-SH obtained was purified by passing through a Sephadex G25 column (NAP-10, Pharmacia Biotech). After elution with Milli-Q water, the concentration of the thiolated oligonucleotide was measured spectrophotometrically at 260 nm and subsequently stored at −20 °C. Stock solutions of 5.5 μM oligonucleotide were stored at 4 °C and diluted with 2 × SSPE buffer solution prior to use as needed for each specific experiment. The 2 × SSPE solution was prepared by dilution of concentrated saline sodium phosphate EDTA 20 × SSPE; 0.2 mol L^{-1} sodium phosphate, 3 mol L^{-1} NaCl, 0.02 mol L^{-1} EDTA from Sigma, Spain. 6-Mercapto-1-hexanol (MCH) obtained from Sigma was also dissolved in 2 × SSPE. Streptavidin-alkaline phosphatase ($Strp_2$−ALP) and hexaminerutenium (III) chloride were obtained from Sigma (Spain). 1-Naphthyl phosphate, 1-naphthol, 4-nitrophenol, 4-aminophenol, bovine serum albumin (BSA), and Tween 20 were purchased from Aldrich (Spain). All solutions were prepared with water purified with a Milli-Q (Millipore) system. Other chemicals employed were of analytical grade.

Instrumentation. Voltammetric and chronocoulometric experiments were performed using an Autolab PGSTAT10 electrochemical analyzer (Eco Chemie B.V., Utrecht, The Netherlands). A microcell that requires less than 500 μL of solution for analysis was designed by our group and made with methacrylate by Adepro (Avilés, Spain). The conventional three-electrode system was employed for all the measurements. A platinum wire served as counter electrode and a Ag/AgCl/KCl(sat.) electrode as reference. The working electrode was a 1.6-mm diameter polycrystalline gold electrode (Bioanalytical Systems, UK). Elimination of organic matter from the gold surface was achieved by cleaning it in piranha solution (30% H_2O_2 and 70% concentrated H_2SO_4; Caution: piranha solution is strongly oxidizing and should be handled with care!) for 10 min. Then the electrode was rinsed with Milli-Q water, and the surface was mechanically polished using slurries of alumina (1, 0.3, and 0.05 μm diameter successively, Buehler, Germany) on a polishing pad and ultrasonicated with Milli-Q water. To obtain reproducible surface conditions,[27] the electrode was subsequently immersed for 1 h in a hot 2 M KOH solution, rinsed with water, immersed in concentrated

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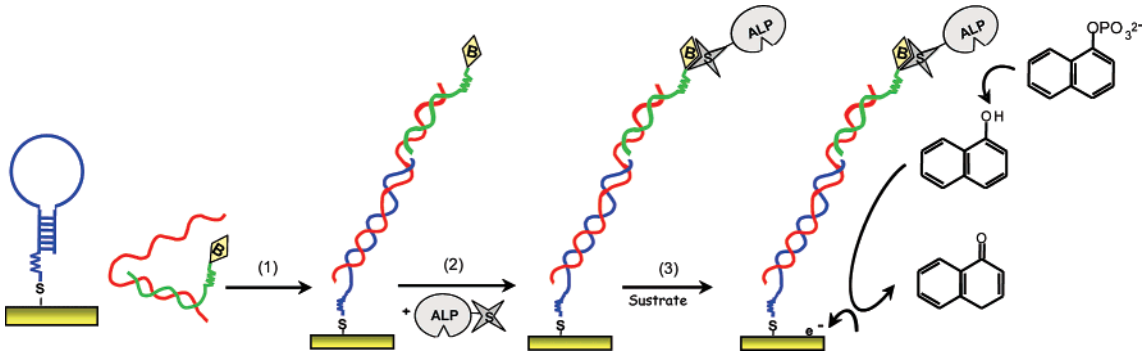

Figure 1. Schematic drawing of the setup for the recognition and electrochemical detection of DNA sequences specific of *L. pneumophila*.

