Anal Bioanal Chem (2012) 402:413–421
DOI 10.1007/s00216-011-5361-0

ORIGINAL PAPER

Probe accessibility effects on the performance of electrochemical biosensors employing DNA monolayers

Vanessa Biagiotti · Alessandro Porchetta · Sara Desiderati · Kevin W. Plaxco · Giuseppe Palleschi · Francesco Ricci

Received: 5 July 2011 / Revised: 23 August 2011 / Accepted: 23 August 2011 / Published online: 19 September 2011

Abstract Surface-confined DNA probes are increasingly used as recognition elements (or presentation scaffolds) for detection of proteins, enzymes, and other macromolecules. Here we demonstrate that the density of the DNA probe monolayer on the gold electrode is a crucial determinant of the final signalling of such devices. We do so using redox modified single-stranded and double-stranded DNA probes attached to the surface of a gold electrode and measuring the rate of digestion in the presence of a non-specific nuclease enzyme. We demonstrate that accessibility of DNA probes for binding to their macromolecular target is, as expected, improved at lower probe densities. However, with double-stranded DNA probes, even at the lowest densities investigated, a significant fraction of the immobilized probe is inaccessible to nuclease digestion. These results stress the importance of the accessibility issue and of probe density effects when DNA-based sensors are used for detection of macromolecular targets.

Keywords Density · DNA sensor · SAM · Protein · Electrochemical

Vanessa Biagiotti and Alessandro Porchetta contributed equally to this work.

Published in the *10th Anniversary Issue*.

V. Biagiotti · A. Porchetta · S. Desiderati · G. Palleschi · F. Ricci
Dipartimento di Scienze e Tecnologie Chimiche,
University of Rome,
Tor Vergata, Via della Ricerca Scientifica,
00133 Rome, Italy

G. Palleschi · F. Ricci (✉)
Consorzio Interuniversitario Biostrutture e Biosistemi "INBB",
Viale Medaglie d'Oro 305,
00136 Rome, Italy
e-mail: Francesco.ricci@uniroma2.it

K. W. Plaxco
Department of Chemistry and Biochemistry,
University of California,
Santa Barbara, CA 93106, USA

Introduction

The use of biomolecules, for example DNA, antibodies, and enzymes, as effective recognition elements for molecular sensing has led, during recent years, to analytical devices with exceptional characteristics of versatility, specificity and affinity [1–4]. Such recognition elements are usually immobilized on a solid support to gain better sensitivity, reduce interferences, and enable reusability [5–9]. Optical techniques, for example SPR and ELISA, and electrochemical biosensors all share this common feature and are all based on the use of surface-confined biomolecular recognition elements. Simple adsorption, cross-linking with glutaraldehyde, and physical entrapment on membranes are some of the procedures adopted to immobilize biomolecules on solid supports [10]. Among these, use of thiol end-labelled biomolecules to form self-assembled monolayers on gold electrodes has increased impressively in recent years, especially for DNA-based sensors [11]. These are primarily focused on the development of sensors for detection of complementary DNA or RNA sequences [12–16]. However, in recent years, DNA probes, especially aptamers, have been also applied to the detection of non-nucleic acid targets [17–20], including macromolecular targets such as thrombin [21, 22], VEGF [23] and C-reactive protein [24]. Another broad and important class of analytes that can be detected via their interactions with DNA are naturally occurring DNA binding proteins,

transcription factors, and anti-DNA antibodies [25–27]. Finally, DNA probes have been also used as physical scaffolds for presentation of small recognition elements (usually antigens) to antibodies, thus expanding the range of targets recognized by use of these techniques [28–31].

In all recent examples by our group and by other groups that developed E-DNA signal-off sensors for electrochemical detection of macromolecular targets a common feature was always observed—a significant residual current signal is obtained for all sensors, even at saturating target concentrations [25, 26, 28, 29]. This raises the question whether the residual current signal is because of a fraction of the probes that are not accessible to target binding or because of other reasons (for example, target bound probes could still give a specific redox signal). Moreover, the effect that packing density can have on the sensitivity and efficiency of the DNA–target interaction could be of importance because of the increasing use of surface-confined DNA probes as recognition elements (or presentation scaffolds) for detection of proteins, enzymes, and other macromolecules. Recently, we and others have stressed the importance of the density with which the DNA probes are packed on the sensor for detection of the hybridization event [32–37]. However, because of different steric and charge properties between proteins and DNA sequences, earlier reports do not seem directly applicable to the optimization of DNA sensors targeting macromolecules. For this reason here we expand on this issue and address the accessibility of proteins on DNA monolayers and the effect of DNA probe density on the detection of DNA/protein interactions using electrochemical DNA sensors.

Materials and methods

Reagents and DNA probes

Reagent-grade chemicals, including 6-mercapto-1-hexanol, sulfuric acid, monobasic and dibasic potassium phosphate, and sodium chloride (all from Sigma–Aldrich, St Louis, Missouri, USA) were used without further purification. Bovine pancreatic nuclease (deoxyribonuclease I, denoted DNase I) was purchased from Sigma–Aldrich. This is an endonuclease that hydrolyses double-stranded and single-stranded DNA in the presence of divalent cations. DNase I attacks each strand of DNA independently and the cleavage sites are random [38].

