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ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 2005

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Anal. Chem. **2005,** *77,* 6324−6330

Steric Factors Controlling the Surface Hybridization of PCR Amplified Sequences

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This study elucidated the hybridization behavior of surface-bound oligonucleotides to their longer PCR-amplified targets. The screen-printed gold surface of disposable electrodes was the platform onto which thiol-tethered oligonucleotides (21-mer) were immobilized by chemisorption. As a model case, ~600-bp amplicons were studied. Surface hybridization was monitored by means of an enzyme-linked assay with electrochemical detection. Use of different surface-tethered probe sequences over a wide range of surface densities was explored to achieve the highest duplex yield. Both the surface coverage by the probe and its relative position on the target strand were found to control the efficiency of capture of the target sequence. Interfacial hybridization occurred with the highest efficiency for a probe coverage of $\sim 2.9 \times 10^{12}$ molecules/cm^2 and when the 3′ end of the amplicon was involved. An unusual (bell-shaped) response/amplicon concentration profile was additionally found. It was hypothesised that when the amount of solution-phase target is relatively high, random collisions make reannealing of the ~600-bp strands favored over formation of the surface-tethered probe−amplicon complex. This paper also describes a strategy to enhance the sensitivity of enzyme-linked hybridization assays. Such a strategy relies on formation, around the long target sequence, of dendritic-like structures, which could offer multiple anchoring points for the enzyme conjugate. The results shown in this work might have great significance for the practical application of hybridization to oligonucleotide chips.

In the past few years, an increasing number of researchers have exploited surface-immobilized oligonucleotides[1] for a variety of applications, including drug discovery,[2] study of gene expression,[3] screening of genetic material for mutations,[4] investigation of the molecular basis of infectious diseases,[5] and sequencing of particular genes of interest among complex DNA samples.[6] In contrast to solution-phase hybridization, in which the concentration and the diffusion of all reactants affect the thermodynamics and the kinetics of the process, chip-based hybridization is heavily dependent on the construction of the probe layer at the surface. During hybridization, immobilized oligomers experience environmental conditions which significantly differ from those of analogous solution-phase reactions. Therefore, to elucidate how hybridization of such probes is influenced by nearest-neighbor interactions between immobilized strands and between immobilized strands and the solid surface, several investigations have been performed.

Shchepinov et al. explored the use of different spacer arms to mitigate the influence of the solid support (amino-modified polypropylene) on the hybridization behavior of immobilized 12-mer probes.[7] The optimal spacer length was determined to be at least 40 atoms in length, giving an up to 150-fold increase in the yield of hybridization, as compared to nontethered probes. Surface coverage was modulated using a combination of stable and cleavable linkers, giving the highest hybridization yields for surfaces containing ~50% of the maximum concentration of oligonucleotides.

Chemisorption of 25-mer thiol-derivatized oligonucleotides onto gold substrates was extensively characterized using a number of methods, including XPS, ellipsometry, ^{32}P-radiolabeling,[8] neutron reflectivity,[9] and electrochemical methods.[10] The ionic strength of thiolated probe solutions was found to have a profound effect on surface coverage, with chemisorption greatly enhanced at high salt concentrations. The authors attributed this trend to minimization of intermolecular electrostatic repulsion between neighboring strands, which were efficiently shielded under the high ionic strength conditions. Precise control over surface coverage and

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10.1021/ac0506175 CCC: $30.25
Published on Web 08/27/2005

probe availability was additionally achieved by creating mixed monolayers of the thiol-tethered DNA probe and a spacer thiol (MCH). MCH largely displaced nonspecifically adsorbed oligos that interacted with the gold surface also or only through nitrogen-containing bases, thus dramatically increasing the availability of the surface-bound probes for specific hybridization. Neutron reflectivity measurements by Levicky's group[9] confirmed that after treatment with MCH, probe molecules "stand up" consistently with the primary attachment through the thiol group. Moreover, assembly of MCH created a compact layer, which efficiently prevented the nonspecific adsorption of the complementary or noncomplementary sequences on the gold surface. The authors also observed that hybridization of the surface-bound probe was highly dependent on surface coverage. A combination of steric and electrostatic hindrance, arising from too tightly packed thiol-tethered probes, was found to severely inhibit the hybridization of the complementary sequence. In contrast, a hybridization efficiency of ~100% was estimated for the optimized thiolated probe/MCH mixed monolayer.

The influence of probe surface coverage on both hybridization kinetics and efficiency of the process of capture of the 25-mer target was investigated by Georgiadis and co-workers using SPR spectroscopy.[11]

Krull and co-workers used an optical fiber biosensor to study the thermodynamics of surface hybridization for 20-mer oligonucleotides.[12] The density of the immobilized probe was found to govern the thermodynamic stability of the duplexes. Interestingly, the melting temperature observed in the presence of a central single-base mismatch was found to be significantly lower when thermal denaturation occurred at the surface of the optical fibers relative to that observed in bulk solution.