H_2SO_4 for 10 min, rinsed with water, immersed in concentrated HNO_3 for 10 min, and thoroughly washed with Milli-Q water. The cleaned gold surface was checked by cyclic voltammetry in an oxygen-free 0.1 M H_2SO_4 solution, between 0 and 1.6 V, until an ideal redox wave of polycrystalline gold was obtained. The electrochemical surface area was calculated from the charge corresponding to the reduction of gold oxides.[28] In order to compare all the signals, the results were expressed as current densities.

Procedures. The DNA self-assembly process was performed under potential control because it was shown that the application of low positive potentials to the gold surface accelerates the chemisorption process and may assist in organizing the monolayer.[29] The clean gold electrode was initially subjected to 0.3 V for 5 min in order to ensure a reduced gold surface. Subsequently the electrode was immersed in 400 μL of 0.2 μM capture probe and incubated for 5 min under an applied potential of 0.4 V. The electrode was then thoroughly rinsed with water to remove the weakly adsorbed DNA and immersed in 4.5 mM MCH for 1 h to block the uncovered gold surface. Finally, the modified electrode was rinsed with Milli-Q water. The surface concentration of DNA immobilized on the electrode was quantified by means of chronocoulometry in the presence of 100 μM of hexaammineruthenium-(III) in 10 mM Tris-HCl pH 7.4.[30]

The sandwich-type format assay used consists of two steps. First, a homogeneous hybridization reaction takes place between 2 μM biotinylated signaling probe and variable concentrations of target in 2 × SSPE buffer. This hybridization solution was heated at 90 °C for 5 min and then allowed to cool to room temperature over 1 h, after which it was employed in the heterogeneous hybridization reaction. For this step the modified electrode was washed with 2 × SSPE buffer for 2 min and then immersed into the above-mentioned hybridization solution under stirring for 1 h. Rinsing the electrode surface with water after hybridization is an important step to remove nonspecifically adsorbed sequences.

The biotinylated sandwich complex formed at the electrode surface was labeled with ALP. With this aim, the modified electrode was pre-equilibrated for 2 min with a 5 × SSPE solution containing 2% (w/v) BSA and 0.1% Tween 20 and then incubated in 4.3 × 10^{-3} g/L $Strp_2$−ALP in the same solution for 10 min. This incubation step was followed by thoroughly rinsing of the surface with 2 × SSPE buffer to eliminate detergents.

To perform the voltammetric detection, the electrode modified with the ALP-labeled sandwich complex was incubated at 37 °C with 400 μL of 1-naphthyl phosphate solution (4 × 10^{-3} M in 0.5 M Tris-HCl pH 9.8 containing 1 mM $MgCl_2$). After 2 min, a DPV voltammogram from 0 to 0.5 V was recorded (modulation amplitude 50 mV, scan rate 10 mV s^{-1})

RESULTS AND DISCUSSION

Sensor Design. The strand chosen as target takes part in the 16S r-RNA subunit sequence of *L. pneumophila* that is included in the GenBank database (http://www.ncbi.nim.nih.gov), accession number M59157. The target is a 52-mer oligonucleotide whose bases appear between numbers 595 and 645, both included; this is the specific sequence used to detect this pathogenic bacterium by PCR protocols.[31]

For this target sequence a capture probe with a stem-loop structure was designed. Generally, only the loop sequence is complementary to the target, but problems could arise if the target sequence is very long, because a very long stem would be needed to maintain hairpin closed, and this could cause the stem not to be open in the presence of the target. For that reason a 19-base loop sequence and 6-base pair stem was designed in which the overall loop and three bases of the stem were complementary to the target at the 3′ terminal. Mfold Web Server[32] was used to predict the solution-phase conformation of this sequence. This program predicts that under the hybridization conditions adopted for the analysis, only one secondary structure (the hairpin one) is thermodynamically stable, confirming the suitability of the chosen sequence. According to this program, one extra T at the 3′-end was added to avoid the special separation of the terminal base-pair of the stem.