The single stranded (ss) and double stranded (ds)-DNA probes employed in this work were synthesized, labelled, and purified by BioSearch, Tech (Novato, CA, USA) and used as received. Their sequences are as follows:

1. ss-DNA probe: a 27-base, 5′ thiol-modified, 3′ methylene blue (MB)-modified probe DNA was used as ss-DNA probe. The 17 internal bases of this sequence are complementary to the *Salmonella gyrB* gene [35]. The MB redox moiety was conjugated to the 3′ end of the oligonucleotide via succinimide ester coupling to a 3′ amino modification producing the probe sequence 5′-HS-$(CH_2)_6$-CGTCAATCTTCTATTTCTCCACACTGC-$(CH_2)_7$-NH_2-MB-3′.

2. ds-DNA probe: this probe is designed to have a two self-complementary sequences forming a 10-base double-stranded stem. The probe is modified with a thiol group at the 5′ end and with a methylene blue redox tag on a thymine base along the double-stranded stem (underlined below) and has the sequence 5′-HS-$(CH_2)_6$-CGGGCTA TAT*(MB)AAGGGGCGTTTTCTTATATAG-3′. Of note, the double stranded region of the probe is specifically recognized by the eukaryotic TATA-box binding protein (TBP, a core component of the eukaryotic transcriptional machinery).

TBP was obtained by expression of recombinant, his-tagged proteins in *Escherichia coli*, as described previously [25].

Sensor fabrication

The sensors were fabricated using standard approaches [39] that we reiterate in brief here. E-DNA sensors were fabricated on rod gold disk electrodes (2.0 mm diameter, BAS, West Lafayette, IN, USA). The disk electrodes were prepared by polishing with diamond and alumina (BAS), followed by sonication in water, and electrochemical cleaning (a series of oxidation and reduction cycles in 0.5 mol L^{-1} H_2SO_4, 0.01 mol L^{-1} KCl/0.1 mol L^{-1} H_2SO_4, and 0.05 mol L^{-1} H_2SO_4). Effective electrode areas were determined from the charge associated with the gold oxide reduction peak obtained after the cleaning process. The thiol-containing oligonucleotides we used are supplied as a mixed disulfide of 6-mercapto-1-hexanol in order to minimize the risk of oxidation. Thus the first step in sensor fabrication is reduction of the probe DNA (100 μmol L^{-1}) for 1 h in a solution of 0.4 mmol L^{-1} Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in 100 mmol L^{-1} NaCl, 10 mmol L^{-1} potassium phosphate, pH 7. The so-reduced relevant probe DNA was immobilized on the freshly cleaned electrodes by incubating for 1 h in a solution of 1 mol L^{-1} NaCl, 10 mmol L^{-1} potassium phosphate buffer, pH 7. Different probe densities were obtained by controlling the concentration of probe DNA used during the fabrication process, from 0.01 μmol L^{-1} to 1 μmol L^{-1}. We note here that our previous study with similar probes confirmed that probe density is linearly dependent on probe concentration used during sensor fabrication in this range of concentrations [35]. After probe immobilization the electrode surface was rinsed with distilled, de-ionized water passivated with 1 mmol L^{-1} 6-mercaptohexanol in

1 mol L^{-1} NaCl, 10 mmol L^{-1} potassium phosphate buffer, pH 7, for 2 h and followed by further rinsing with deionized water. Sensors were stored in the dark in buffer, under air, conditions that enable multi-month storage stability [40].

Calculation of probe surface density

Probe surface density (i.e., the number of electroactive probe DNA molecules per unit area of the electrode surface, N_{tot}) was determined by use of a previously established relationship with ACV peak current [41] described by Eq. (1):

$$I_{avg}(E_0) = 2nfFN_{tot}\frac{\sinh(nFE_{ac}/RT)}{\cosh(nFE_{ac}/RT)+1} \quad (1)$$

where $I_{avg}(E_0)$ is the average AC peak current in a voltammogram, n is the number of electrons transferred per redox event (with our MB label n=2), F is the Faraday current, R is the universal gas constant, T is the temperature, E_{ac} is the peak amplitude, and f is the frequency of the applied AC voltage perturbation. Perfect transfer efficiency was assumed (i.e., that all of the redox moieties participate in electron transfer); errors in this assumption would lead us to underestimate probe density. Experimentally, four different frequencies were used (5, 10, 50, and 100 Hz), and the average peak current was calculated to give the value of N_{tot} [42, 43]. To calculate mean probe densities from N_{tot}, we used the apparent surface area (see above).

Electrochemical measurements

The sensors produced as described above were tested at room temperature using an Autolab (EcoChemie, Utrecht, The Netherlands). Square-wave voltammetry (SWV) was recorded from −0.45 V to −0.05 V versus an external Ag/AgCl reference electrode and a platinum counterelectrode. The sensors were first left to equilibrate for ca. 30 min in a buffer solution (10 mmol L^{-1} phosphate+1 mol L^{-1} NaCl+ 5 mmol L^{-1} $CaCl_2$, pH 7.0). Of note, the presence of calcium ions is essential for nuclease activity and tests performed without this ion failed to furnish any evidence of hydrolysis of the DNA probe (data not shown). Once the sensor's signal was stable the desired nuclease concentration (3 mg mL^{-1}) was added to the solution and the resulting signal decrease was evaluated in real time by interrogating the electrode at regular intervals. Despite the different equilibration times for low and high probe-density sensors, signal suppression was evaluated for a maximum of 90 min for direct comparison. Unless otherwise specified the sensors were used a single time and then discarded. For experiments with ds-DNA probe and TBP the buffer was implemented with 10 mmol L^{-1} $MgCl_2$, because magnesium is essential for TBP binding [25].