Mir and Southern used arrays of short oligonucleotide probes immobilized on a glass plate to study how the secondary structure of a tRNA target (76-mer) influences the formation of the corresponding surface duplex.[13] The authors found reasonably high hybridization yield only when regions which were not involved in intramolecular base-pairing formed the heteroduplex with the surface-bound probe.

The work of Huang et al. constitutes a rare example in which the hybridization efficiency of surface-tethered probes was investigated with respect to a polynucleotide target.[14] However, such a molecule (i.e., M13 phage DNA, circular, 7249 bases) was a single-stranded sequence, and the problems arising from DNA strands' reannealing were not encountered.

Despite the considerable research efforts devoted to the characterization of interfacial oligonucleotide-oligonucleotide interactions, to these authors' knowledge, no efforts have yet been made to extend these studies to the detection of longer, double-stranded sequences. This paper investigated the hybridization behavior of 21-mer surface-bound probes to their longer PCR-amplified targets. Specifically, the surface used in this study was that of disposable screen-printed gold electrodes, for which thiol-tethered oligonucleotides were immobilized by chemisorption. As far as the PCR products are concerned, a case of species-specific analysis is illustrated in this paper. The aim of this analysis was to discriminate Tetraodontidae (puffer fish), which could be toxic during a certain period of the year, from Lofhiidae (angler fish), which are not. Careful selection of a unique couple of primers allowed amplification of a region of mitochondrial DNA of both *Lophius budegassa*, which belongs to the Lophiidae family, and *Takifugu niphobles*, which belongs to the Tetraodontidae family. Fragments (605- and 599-bp) of the gene encoding the 16S ribosomal subunity were amplified from *L. budegassa* and *T. niphobles*, respectively. Interestingly, this region of the mitochondrial DNA shows significant changes in the base sequences (polymorphisms) among these two species. Surface hybridization was detected by means of electrochemical measurements. The sequences belonging to each fish species were selectively identified through a sandwich hybridization assay[15−18] using biotinylated signaling probes specific for either one or the other species. Coupling of a streptavidin−alkaline phosphatase conjugate and subsequent incubation with its commercially available substrate, α-naphthyl phosphate, allowed an electroactive compound (α-naphthol) to be produced. Differential pulse voltammetry[19,20] was finally used to detect the α-naphthol oxidation signal.

The results of this study, which might have great significance for the practical application of hybridization to oligonucleotide chips, are described in the following sections. Additionally, a strategy to enhance the sensitivity of the enzyme-linked hybridization assay is presented. Such a strategy relies on formation, around the target sequence, of dendritic-like structures which can offer multiple anchoring points for the streptavidin-conjugated enzyme.

EXPERIMENTAL SECTION

Materials. Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), streptavidin−alkaline phosphatase, α-naphthyl phosphate, bovine serum albumin (BSA), Tris/HCl, magnesium chloride, and diethanolamine were obtained from Sigma-Aldrich. Disodium hydrogenphosphate, ethylenediamine tetraacetic acid (EDTA), and potassium chloride were purchased from Merck. MilliQ water was used throughout this work. TE buffer 20× (200 mmol/L Tris/HCl; 20 mmol/L EDTA; pH 7.5), Picogreen, and λ-DNA standard solution (100 μg/mL), used for fluorescence measurements, were obtained from Molecular Probes. NAP-10 columns of Sephadex G-25 were obtained from Amersham Pharmacia Biotech.

Synthetic oligonucleotides were obtained from MWG Biotech AG. The sequences of thiol-tethered probes and biotinylated signaling probes are listed in Table 1. Prior to use, thiol-modified probes were treated with DTT and then purified by elution through a NAP-10 column of Sephadex G-25. All oligonucleotide stock solutions were prepared in 0.5 mol/L phosphate buffer (pH 7) and stored frozen.

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Table 1. PCR Amplified *Lophius budegassa* and *Takifugu niphobles* Sequences and Synthetic Oligonucleotide Probes[a]