The 5′ thiolated hairpin probe was immobilized on the gold surface through the formation of a self-assembled monolayer (SAM). For this, the gold electrode was immersed into 2 × SSPE buffer solutions containing different amounts of the probe from 0.02 μM to 2 μM for 5−30 min. The extent of blocking and the presence of pinholes were qualitatively assessed by cyclic voltammetry measurements in 0.1 M phosphate, 0.1 M KCl (pH 7) in the presence of 2 × 10^{-3} M hydroquinone as a redox probe. We

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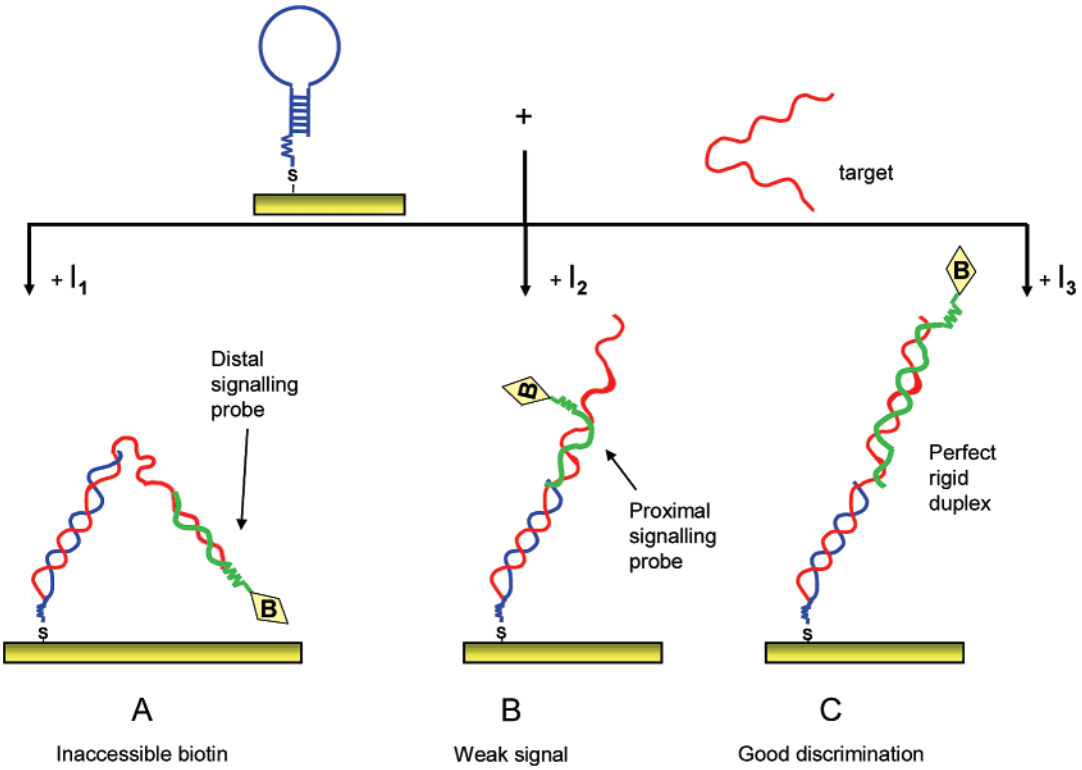

Figure 2. Strategies used in the present study for the introduction of biotin label in the sandwich complex.

found that a concentration of 0.2 μM for 20 min is sufficient to obtain a reproducible monolayer. When the formation of the monolayer is performed under a constant applied potential of +0.4 V, the time of deposition can be reduced to only 5 min. Thus, these conditions were used for all subsequent experiments. Blocking and reorganization of the DNA monolayer was achieved by means of a posterior treatment of the modified surface with MCH (4.5 mM for 1 h) as spacer thiol. Under these conditions a hairpin probe surface coverage of $(1.5 \pm 0.5) \times 10^{12}$ molecules/cm^2 was estimated by chronocoulometry.