Results and discussion

As a test bed for our study we employ here E-DNA sensors, a class of electrochemical device based on the use of electrode-bound, redox-tagged oligonucleotide probes [9]. The stable and reversible electrochemical signal generated by the redox label (here methylene blue) conjugated to the probe, gives a measure of the probe density on the sensor surface and of the efficiency of redox transfer. Upon binding of the target to the DNA probe, a signal decrease is observed because of the reduced efficiency with which the attached redox tag strikes the electrode and transfers electrons [9]. We and others have demonstrated the possible use of this type of sensor for the detection of DNA

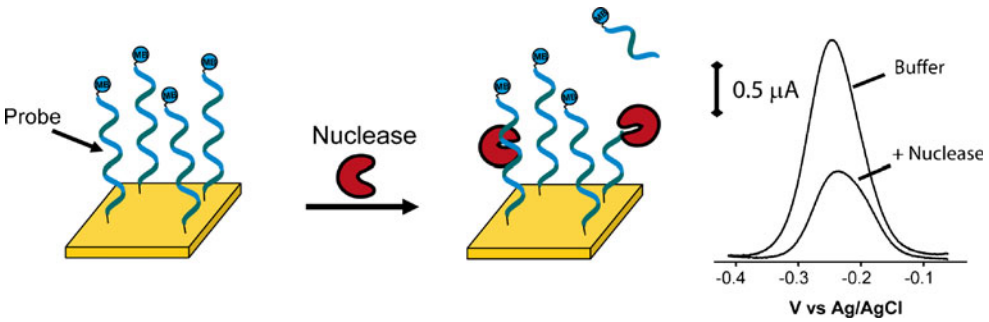

Fig. 1 To investigate the effect of probe density on the efficiency of the interaction between a DNA probe self-assembled on the surface of a gold electrode and a macromolecular target recognizing this probe, we have used here a classic E-DNA sensor based on the use of an electrode-bound, redox-tagged oligonucleotide probe. The stable and reversible electrochemical signal generated by the redox label (here methylene blue) conjugated to the probe is reduced on binding of the target macromolecule (here nuclease enzyme). Because the interaction between the DNA probe and the enzyme leads to complete loss of the signal (because the redox tag is removed from the electrode surface), the residual signal observed after nuclease digestion gives a measure of the percentage of the probe not accessible to protein binding. The figure depicts the sensor obtained with the ss-DNA probe (27 base) and the signals observed before and after (90 min) incubation with 3 mg mL^{-1} nuclease (density of the sensor shown here is 2.5 (±0.3)× 10^{12} molecules cm^{-2})

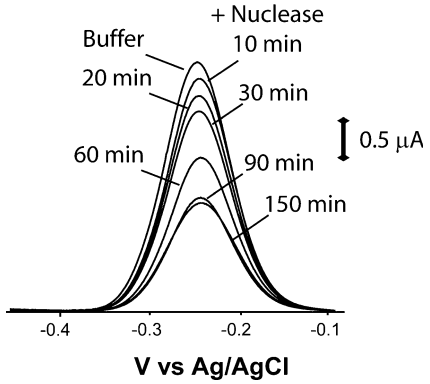

Fig. 2 E-DNA sensor modified with a ss-DNA probe (27 bases) gives a well defined SWV redox peak at the potential expected for methylene blue (~−0.25 V vs Ag/AgCl). In presence of a saturating concentration of nuclease (3 mg mL^{-1}) this signal is progressively reduced as nuclease leads to digestion of the probe and subsequent separation of the redox label from the electrode surface. The signal can be followed in real time during the reaction. Density of the sensor shown here is 2.5 (±0.3)×10^{12} molecules cm^{-2}

complementary sequences, proteins, or antibodies [9]. The reagentless nature of such devices results in unprecedented advantages over other techniques and has been demonstrated to be sensitive, selective, reusable, and suitable for use with complex samples, for example blood serum [9, 44]. By use of this technique we evaluated the accessibility of single-stranded and double-stranded oligonucleotide probes to interaction with macromolecular targets.

For our studies we used E-DNA sensors comprising either a 27-base, single-stranded probe (ss-DNA probe) or a 31-base, self-complementary hairpin probe (ds-DNA probe). Both were modified with a 5′ thiol group, supporting strong chemisorption to the interrogating gold electrode, and with a methylene blue redox tag at the 3′ end of the linear probe or pendant on a thymine base along the 10-base double-stranded stem of the hairpin probe. We interrogated both sensors with a non-specific nuclease enzyme (deoxyribonuclease I). Hydrolysis of the probe catalysed by the enzyme results in removal of the methylene blue label from the electrode surface and a subsequent decrease of the observed voltammetric signal (Fig. 1). Because only probes accessible to the nuclease will be hydrolysed, this test will give a valid measure of the percentage of probes in the monolayer that are accessible to this macromolecule.

In the absence of nuclease, the ss-DNA probe produces a large Faradaic peak at the potential expected for the methylene blue redox tag (Fig. 2). In the presence of high concentrations of nuclease (3 mg mL^{-1}) this current is progressively reduced as the DNA probe hydrolyses and the redox tag dissociates from the electrode surface. We find, however, that the reaction proceeds only until a residual current of about 50% of the initial current is reached after approximately 90 min of reaction (Fig. 2). This residual current signal remains even after prolonged incubation in the presence of nuclease (Fig. 2). We believe that this residual signal is because of the DNA probes that are inaccessible to the nuclease. Of note, diffusion to the electrode surface of the redox tags coming from hydrolysed probes does not produce any significant current signal.