	Lophius budegassa (605 bp)
001	**TCTGTGGCAAAAGAGTGGGAA***GAGCTTTGAGTAGAGGTGACA*GACCTACCGAG
054	CTTAGTTATAGCTGGTT**CCCTGGGAAATGGATAGGAGT**TCAGCCTTCTAAGTTCT
109	TCATTCCAACCCTTCAACAAGGTAAAAAGAAATCAGAAGAGTTAATCAACGGG
162	GGTACAGCCCCCTTGAAAAAAGATACAACTTTAGCAGAAGGATAAAGATCATA
215	CGAAATTTAAAGGAGAGTCCTCTTGGTGGGCCTAAAAGCAGCCACCCCGATAGA
269	AAGCGTTAAAGCTCAAATACGGAACGCCCTATATATTCTGACAACCTAATCTTA
323	ATCCTCTAAAACTACCAGGAAGCCCTATGCAAGCATAGGAATTATACTGCTAAT
377	ATGAGTAATAAGAGAACATAAGATTCTCTCCTAGCACAAGTGTAAATCGGAACG
431	AACCCCCACCGAAAATTGACGGCC**CCAAGAAAAGAGGGAACTG**GATGAAAAAT
484	AAAAAACTAGAAGAACACCCAACAATTAACCGTTAACCTCACACCAGAGTGCC
537	ACCAGGGAAAGACTAAAGGGGGGAGAAGG**AACTCGGCAAACACACCCA**AGCC
589	TCGCCTGTTTACCAAAA
	Takifugu niphobles (599 bp)
001	**TCTGTGGCAAAAGAGTGGAAA***GAGCTTTGAGTAGAGGTGAC*AAACCTACCGAA
054	TTCAGTTATAGCTGGTT**GCCCGTGAATTGAGTATAAGC**TCAGCCTTTTGGCTTCT
109	TAGCTCCACAGCTATATTCATAACCCCGACTTTAAGAAACCAAAAGAGTTAATC
163	AAAGGGGGTACAGCCCATTTGATACAAGAAACAACTTTTAACAGGAGGATAAG
216	GATTATAAAAACCAAGGTATCGCGCTTAAGTAGGCTTAGAAGCAGCCATCACA
269	AGAAAGCGTTAAAGCTCTAGCACATCCCTACCACAAATACCAATAAACTACTCC
323	TAACCCCTCTCCCTACCGGGCTTTTCTATGCCCCCATAGAAAAAATTATGCTAAA
378	ATGAGTAATAAGGGGCCGACCCCCTCCAAGCACAAGTGTACATCGGAACGAAC
431	CCCCACCGAAATTTAACGGACCCATACAAAGAGGGAAGTAAATACTAAACTCA
484	CGACAAGAAAAACATTTAATAATCCTCCGTTACCCCTACACTGGTGTGCCAAAT
538	AGGAAAGACTAAAAGAAAAAGAAGGAACTCGGCAAACTCAAAGCCTCGCCTG
590	TTTACCAAAA
	Capture Probes
CP0	5′ HS − (CH2)6 − TCT GTG GCA AAA GAG TGG GAA − 3′
CP1	5′ HS − (CH2)6 − GAG CTT TGA GTA GAG GTG ACA − 3′
CP2	5′ HS − (CH2)6 − CCC TGG GAA ATG GAT AGG AGT − 3′
	Signaling Probes
SP2	5′-CCC TGG GAA ATG GAT AGG AGT−TEG−biotin-3′
SP3	5′-CCA AGA AAA GAG GGA ACT GG−TEG−biotin-3′
SP4	5′-AAC TCG GCA AAC ACA CCC AA−TEG−biotin-3′
SP5	5′-GCC CGT GAA TTG AGT ATA AGC−TEG−biotin-3′

[a] The bold and italic areas help in finding the position of the probes within the amplicons. CP2 and SP2 identified the first differentiating domain on *Lophius* sequences; SP3, the second one. The first differentiating region on *Takifugu* sequences was recognized by SP5.

PCR products obtained from the mitochondrial DNA of *L. budegassa* and *T. niphobles* were prepared at the Laboratorio di Sicurezza Alimentare ed Ambientale, Dipartimento di Chimica e Chimica Industriale, Università di Genova, Italy. Fragments (605- and 599-bp) of this DNA were amplified using primers that are common to both species and bound the zones characterized by polymorphisms. A biotin-labeled reverse primer was used to obtain biotinylated amplicons.

Prior to analysis, the PCR samples were purified using Millipore Montage PCR centrifugal filter devices according to the manufacturer's protocol.

The concentration of the amplicons was finally determined by fluorescence measurements using the Picogreen dye and a TD-700 fluorometer (Analytical Control).

Biomodification of the Sensor Surface. Materials and procedures to screen-print the gold electrode transducers are described in our previously published paper.[20] The morphology of the screen-printed gold layer was examined by AFM. AFM measurements highlighted the roughness of the working electrode, which is characterized by canyon-like depressions 1−2 μm deep. An electrochemically active surface of 7.05 ± 0.35 mm^2 was previously evaluated for such sensors.[18]

The gold surface was modified by interaction with 10 μL of the thiolated probe solution (0.15 μmol/L in 0.5 mol/L phosphate buffer). Chemisorption was allowed to proceed overnight (≈16 h) with the electrodes stored in Petri dishes to protect the solutions from evaporation. The immobilization step was then followed by treatment with a spacer thiol.[10] A 10-μL drop of the 1 mmol/L aqueous solution of MCH was placed onto the probe-modified surfaces for 1 h. Prior to hybridization reaction, the modified electrodes were washed twice with 15 μL of 0.5 mol/L phosphate buffer.