The method for the electrochemical detection of a specific DNA sequence (Lp) using the above-described modified surface is schematically depicted in Figure 1. The target-containing sample is pretreated with the biotinylated oligonucleotide (signaling probe, I). Then, the sensing interface interacts with the double-stranded complex between the target DNA and biotinylated oligonucleotide, yielding a biotin-labeled three-component double strand on the transducer (step 1, Figure 1). Subsequent association of the Strep$_2$−ALP conjugated to the sensing interface (step 2, Figure 1) was followed by the enzyme-amplified electrochemical detection (step 3, Figure 1). ALP catalyzes the conversion of an electrochemically inactive monoester of phosphoric acid into an electrochemically active phenol whose oxidation peak is used as the biosensor response. Three commercially available substrates, i.e., 1-naphthyl phosphate, 4-aminophenyl phosphate, and 4-nitrophenyl phosphate, were tested. These compounds are enzymatically transformed into 1-naphthol, 4-aminophenol, and 4-nitrophenol, respectively. Cyclic voltammetry was used to examine the electrochemical properties of the enzymatic products on a mixed-SAM modified gold electrode. While 4-nitrophenol does not show electroactivity up to 600 mV, the voltammetric behavior of 4-aminophenol is affected by the mixed-SAM as is apparent from

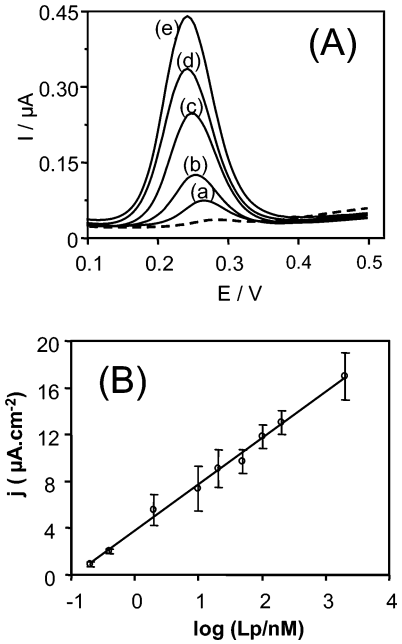

Figure 3. (A) DPV responses after hybridization with (dashed line) interference nHI sequence (2 μM) and (solid line) *L. pneumophila* nucleic acid sequence at concentrations of (a) 0.4 nM, (b) 2 nM, (c) 20 nM, (d) 100 nM, and (e) 2 μM. Background electrolyte: 0.5 M Tris/HCl pH 9.8, $MgCl_2$ 10^{-3} M. Scan rate 10 mV s^{-1}; pulse amplitude 20 mV. (B) Calibration plot.

the increased anodic to cathodic peak separation that makes this process difficult to use. 1-Naphthol exhibits an irreversible oxidation peak around 310 mV, which remains nearly unaffected by the mixed DNA−MCH monolayer. Additionally, 1-naphthyl phosphate is not electrochemically active in the potential window used (−0.3 to 0.6 V). Thus, 1-naphthyl phosphate was selected