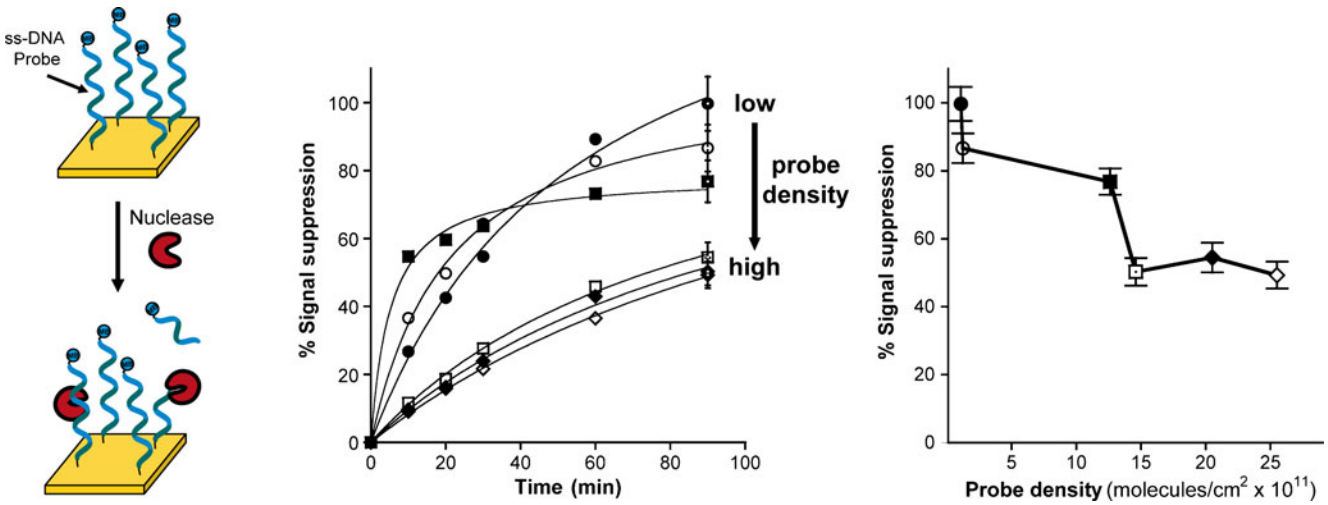

Fig. 3 DNA–protein interaction is strongly dependent on DNA probe density. We demonstrate this by fabricating E-DNA sensors of different probe densities by varying the concentration of ss-DNA probe employed during sensor fabrication and testing these sensors for 90 min in a solution containing a non-specific nuclease enzyme (3 mg mL^{-1}). At high probe densities only half of the immobilized probes are accessible to digestion by nuclease. The percentage of hydrolysed probes increases monotonically with the decrease of the probe density until it levels off at ~100% (*right*) at the lowest probe density we have investigated (1.1 (±0.1)×10^{11} molecules cm^{-2}). Percentage values of signal suppression as a result of nuclease digestion are the average and standard deviation of measurements performed with four independent sensors. Signal suppression values (*right*) were taken after incubation for 90 min

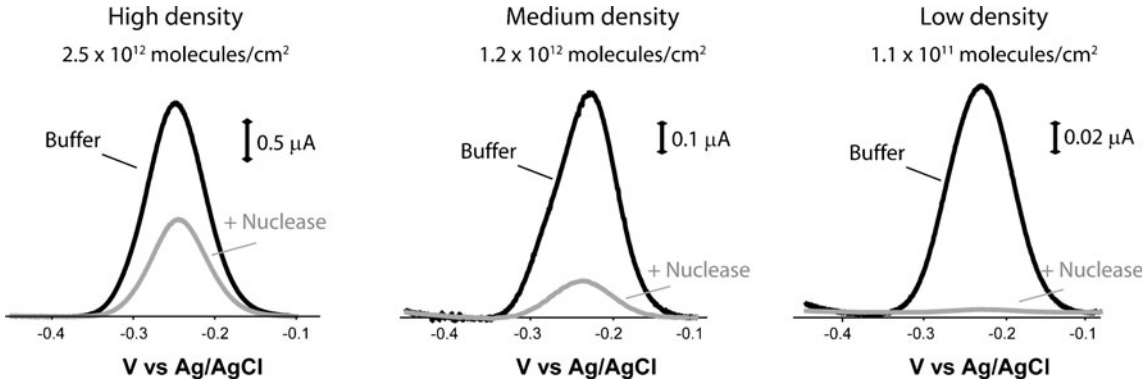

Fig. 4 Representative SWVs illustrating the dependence of signal suppression (after nuclease digestion) on probe surface density

To investigate the effect of the probe surface coverage on this interaction (and, eventually, on this residual signal) we fabricated sensors with different probe DNA densities. We controlled probe density by changing the concentration of the probe DNA used during sensor fabrication [35–37]. Using this approach, we can readily and reproducibly fabricate E-DNA electrodes with probe densities ranging from 1.1 $(\pm 0.1)\times 10^{11}$ to 2.5 $(\pm 0.3)\times 10^{12}$ molecules cm^{-2} (corresponding to packing of 1.7 $(\pm 0.2)\times 10^{-13}$ to 4.3 $(\pm 0.4)\times 10^{-12}$ mol cm^{-2}) by using probe DNA concentrations of 0.01 to 1 μmol L^{-1} during fabrication. Attempts to fabricate sensors with lower probe densities fail to produce stable, active films, and no electrochemical signal was detected. The observed probe density increases monotonically with increasing probe concentration until a density of 2.5 $(\pm 0.3)\times 10^{12}$ molecules cm^{-2} is obtained using a fabrication probe concentration of 1 μmol L^{-1}. Above this concentration, no further increases in probe density are observed. We find that the highest probe densities we investigated (2.5×10^{12} molecules cm^{-2}) produce the *smallest* signal suppression in the presence of nuclease (~50% at our test nuclease concentration) (Figs. 3 and 4). As probe density is reduced, the observed signal suppression increases before reaching a value of ~100% at 1.1×10^{11} molecules cm^{-2}, the lowest probe density we used (Figs. 3 and 4). Of note, the signal is completely suppressed only at the lowest density investigated where the average distance between the probes is high enough to enable DNA–protein interaction free from steric constraints. We also note that, as expected, equilibration time is faster and we observe plateau signal suppression after 90 min only at the lower probe densities we have investigated. The response times of sensors are in fact dependent on probe density and we observe equilibration time constants ranging from 5 to 25 min ranging from low to high probe densities (Fig. 3).