Hybridization with Synthetic Oligonucleotides and PCR-Amplified Samples. Hybridization experiments were carried out in both a direct and a sandwich-like format.

Biotinylated oligonucleotide sequences and biotinylated amplicons were directly hybridized with the surface-tethered probes. A 10-μL drop of the synthetic target solution (in 0.5 mol/L phosphate buffer) was interacted with the probe-modified electrodes for 1 h. A biotinylated noncomplementary sequence was used as the negative control. Biotinylated PCR products were diluted to the desired concentration using 0.5 mol/L phosphate buffer. The double-stranded DNAs were then thermally denatured by using a boiling water bath (5 min at 100 °C); amplicon strand reannealing was retarded by cooling the sample in an ice−water bath for 5 min. A 10-μL aliquot of this solution was finally placed directly onto the probe-modified electrodes for 1 h. Both the PCR

blank and noncomplementary PCR products were used as negative controls.

Unmodified PCR products were analyzed using the sandwich format. Biotinylated signaling probe (0.15 μmol/L) was included in the phosphate buffer used for diluting the samples. The analysis was then carried out according to the procedure described for biotinylated samples.

After hybridization, the electrode surface was washed twice with 15 μL of DEA buffer (0.1 mol/L diethanolamine, 1 mmol/L $MgCl_2$, 0.1 mol/L KCl; pH 9.6), to remove nonspecifically adsorbed sequences.

Labeling with Alkaline Phosphatase and Electrochemical Detection. The biotinylated hybrid obtained at the electrode surface was reacted with 10 μL of a solution containing 1 U/mL of the streptavidin−alkaline phosphatase conjugate and 8 mg/mL of BSA (blocking agent) in DEA buffer. After 20 min, the sensors were washed twice with 15 μL of DEA buffer.

The planar electrochemical cell was then incubated with 150 μL of an α-naphthyl phosphate solution (1 mg/mL in DEA buffer). After 20 min, the electrochemical signal of the enzymatically produced α-naphthol was measured by DPV (modulation time, 0.05 s; interval time, 0.15 s; step potential, 5 mV; modulation amplitude, 70 mV; potential scan, from 0 to +0.6 V). Upon scanning the potential, the α-naphthol was irreversibly converted into an electropolymerized derivative;[21] the height of its oxidation peak was taken as the analytical signal. All electrochemical measurements were performed with a μAutolab type II digital potentiostat/galvanostat (Eco Chemie). All potentials were referred to the silver pseudoreference electrode. The experiments were carried out at room temperature (25 °C).

RESULTS AND DISCUSSION

Selection of the Capture Probe: Role of Sequence Position and Probe Concentration. This study elucidated the hybridization behavior of immobilized oligonucleotides to longer amplicons. As a model case, the sequences from the edible species *L. budegassa* were differentiated from those of the toxic *T. niphobles* through the electrochemical analysis of the polymorphisms of the gene that encodes the 16S ribosomal subunity of their mitochondrial DNA. The analyses were performed on PCR-amplified samples obtained using primers that are common to both species and bound two regions characterized by several changes in the base sequences. The oligonucleotide probes used in this study and the sequences amplified from both fish species are shown in Table 1.

Several variables are known to affect both the solution-phase and the heterogeneous (interfacial) hybridization of DNA. These variables include salt (sodium) concentration, temperature, the presence of accelerating agents, contact time, length, and GC content of the probe sequence.[22] All hybridization experiments reported in this paper were performed at room temperature and using a 0.5 mol/L phosphate buffer, pH 7 (sodium concentration ~ 0.75 mol/L). Some control measurements were also performed using saline sodium citrate buffers (e.g., 4 × SSC = 60 mmol/L sodium citrate, 600 mmol/L sodium chloride), as widely described in the literature.[22] No evident advantages were found with the use of such buffers (data not shown), thus confirming the suitability of the phosphate one as the hybridization medium.

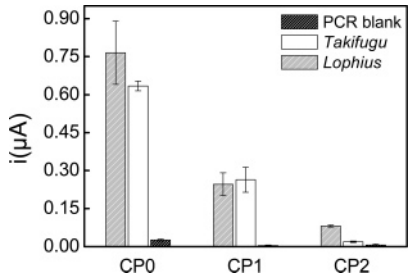

Figure 1. Influence of the relative position of the surface-bound probes with respect to the target strand on hybridization signals. Thiol-tethered probes CP0, CP1, and CP2 immobilization: 10 μL (0.15 μmol/L in 0.5 mol/L phosphate buffer) onto the gold working electrode surface overnight. Hybridization: 10 μL of PCR blank, *Lophius* and *Takifugu* biotinylated amplicon solutions (12 nmol/L in phosphate buffer) onto the probe-modified surface for 1 h. Other conditions as described in the Experimental Section. The graph bars are the average of at least three measurements, and the error bars are the corresponding standard deviation.