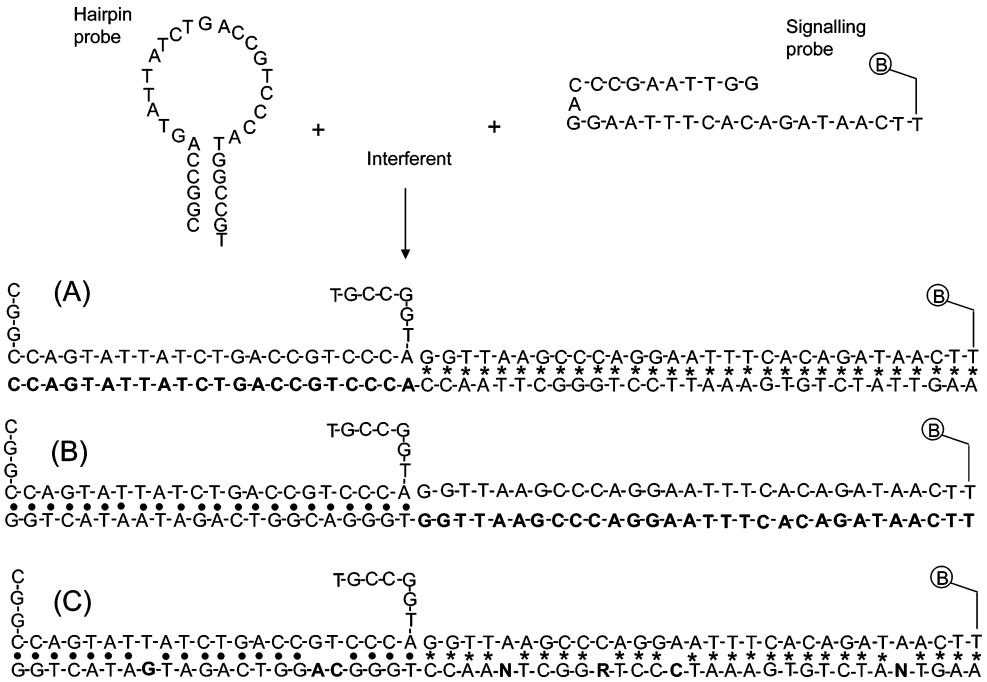

Figure 4. Resulting structure of the Watson−Crick duplex between the hairpin and signaling probes and three interference targets used in this study: (A) nH, (B) nI, (C) LI. The mismatched bases are depicted in bold. • represents matches with the hairpin probe and ∗ represents matches with the signaling probe. R: A or G; N: A, C, G, or T.

as substrate of ALP to measure the activity of the immobilized enzyme.

Three different signaling probes were tested (Figure 2). I_1 was a 12-mer complementary to the 5′ end of the target, I_2 was a 12-mer complementary to the first 12 bases of the target located immediately after the capture probe, and I_3 was the 30-mer fully complementary to the nonhybridized bases of the target. When using I_1, it was not possible to distinguish between sample and background. The highest target/background signals were obtained with I_3 (Table 1), which forms a perfect rigid duplex from the electrode surface to the biotin molecule. This can be explained by taking into account that the presence of single-stranded fragments contribute to the flexibility of the immobilized DNA molecule. This could allow biotin to reach the monolayer, making difficult its binding to the $Strep_2$−ALP conjugate and lowering the signal.

The voltammetric signal also depends on the time of incubation with the enzyme conjugate (step 2, Figure 1). The signal increases with time up to 10 min after which the catalytic current remains virtually unchanged. Therefore, 10 min was selected as the time of incubation in the enzyme conjugate solution for further studies. To determine the immobilized enzyme activity (step 3, Figure 1), a 0.5 M Tris/HCl buffer solution (pH 9.8), containing 1 mM $MgCl_2$ as enzyme activator, was selected because it promotes the transphosphorylation of the substrate. The amount of 1-naphthol generated after a fixed reaction time (reaction rate) can be used to determine the enzyme activity whenever the concentration of 1-naphthyl phosphate is superior to 10 K_M. Since the K_M value reported for ALP is 0.3 mM,[33] 4 mM 1-naphthyl phosphate was used for all experiments. Enzymatic reaction times varying from 1 to 20 min were tested. The highest signal/background ratio was obtained for 2 min at 37 °C, and this value was used for all subsequent experiments.

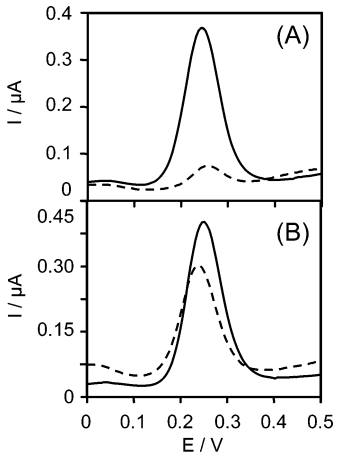

Figure 5. DPV for (solid line) *L. pneumophila* and (dashed line) *L. longbeachae* using (A) hairpin and (B) linear capture probe. The concentration of both sequences was 200 nM. Other experimental conditions are the same as in Figure 3.

Analytical Characteristics. Figure 3 shows representative DPV signals for different sensors hybridized under optimal experimental conditions with increasing concentrations of target DNA. The response signal increases with the increase of target concentration in the range 2×10^{-10} M to 2×10^{-6} M. The calibration curve is shown in Figure 3B. A linear relationship between the peak current density and the logarithm of the target concentration was obtained ($j/\mu A\ cm^{-2} = (3.9 \pm 0.1)\ \log([Lp]/nM) + 3.7 \pm 0.2$; $r = 0.996$; $n = 9$) as expected for an affinity interaction when the calibration is extended over a wide range of target concentrations. The background signal, obtained in the presence of a random, noncomplementary sequence (2 μM of interference R in Table 1) instead of the target DNA, was 0.62 ±