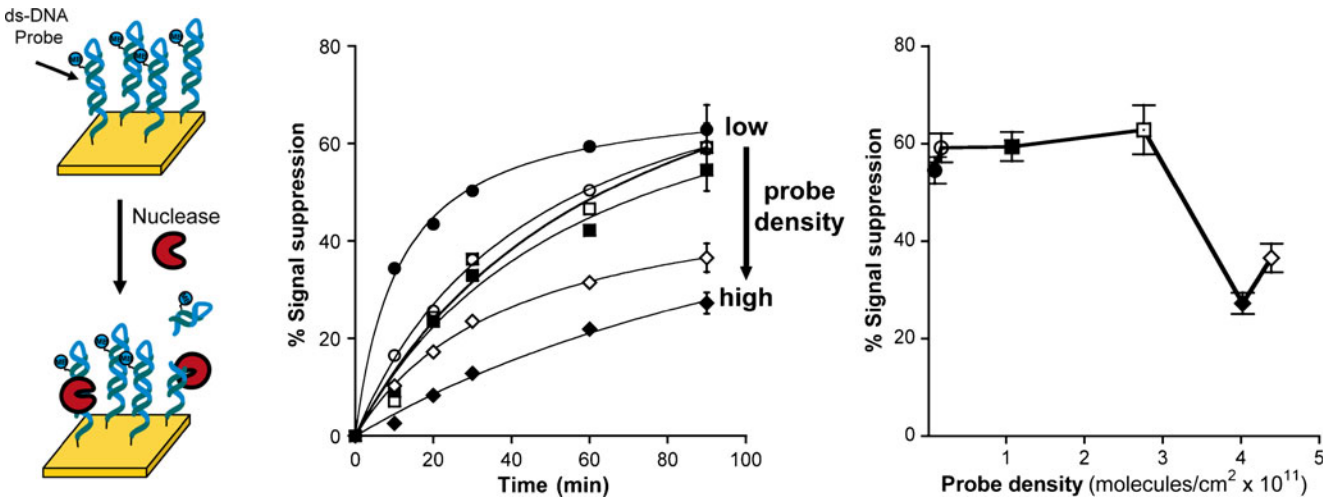

Fig. 5 Under our conditions the accessibility of double-stranded DNA probes with protein targets is somewhat more limited than that of their single-stranded counterparts. In fact, even at the lowest probe densities investigated (i.e. 0.09×10^{11} molecules cm^{-2}) only 60% of final signal suppression is observed, probably suggesting that steric and electrostatic repulsion between double-stranded probes do not allow the formation of a sufficiently well-ordered monolayer. Percentage values of signal suppression as a result of nuclease digestion are the average and standard deviation of measurements performed with four independent sensors. Signal suppression values (*right*) were taken after incubation for 90 min

Table 1 Limited accessibility of ds-DNA probes to macromolecular targets. The limited accessibility of double-stranded DNA probes to the binding of macromolecular targets is a common feature of other DNA-based sensors recently developed for the detection of macromolecular targets. Here we summarize the results obtained with E-DNA sensors recently optimized for detection of antibodies, transcription factors, and other proteins, and highlight that none of these sensors has ever achieved signal suppression higher than 65%. This, in our opinion, calls for optimization of ds-DNA monolayer formation to provide better accessibility to macromolecular targets

Target	Type of DNA probe	Maximum signal suppression (%)	Ref.
Endonuclease I	Self-complementary hairpin probe	60	This work
TATA-box binding protein	Self-complementary hairpin probe	65	[25]
M. HhaI	Self-complementary hairpin probe	50	[25]
Anti-DNA antibodies	ds-DNA (two complementary probes)	50	[26]
Generic antibodies	Antigen conjugated scaffold ds-DNA	55	[28, 29]
Thrombin	Single stranded G-quadruplex aptamer	40	[21, 34]

When the ds-DNA probe was used a trend similar to that observed with the single-stranded counterpart was observed. More specifically, higher probe densities enable only limited interaction with nuclease and result in low signal-suppression values (20–30%). Lower densities result in greater signal suppression, although, in this case, even at the lowest densities tested complete suppression of the signal was not obtained and we observed a plateau at approximately 60% (Fig. 5). Of note, in this case a steeper transition is observed as the probe density changes from 4.1 $(\pm 0.4)\times 10^{11}$ to 2.8 $(\pm 0.3)\times 10^{11}$ molecules cm^{-2}. This behaviour can be explained in terms of accessibility of the DNA probes to nuclease enzyme. It is likely that ds-DNA probes, because of their greater steric and electrostatic repulsion, will form more poorly ordered monolayers than their single-stranded counterparts. This would result in partial inaccessibility of some of the surface-confined probes even at the lowest densities investigated.