The hybridization experiments were first performed on biotinylated amplicons. Three different capture probes (CP), selected from the amplified sequences, were tested. As shown in Figure 1, CP2, which corresponded to the first discriminating region and was specific for *L. budegassa*, allowed selective identification of *Lophius* sequences; however, the magnitude of the specific signal (only 81 nA, ~4 times higher than the nonspecific one) demonstrated the limited hybridization capabilities of such a probe. Interestingly, the number of CP2 molecules undergoing hybridization remained poor, independent of their packing on the sensor surface (data not shown).

In contrast, significantly enhanced sensitivity was observed when using capture probes CP1 and CP0, respectively (see central and left bars in Figure 1). An overview of the results shown in Figure 1 seemed to suggest an interesting correlation between the position occupied by the surface-tethered probes (with respect to the total length of the amplified strand) and the magnitude of the analytical signals. The lowest signals were obtained using CP2, which recognized an inner region of the amplicon. CP1 (which allowed 3.0-fold enhanced signals) was located in the region which immediately follows the top primer used for PCR amplifications. CP0 (9.4-fold higher signals) largely overlapped such a primer and, therefore, recognized and bound the 3′ end of the biotinylated target. In contrast to solution-phase hybridizations, it appeared reasonable that the interfacial interaction—probe/PCR amplified target—occurred with a higher efficiency when one of the termini of the long amplicon was involved. In that case, the steric interference of the electrode surface, which clearly hinders access to target DNA and inhibits the recognition of an inner region of the amplicon, was minimized. Interestingly, the relative position of the probe on the target strand was not found to significantly affect the surface hybridization of relatively shorter PCR products (~200 bp or smaller; data not shown).

In an attempt to further elucidate the trend observed using CP0, CP1, and CP2, Mfold web server[23] was used to predict the

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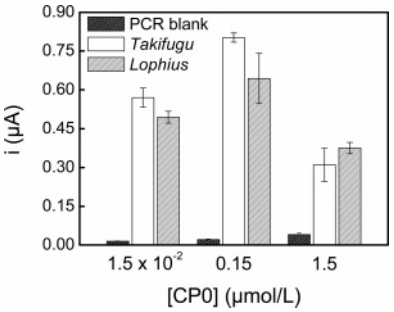

Figure 2. Influence of CP0 concentration on hybridization yield. CP0 immobilization: 10 μL (1.5×10^{-2}, 0.15 and 1.5 μmol/L in 0.5 mol/L phosphate buffer) onto the gold working electrode surface overnight. Other conditions as described in Figure 1.

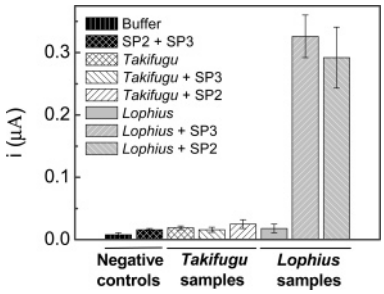

Figure 3. Selective identification of *Lophius* amplicons by means of a sandwich hybridization assay. Comparison between species-specific signaling probes SP2 and SP3. CP0 immobilization: 10 μL (0.15 μmol/L in 0.5 mol/L phosphate buffer) onto the gold working electrode surface overnight. Hybridization: probe-modified sensors were exposed for 1 h to the hybridization buffer, a mixture of both biotinylated sequences (each 0.15 μmol/L) and *Lophius* and *Takifugu* amplicons (12 nmol/L in phosphate buffer) in the absence and presence of SP2 or SP3. Further details are described in the Experimental Section. The graph bars are the average of at least three measurements, and the error bars are the corresponding standard deviation.

solution-phase conformation of the ~600-bp amplified sequences. This program showed that, under hybridization conditions adopted for the analysis, several secondary structures, due to intramolecular base-pairing, were thermodynamically stable. However, since such stable secondary structures involved the total length of the amplicon, the trend observed when using the different probes was unlikely to be attributed to the unavailability (or partial availability) of a specific recognition site.

A possible dependence of the observed trend from the different GC content of the three capture probes (52.4% for CP2 and 47.6% for CP0 and CP1) could also be excluded. Although CP2 should form the duplex with the highest thermodynamic stability, its hybridization signals were the less favorable.

CP0 was then chosen as the capture probe for subsequent experiments by virtue of its higher hybridization efficiency. Its optimal surface coverage was additionally assessed. The results obtained by varying the concentration of probe solution over 2 orders of magnitude are shown in Figure 2.