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0.07 μA cm^{-2}. DPV signal increases by 3.5 fold in the presence of 0.4 nM target. With these data the estimated limit of detection for the proposed assay is 340 pM, 170 fmol in 500 μL. The background is not dependent on the length of the noncomplementary strand, as the same value is obtained for a 52-mer, noncomplementary sequence (interference nHI). In addition, a similar voltammetric response was obtained when a modified electrode was immersed, prior to hybridization, in a 4 mM 1-naphthyl phosphate solution, suggesting that the presence of 1-naphthol in the commercially available 1-naphthyl phosphate is the main contribution to the background. Contribution of alkaline nonenzymatic hydrolysis of 1-naphthyl phosphate was evaluated experimentally under the assay conditions and was found to be negligible.

The reproducibility of the measurements, estimated as the relative standard deviation of four measurements with different electrodes at 0.4 nM concentration level, was 10%.

To evaluate the selectivity of the assay, we prepared three types of mismatched monolayer (Figure 4). The first one possesses the interference strand nI, 52-mer that contains 30 bases in the 5′ terminal that are noncomplementary to the signaling probe, resulting in a mismatch monolayer distal to the electrode surface. The second interference strand, nH, possesses 22 bases in the 3′ terminus that makes this sequence noncomplementary to the capture probe. Ll is a 52-mer sequence specific to *L. longbeachae* that contains between 4 and 7 mismatches when compared with Lp, giving rise to a monolayer with the mismatch in the middle of the three-component duplex adduct. Typical DPV of a perfect duplex and a duplex containing the Ll sequence at 200 nM are shown in the Figure 5A. We found that high selectivity could be obtained with no changes in assay stringency because the signal measured for 200 nM of nH, nI, or Ll were equivalent to that obtained for 0.3 nM, 0.4 nM, or 1 nM of Lp, respectively.

The ability of the sensor to distinguish between *L. pneumophila* and *L. longbeachae* specific sequences was further confirmed by incubating the sensor in a solution containing both sequences at a 200 nM concentration level. From this solution the same signal (13 μA cm^{-2}) as that from a solution containing 200 nM Lp (13 $\pm$ 1 μA cm^{-2}) was obtained.

Hairpin versus Linear Capture Probes. To clarify the role of the structured probe, we tested the same sensing scheme immobilizing a linear probe, S, instead of the hairpin probe H on the electrode surface. The linear probe surface coverage, (1.4 $\pm$ 0.2) $\times$ 10^{12} molecules cm^{-2}, is very close to that obtained for the hairpin probe. To comparatively evaluate the sensitivity of the hairpin and linear probes, electrodes containing surface immobilized H or S were prepared at the same time and then incubated into 2 μM random sequence (background) or 200 nM Lp. The peak current measured for the target increases by a factor of 21 with respect to the background for the hairpin probe, while the sensor modified with the linear probe only showed an 8-fold increase. With the purpose of investigating whether or not the hairpin probe improves the selectivity of the assay, a similar experiment was performed with 200 nM Ll sequences. Using the sandwich-assay with linear capture probes (Figure 5B) the voltammetric signal obtained for Lp was found to be only about 1.7 times higher than the signal obtained for the same concentration of Ll. At the same time, the signal for the interference Ll was significantly decreased when a hairpin probe was used. These data clearly show the high selectivity of hairpin probes compared with linear probes. The enhanced selectivity can be attributed to the loop-stem structure of the former, which stabilizes the dissociated state of the probe−analyte duplex, especially in the presence of mismatched base-pairing.

CONCLUSIONS

We have described, for the first time, a DNA electrochemical sensor based on an immobilized hairpin as capture probe in a sandwich-hybridization format assay combined with enzymatic amplification. This scheme allowed *L. pneumophila* to be detected at 340 pM level. We have shown that the improved selectivity is due to the higher ability of structured hairpin probes, compared to linear ones, to distinguish target from mismatched sequences. Thus *L. pneumophila* sequences can be detected in the presence of similar amounts of *L. longbeachae* ones under nonstringent assay conditions. In addition to this, the hairpin-based assay exhibits a signal to background ratio three times larger than the assay using linear capture probes.

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