It is interesting to note that the plateau suppression percentage value we have observed in this study (i.e., ca. 60%) with ds-DNA probes compares well with the values observed in previous studies with E-DNA sensors and similar DNA probes tested with macromolecular targets. For example, when ds-DNA probes were used to detect DNA binding proteins or anti-DNA antibodies in two different embodiments of the E-DNA sensor, signal suppression of approximately 60% was observed with all the targets tested (more precisely, 65% with TBP, 50% with M. Hhal, and 50% with anti-DNA antibodies) [25, 26]. A very similar value was also observed with scaffold E-DNA sensors in which the binding of an antibody to a ds-DNA probe conjugated with an antigen was tested (average

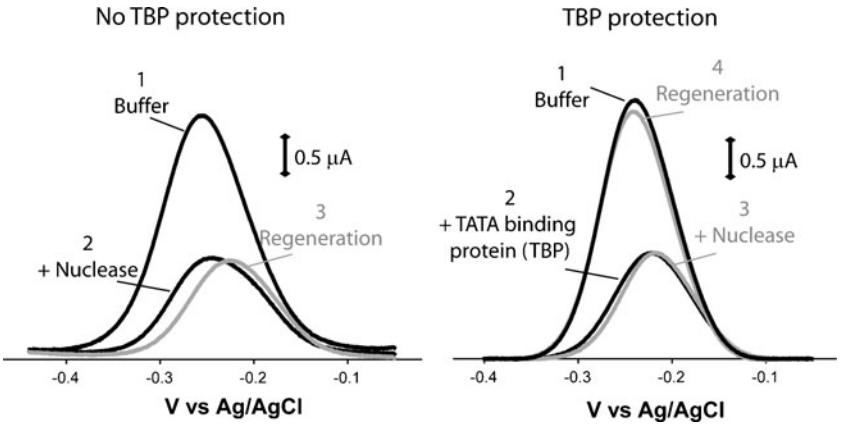

Fig. 6 Limitation of the accessibility of double-stranded DNA probes to macromolecular targets is not only limited to nuclease digestion. Here we demonstrate this by using a ds-DNA probe which is specifically recognized by a DNA binding protein (i.e. TATA-box binding protein, TBP). *Left*. In the absence of TBP, upon the addition of nuclease we observe a maximum signal suppression of approximately 60%. Because the probe is hydrolysed the signal cannot be restored (here regeneration is performed with a wash in 8 mol L^{-1} guanidinium chloride). *Right*. When the probe is first incubated with TBP we observe signal suppression comparable with that previously obtained with nuclease. However, in this case, injection of nuclease on to this sensor does not lead to any further signal decrease, because the TBP-bound probe is protected against digestion. Because the signal suppression observed is due to reversible binding of TBP only, a simple wash with 8 mol L^{-1} guanidinium chloride is sufficient to restore the initial signal and regenerate the sensor. This experiment demonstrates that a significant portion of ds-DNA probes is not accessible to protein interaction and is responsible for the residual current signal observed after the binding of the macromolecular target

suppression signals, 50%) [28, 29]. Also, the 60% maximum signal suppression observed is somewhat similar to the value obtained using the thrombin aptamer, a single-stranded DNA probe capable of forming a well-ordered secondary structure which is comparable in size and steric hindrance with a ds-DNA probe (Table 1) [21, 34]. In our opinion this suggests that in these previous examples binding of the macromolecular targets to their recognition DNA sequences completely suppresses the signal of the redox label conjugated to the bound DNA probes. The residual current can then be ascribed to DNA probes that are not accessible to their targets and that, even at a saturating concentration of the macromolecular target, give a non-trivial redox signal. To further support this hypothesis we have tested a ds-DNA probe which contains the cognate sequence recognized by a DNA binding protein (i.e. TATA-box binding protein, TBP) and have challenged it with its DNA binding protein target (i.e. TBP). As expected [25], the maximum signal change we observed under this condition plateaus at ca. 60% of the initial current signal (Fig. 6, right). Addition of nuclease to this solution does not lead to any further decrease of current signal, suggesting that the TBP binds to *all* the accessible probes on the electrode surface and that the residual current is due to unbound/inaccessible probes. In fact, it is well known that the binding of TBP and, more generally, of transcription factors to their cognate sequences results in protection from nuclease action. The fact that no decrease in current is observed after nuclease injection suggests the presence in the monolayers of a non trivial percentage of probes which is not accessible to protein interaction. Successful regeneration of the sensor by washing with 8 mol L^{-1} guanidinium chloride is an additional demonstration of this (Fig. 6, right). In fact, while the probes hydrolysed by nuclease cannot be regenerated (Fig. 6, left), it was demonstrated that TBP bound probes can be easily brought back to their unbound configuration by a simple distilled water wash [25].

Because most DNA-binding proteins target specific double stranded DNA sequences and because scaffold DNA-based approaches are achieved by hybridization of an anchoring and a recognition DNA strand, most DNA-based sensors (either based on electrochemistry or other surface analysis techniques) developed for detection of non-DNA targets make use of double-stranded DNA probes. For this reason, knowledge of the extent to which probe density affects the DNA–protein interaction would provide a general tool for optimization of other similar sensing devices [25, 26, 28]. We note here that several reports have previously demonstrated the effect of probe density on the hybridization efficiency and sensitivity of DNA sequence detection. However, there are very few reports dealing with the investigation of DNA–protein interaction on surfaces [34, 45–49]. Because the use of DNA monolayers for detection of macromolecules has recently been investigated by many research groups we believe this report could be of value in this perspective. The results we present in this work suggest, in fact, that the accessibility issue is crucial and should be carefully taken in consideration when DNA-based sensors are used for the detection of macromolecular targets. It should also be noted that, for analytical applications, it would be preferable to have a slightly higher density which could produce better defined and more reproducible current peak signals. For this reason, we believe it would be advisable to use probe packing densities between 0.5×10^{11} and 2×10^{11} molecules cm^{-2} by using probe concentrations between 10 and 50 nmol L^{-1} during fabrication.