The highest hybridization yield was observed at the electrodes modified with 0.15 μmol/L of the thiolated probe solution; accordingly, this concentration was used for all subsequent experiments. Analogous immobilization accomplished on the screen-printed gold surfaces using a slightly different probe concentration (0.13 μmol/L) led to a probe coverage of $(2.9 \pm 0.4) \times 10^{12}$ molecules/cm^2.[18] Interestingly, this value closely approaches theoretically and empirically determined optimal probe surface densities for DNA hybridization of glass surfaces.[24,25] Concerning the diminished analytical signals observed using both lower (1.5×10^{-2} μmol/L) and higher (1.5 μmol/L) concentrations of the thiolated probe, they can be explained as follows. Electrodic surfaces characterized by low probe densities clearly possessed a limited number of biorecognition sites so that only a few amplified strands could be captured. In contrast, higher surface densities caused steric and electrostatic interference between tightly packed probes and incoming target DNA.[14]

Analysis of Unmodified Amplicons through Sandwich Hybridization. CP0 recognized a highly conserved region of *Lophius* and *Takifugu* mitochondrial DNA, and therefore, it was not suitable for a direct discriminating assay. Selective detection of each (unmodified) amplicon was achieved using biotinylated signaling probes (SP), which corresponded to highly differentiated (polymorphic) regions and, therefore, exclusively identified either one or the other species.

Signaling probes SP2 and SP3 were used in the case of *Lophius* amplicons. These secondary probes were, respectively, located 70 and 454 bases away from the 3′ end, the one which is bound by the surface-tethered probe. The analytical results obtained using either one or the other signaling probe are summarized in Figure 3.

A series of controls were performed to verify, first, the specificity of the response. The voltammetric signals obtained by exposing the CP0-modified surfaces to the hybridization buffer were comparable with those obtained for a mixture of both signaling probes. This demonstrated the absence of any nonspecific interaction of such biotinylated sequences with the capture probe. As further negative controls, both amplified samples were interacted with the CP0-modified surfaces in the absence of any signaling probe. Both amplicons were captured at the electrode surface; however, since none of them is directly able to bind the enzymatic conjugate, negligible analytical signals were detected. As expected, no signal was observed for *Takifugu* samples in the presence of one or the other signaling probe, because these amplicons do not possess any region complementary to the biotin-labeled sequences. In contrast, clear voltammetric signals were obtained when analyzing *Lophius* amplicons. Interestingly, the magnitude of the analytical signals obtained using SP2 and SP3 was nearly the same, irrespective of their relative position on the target strand (first or second discriminating region). Apparently, both the hybridization of the biotinylated sequences with the target strand and the subsequent binding of a single enzymatic conjugate in different domains of the hybrid were not sterically hindered and occurred with the same efficiency, independent of the conformation of the biomodified interface. Provided this interpretation is correct, the reliability of the enzyme-linked assay for evaluating the hybridization efficiency of the different surface-bound probes (see Figure 1) was demonstrated, because the magnitude of the electroanalytical signals would exclusively reflect the amount of target sequence bound by the capture probe.

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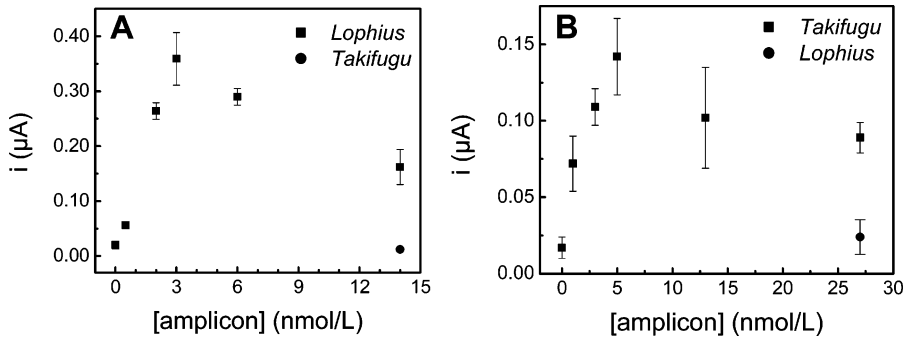

Figure 4. Influence of amplicon concentration on analytical signals. A. CP0-modified electrodes were exposed for 1 h to 10 μL of *Lophius* solutions (0, 0.5, 2, 3, 6, 14 nmol/L; 14 nmol/L only for *Takifugu*) in the presence of 0.15 μmol/L of SP3. B. CP0-modified electrodes were exposed for 1 h to 10 μL of *Takifugu* solutions (0, 1, 3, 5, 13, 27 nmol/L; 27 nmol/L only for *Lophius*) in the presence of 0.15 μmol/L of SP5. Further details are available in the Experimental Section. Each point is the mean of at least three measurements, and the error bars are the corresponding standard deviation.