It is also important to note that, while the results reported here were obtained with sensors fabricated with gold rod macroelectrodes where DNA–thiol mixed monolayers were produced by simple adsorption, it is likely that the use of gold microelectrodes, nanobeads, or ultraflat surfaces and the adoption of more accurate and precise techniques for SAMs preparation and density control (for example, nano-grafting [4, 46]) could lead to better accessibility of DNA probes to protein interaction. Moreover, in our opinion, the effect of the coadsorbate (in this work mercaptohexanol) could be an important determinant in this perspective and should be carefully taken into consideration to improve the accessibility of DNA monolayers to protein interaction [50]. These issues are currently under evaluation in our laboratory.

Acknowledgements The authors acknowledge members of our research groups for helpful discussions and comments on the manuscript. This work was supported by the Italian Ministry of University and Research (MIUR) through the project *FIRB* "Futuro in Ricerca".

References

1. Katz E, Willner I (2003) Probing biomolecular interactions at conductive and semiconductive surfaces by impedance spectroscopy: Routes to impedimetric immunosensors, DNA-sensors and enzyme biosensors. Electroanalysis 15(11):913–947
2. Liu J, Cao Z, Lu Y (2009) Functional nucleic acid sensors. Chem Rev 109(5):1948–1998
3. Wang J (2000) From DNA biosensors to gene chips. Nucleic Acids Res 28(16):3011–3016
4. Ricci F, Volpe G, Micheli L, Palleschi G (2007) A review on novel developments and applications of immunosensors in food analysis. Anal Chim Acta 605(2):111–129
5. Drummond TG, Hill MG, Barton JK (2003) Electrochemical DNA sensors. Nat Biotechnol 21(10):1192–1199
6. Wang J (2002) Electrochemical nucleic acid biosensors. Anal Chim Acta 469(1):63–71
7. Homola J (2003) Present and future of surface plasmon resonance biosensors. Anal Bioanal Chem 377(3):528–539