Selective identification of *Takifugu* amplicons was accomplished at CP0 modified surfaces simply using a suitable signaling probe. The biotinylated probe SP5 was located in the first discriminating region, analogously to SP2 on *Lophius* amplicons. As in the previously discussed case, the experiments demonstrated the absence of any nonspecific interaction of SP5 with both the surface-tethered probe (signal = 2 ± 1 nA) and *Lophius* amplicons (signal = 1 ± 1 nA). The sequences amplified from *Takifugu* mitochondrial DNA were, on the contrary, unambiguously recognized (signal = 21 ± 4 nA). Currently, the reason these currents were significantly lower than those obtained for *Lophius* amplicons is not fully understood.

The influence of amplicon concentration on analytical signals was additionally assessed. The experiments shown in Figure 4A were carried out on *Lophius* amplicons in the presence of SP3. As the negative control, a sample from *Takifugu* was also analyzed. Although a negligible signal was observed for the nonspecific amplicon, thus confirming the selectivity of the genosensor, what was obtained by varying the target concentration was somewhat unexpected. The range of linear response, found between 0 and 3 nmol/L ($R^2 = 0.97$), was followed by a marked decrease in the signal. Such an unusual influence of sample concentration on the genosensor response was confirmed by analyzing different *Takifugu* samples in the presence of SP5 (Figure 4B). The electrochemical response rapidly rose with the sample concentration up to 5 nmol/L, decreased slowly, and then seemed to level off. The trend observed for both amplicons could not be explained in terms of saturation of all available surface-tethered probes. When analyzing both *Lophius* and *Takifugu* amplicons, the most favorable analytical signal was obtained for concentrations in the range 3−5 nmol/L. In contrast, using oligonucleotide targets, the analytical signals reached a plateau, but only for concentrations higher than 25 nmol/L (data not shown). A few hypotheses were formulated to explain the bell-shaped response/concentration profile. One of these attributed the decline of the signal to some fouling of the electrode surface by the huge nucleic acid target. However, the existence of a route which substantially diminished the number of target molecules available for hybridization to the surface-tethered probe was also considered. DNA hybridization depends on the random collision of the two complementary strands. Therefore, when the amount of amplified target freely moving in solution is relatively high, renaturation of the ~600-bp sequences could be favored over formation of the surface-tethered probe−amplicon complex.

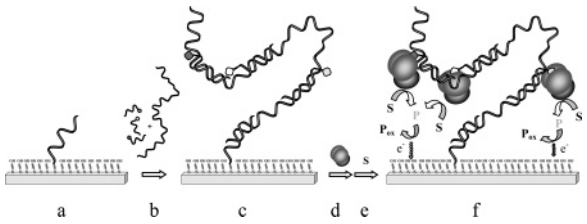

Figure 5. Schematic representation of the dendritic-like signal amplification path. Unmodified PCR products (b) were captured at the sensor interface (a) via sandwich hybridization with the surface-tethered probe and up to three biotinylated signaling probes. The polybiotinylated hybrid (c) was then coupled with streptavidin-alkaline phosphatase conjugates (d) and exposed to the enzyme substrate solution (e). DPV was finally used to detected the oxidation signal of the product of enzymatic hydrolysis (f).

Enhancing the Sensitivity of the Assay through a Dendritic-Like Signal Amplification Path. Over the past few years, optical,[26] microgravimetric,[15,27] and electrochemical genosensors[28−30] based on dendritic-like signal amplification paths have been described. However, several routes that can lead to formation of complex bioarchitectures still remain to be explored. As has been discussed, long amplified sequences require some tricks to be analyzed by surface hybridization, because their detection may be not straightforward. Nevertheless, the length of these amplicons, which often constitutes a problem, was turned into an advantageous means to increase the sensitivity of the electrochemical genoassay. Assembly of DNA/(enzyme)$_n$ superstructures was demonstrated by simultaneously using up to three signaling probes (Figure 5).

Binding of more than one biotinylated oligonucleotide per target sequence thus offered multiple anchoring points for the streptavidin−alkaline phosphatase conjugate. In addition to SP2

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Table 2. Dendritic-Like Signal Amplification Route: Influence of the Binding of Multiple Enzymatic Conjugates per Target Molecule on Analytical Results[a]

sample	i (nA)
phosphate buffer	11 ± 1
SP4	21 ± 2
[SP2 + SP3 + SP4]	29 ± 4
[*Lophius* + SP3]	426 ± 29
[*Lophius* + SP2 + SP3]	776 ± 18
[*Lophius* + SP2 + SP3 + SP4]	965 ± 18

[a] CP0-modified electrodes were exposed for 1 h to 10 μL of phosphate buffer, SP4 (0.15 μmol/L), a mixture of SP2, SP3, and SP4 (each 0.15 μmol/L), and *Lophius* amplicons (12 nmol/L) in the presence of one, two, or three signaling probes (each 0.15 μmol/L). Further details are available in the Experimental Section. Each result is the mean and standard deviation of at least three measurements.