8. Lequin RM (2005) Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin Chem 51(12):2415–2418
9. Ricci F (2008) Plaxco KW (2008) E-DNA sensors for convenient, label-free electrochemical detection of hybridization. Microchim Acta 163(3–4):149–155
10. Cosnier S (1999) Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerized films. Biosens Bioelectron 14(5):443–456
11. Gooding JJ, Mearns F, Yang W, Liu J (2003) Self-assembled monolayers into the 21st century: Recent advances and applications. Electroanalysis 15(2):81–96
12. Fan C, Plaxco KW, Heeger AJ (2003) Electrochemical interrogation of conformational changes as a reagentless method for the sequence-specific detection of DNA. Proc Natl Acad Sci U S A 100(16):9134–9137
13. Kelley SO, Boon EM, Barton JK, Jackson NM, Hill MG (1999) Single-base mismatch detection based on charge transduction through DNA. Nucleic Acids Res 27(24):4830–4837
14. Tosar JP, Brañas G, Laíz J (2010) Electrochemical DNA hybridization sensors applied to real and complex biological samples. Biosens Bioelectron 26(4):1205–1217
15. Park J, Park S (2009) DNA hybridization sensors based on electrochemical impedance spectroscopy as a detection tool. Sensors 9(12):9513–9532
16. Vercoutere W, Akeson M (2002) Biosensors for DNA sequence detection. Curr Opin Chem Biol 6(6):816–822
17. Jayasena SD (1999) Aptamers: An emerging class of molecules that rival antibodies in diagnostics. Clin Chem 45 (9):1628–1650
18. Willner I, Zayats M (2007) Electronic aptamer-based sensors. Ang Chemie Int Ed 46(34):6408–6418
19. Xiao Y, Rowe AA, Plaxco KW (2007) Electrochemical detection of parts-per-billion lead via an electrode-bound DNAzyme assembly. J Am Chem Soc 129(2):262–263
20. Xiao Y, Piorek BD, Plaxco KW, Heeger AJ (2005) A reagentless signal-on architecture for electronic, aptamer-based sensors via target-induced strand displacement. J Am Chem Soc 127 (51):17990–17991
21. Xiao Y, Lubin AA, Heeger AJ, Plaxco KW (2005) Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor. Ang Chemie Int Ed 44(34):5456–5459
22. Radi A, Acero Sánchez JL, Baldrich E, O'Sullivan CK (2006) Reagentless, reusable, ultrasensitive electrochemical molecular beacon aptasensor. J Am Chem Soc 128(1):117–124
23. Strehlitz B, Nikolaus N, Stoltenburg R (2008) Protein detection with aptamer biosensors. Sensors 8(7):4296–4307
24. Qureshi A, Gurbuz Y, Kallempudi S, Niazi JH (2010) Label-free RNA aptamer-based capacitive biosensor for the detection of C-reactive protein. Phys Chem Chem Phys 12(32):9176–9182
25. Ricci F, Bonham AJ, Mason AC, Reich NO, Plaxco KW (2009) Reagentless, electrochemical approach for the specific detection of double- and single-stranded DNA binding proteins. Anal Chem 81 (4):1608–1614
26. Ricci F, Adornetto G, Moscone D, Plaxco KW, Palleschi G (2010) Quantitative, reagentless, single-step electrochemical detection of anti-DNA antibodies directly in blood serum. Chem Comm 46 (10):1742–1744
27. Wang J, Onoshima D, Aki M, Okamoto Y, Kaji N, Tokeshi M et al (2011) Label-free detection of DNA-binding proteins based on microfluidic solid-state molecular beacon sensor. Anal Chem 83 (9):3528–3532
28. Cash KJ, Ricci F, Plaxco KW (2009) An electrochemical sensor for the detection of protein–small molecule interactions directly in serum and other complex matrices. J Am Chem Soc 131 (20):6955–6857
29. Cash KJ, Ricci F, Plaxco KW (2009) A general electrochemical method for label-free screening of protein–small molecule interactions. Chem Commun 41:6222–6224
30. Rant U, Pringsheim E, Kaiser W, Arinaga K, Knezevic J, Tornow M, Fujita S, Yokoyama N, Abstreiter G (2009) Detection and size analysis of proteins with switchable DNA layers. Nano Lett 9 (4):1290–1295
31. Tort N, Salvador JP, Eritja R, Poch M, Martınez E, Samitier J, Marco MP (2009) Fluorescence site-encoded DNA addressable hapten microarray for anabolic androgenic steroids.Trends. Anal Chem 28(6):718–728
32. Chen S, Phillips MF, Cerrina F, Smith LM (2009) Controlling oligonucleotide surface density in light-directed DNA array fabrication. Langmuir 25(11):6570–6575
33. Kjällman THM, Peng H, Soeller C, Travas-Sejdic J (2008) Effect of probe density and hybridization temperature on the response of an electrochemical hairpin-DNA sensor. Anal Chem 80(24):9460–9466
34. White RJ, Phares N, Lubin AA, Xiao Y, Plaxco KW (2008) Optimization of electrochemical aptamer-based sensors via optimization of probe packing density and surface chemistry. Langmuir 24(18):10513–10518
35. Ricci F, Lai RY, Heeger AJ, Plaxco KW, Sumner JJ (2007) Effect of molecular crowding on the response of an electrochemical DNA sensor. Langmuir 23(12):6827–6834
36. Peterson AW, Heaton RJ, Georgiadis RM (2001) The effect of surface probe density on DNA hybridization. Nucleic Acids Res 29(24):5163–5168
37. Lee C, Gong P, Harbers GM, Grainger DW, Castner DG, Gamble LJ (2006) Surface coverage and structure of mixed DNA/Alkylthiol monolayers on gold. Characterization by XPS, NEXAFS, and fluorescence intensity measurements. Anal Chem 78 (10):3316–3325
38. Liao TH, Salnikow J, Moore S, Stein WH (1973) Bovine pancreatic deoxyribonuclease A. isolation of cyanogen bromide peptides; complete covalent structure of the polypeptide chain. J Biol Chem 248(4):1489–1495
39. Xiao Y, Lai RY, Plaxco KW (2007) Preparation of electrode-immobilized, redox-modified oligonucleotides for electrochemical DNA and aptamer-based sensing. Nat Protoc 2 (11):2875–2880
40. Phares N, White RJ, Plaxco KW (2009) Improving the stability and sensing of electrochemical biosensors by employing trithiol-anchoring groups in a six-carbon self-assembled monolayer. Anal Chem 81(3):1095–1100
41. O'Connor SD, Olsen GT, Creager SE (1999) Nernstian electron source model for the ac voltammetric response of a reversible surface redox reaction using large-amplitude ac voltages. J Electroanal Chem 466(2):197–202
42. Creager SE, Wooster TT (1998) A new way of using ac voltammetry to study redox kinetics in electroactive monolayers. Anal Chem 70(20):4257–4263
43. Sumner JJ, Weber KS, Hockett LA, Creager SE (2000) Long-range heterogeneous electron transfer between ferrocene and gold mediated by n-alkane and W-aikyl-carboxamide bridges. J Phys Chem B 104(31):7449–7454
44. Lubin AA, Plaxco KW (2010) Folding-based electrochemical biosensors: The case for responsive nucleic acid architectures. Acc Chem Res 43(4):496–505
45. Castronovo M, Radovic S, Grunwald C, Casalis L, Morgante M, Scoles G (2008) Control of steric hindrance on restriction enzyme reactions with surface-bound DNA nanostructures. Nano Lett 8 (12):4140–4145
46. Castronovo M, Lucesoli A, Parisse P, Kurnikova A, Malhotra A, Grassi M et al (2011) Two-dimensional enzyme diffusion in laterally confined DNA monolayers. Nature Comm 2(1)

47. Palanisamy R, Connolly AR, Trau M (2010) Considerations of solid-phase DNA amplification. Bioconjug Chem 21(4):690–695
48. McCalla SE, Luryi AL, Tripathi A (2009) Steric effects and mass-transfer limitations surrounding amplification reactions on immobilized long and clinically relevant DNA templates. Langmuir 25 (11):6168–6175
49. Bar M, Bar-Ziv RH (2009) Spatially resolved DNA brushes on a chip: Gene activation by enzymatic cascade. Nano Lett 9 (12):4462–4466
50. Ricci F, Zari N, Caprio F, Recine S, Amine A, Moscone D et al (2009) Surface chemistry effects on the performance of an electrochemical DNA sensor. Bioelectron 76(1–2):208–213