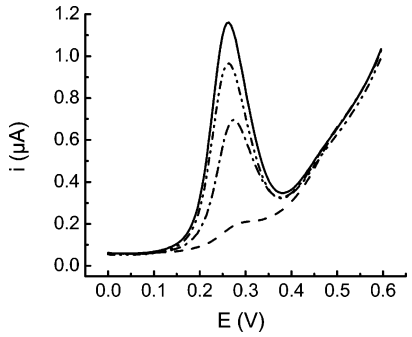

Figure 6. Typical DPV signals for CP0 modified electrodes exposed to the mixture of three signaling probes (— — —) and *Lophius* amplicons in the presence of one (—·), two (—··), or three (—) signaling probes. See Table 2 for further details.

and SP3, SP4 (which was located in a nondifferentiating region) was also included in the experiment to further validate the test. The results shown in Table 2 demonstrate the feasibility of this dendritic-like signal amplification route.

Confirming the specificity of the approach, neither SP4 (alone) nor the mixture of all biotinylated sequences exhibited a significant interaction with the surface-tethered probe. In contrast, simultaneous use of two biotinylated probes led to +82% enhanced signals. An additional +44% signal (+126% relative to the case of a single signaling probe) was observed when using three biotin-labeled sequences. Typical voltammograms from this set of measurements are shown in Figure 6.

The complexity of the surface-bound DNA/enzymatic conjugates assembly was basically limited by the steric hindrance of streptavidin-alkaline phosphatase, the footprint of which was estimated to be ~134 nm^2.[31] Within the 605-bp amplicon, SP2 and SP3 labeled with biotin the discriminating regions, which are sufficiently distant. Therefore, the simultaneous presence of two anchoring points for the enzymatic conjugate resulted in almost twice the analytical signals. In contrast, the change in sensitivity which followed addition of the third signaling probe was significantly lower. SP3 and SP4 occupied relatively close positions, and a gap of, at most, 35−40 nm (even assuming the full extended conformation for the sequences involved in the double-stranded hybrid) separated their biotins. Moreover, the steric and electrostatic interference from the conjugates bound to neighboring duplexes was likely to further inhibit the simultaneous binding of three enzymatic labels per target strand.

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CONCLUSIONS

This study offered valuable insights into the hybridization behavior of surface-bound oligonucleotides to their longer PCR-amplified targets. The results obtained in this work might have great significance for the practical application of hybridization to oligonucleotide chips.

The screen-printed gold surface of disposable electrodes was the platform to which thiol-tethered oligonucleotides (21-mer) were immobilized by chemisorption. Surface hybridization was monitored by means of an enzyme-linked assay with electrochemical detection. As a model case, the sequences from the edible species *L. budegassa* were differentiated from those of the toxic *T. niphobles* through the electrochemical analysis of the polymorphisms of the gene that encodes the 16S ribosomal subunity of their mitochondrial DNA.

Use of different surface-tethered probe sequences was explored to optimize duplex yield. The hybridization capabilities of these oligonucleotides were additionally tested over a wide range of surface densities. An interesting correlation between the position occupied by the immobilized probes on the 605-bp amplified strand and hybridization yield was found. The more peripheric the region of the target hybridized by the probe was, the easier the formation of the probe/amplicon complex was. The interfacial interaction—probe/PCR amplified target—occurred with the highest efficiency when the 3′ end of the amplicon was involved. Under these conditions, the steric interference of the electrode surface, which clearly hinders access to target DNA and inhibits the recognition of an inner region of the amplicon, was minimized.

Selective detection of unmodified amplicons was achieved using biotinylated signaling probes which corresponded to the differentiating regions and, therefore, exclusively identified either one or the other species. Interestingly, the magnitude of the analytical signals obtained using different signaling probes was nearly the same, irrespective of their relative position on the target strand.

An unusual influence of the sample concentration on the genosensor response was additionally found. It was postulated that when the amount of solution-phase target is relatively high, random collisions make reannealing of the ~600-bp strands favored over formation of the surface-tethered probe−amplicon complex.

This paper also described a strategy to enhance the sensitivity of enzyme-linked hybridization assays. Such a strategy relied on formation, around the long target sequence, of dendritic-like structures which could offer multiple anchoring points for the streptavidin-alkaline phosphatase conjugate. The length of the amplicons, which often constitutes a problem hindering the surface hybridization of these molecules, was thus turned into an advantageous means to increase the sensitivity of the genoassay. Simultaneous use of two or three biotinylated probes led to +82 or +126%, respectively, enhanced signals.

ACKNOWLEDGMENT

This work was supported by the Italian Istituto Superiore di Sanità (Contract no. 0M2/C1).

Received for review April 12, 2005. Accepted July 18, 2005.

AC0506175