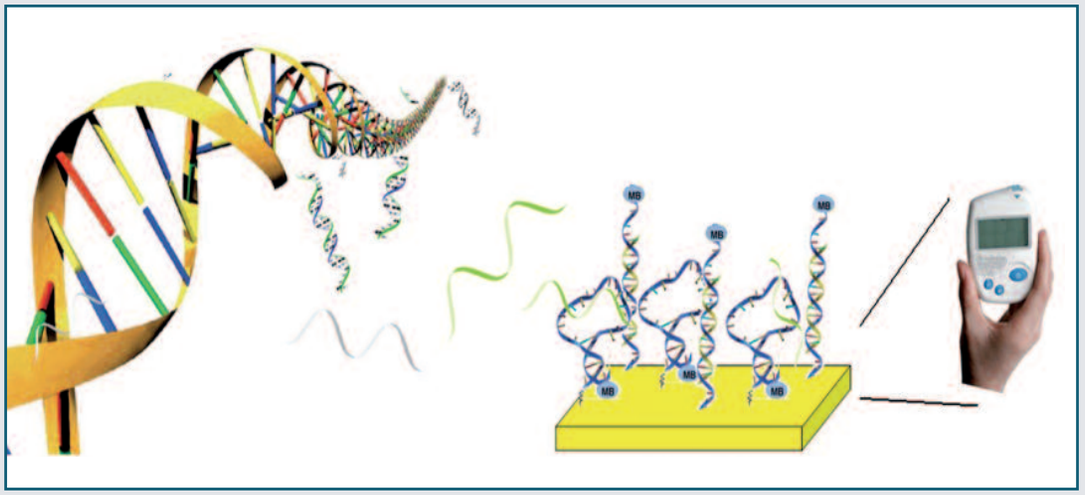

*Francesco Ricci*
*Dipartimento di Scienze*
*e Tecnologie Chimiche*
*Università di Roma*
*Tor Vergata*
*Francesco.ricci@uniroma2.it*

# NOVEL ELECTROCHEMICAL DNA SENSORS FOR CLINICAL APPLICATIONS

*We review here the development, optimization and recent advances of a class of reagentless, electrochemical DNA sensors for the sequence-specific detection of nucleic acids. Novel applications of such platform for the detection of other relevant clinical targets such as transcription factors, anti-DNA antibodies and, potentially, any antibody/antigen will be also discussed.*

Pathogen detection or diagnosis of genetic diseases based on the sequence-specific identification of DNA have attracted significant attention [1] due to the fact that these methods are usually more accurate and much faster than culture based methods. Optical [1], electrochemical [2], acoustic [3] and gravimetric [4] techniques have been applied to this goal. Although each of these approaches has its own advantages, the electrochemical detection of DNA hybridization appears as the most promising due to its rapid response time, low cost and suitability for mass production [5-6]. For this reason many electrochemical DNA detection schemes have been described to date, the best of which achieve limits of detection ranging from picomolar to femtomolar. Recently, Plaxco, Heeger and coworkers, concomitantly with other groups, have introduced a number of single-step, label-free electrochemical biosensors, termed E-DNA sensors, that are based on the target binding-induced folding of electrode-bound DNA probes [7-10]. These sensors, in their original format, were designed as the electrochemical equivalent of optical molecular beacons [7-10]. The original E-DNA sensor is in fact comprised of a redox-modified "stem-loop" probe that is immobilized on the surface of a gold electrode via self assembled monolayer chemistry (Fig. 1). In the absence of target the stem-loop holds the redox moiety in proximity to the electrode, producing a large Faradaic current. Upon target hybridization the stem is broken and the redox moiety moves away from the electrode surface. This, in turn, reduces the efficiency with which electrons are transferred to the electrode, thus decreasing the Faradaic current in proportion to the fraction of DNA probes that have hybridized (Fig. 1).

This approach is particularly promising for oligonucleotide detection because of its rapidity, reagentless nature and operational convenience [7, 10]. E-DNA platform, as its optical counterpart, proved to be highly specific. Unlike other optical similar approaches, the E-DNA platform is very selective and performs well even in real complex matrices such as blood serum, soil extracts and foodstuffs [7, 10, 11].

Also, because the redox-modified probe DNA is strongly bonded to the interrogating electrode via a thiol-gold linkage, the E-DNA sensor is reusable (recovery >99% after a simple, aqueous wash) [7, 10]. Fab-

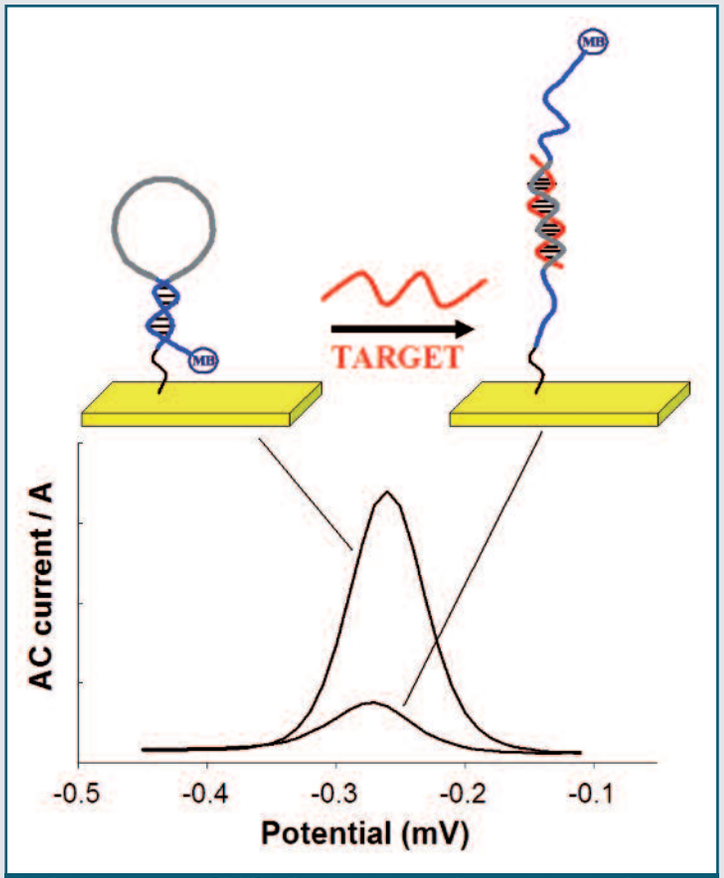


**Fig. 1 - E-DNA sensors [7-10], in their original format, were designed as the electrochemical equivalent of optical molecular beacons comprised of a redox-modified "stem-loop" probe immobilized on the surface of a gold electrode. In the absence of target, the stem–loop structure holds the electrochemical tag (here methylene blue) into close proximity with the electrode surface, thus ensuring efficient redox of the electrochemical label (lower panel). Upon hybridization with the target sequence collisions between the redox tag and electrode are inhibited, decreasing the signaling current**

rication and interrogation procedures for E-DNA sensors are also straightforward [12] and suitable for *in-situ* analysis with portable instrumentation and disposable sensors.

## E-DNA Signaling Mechanism

In an attempt to improve the signaling, reproducibility, and shelf life of the E-DNA platform, in a research carried out in collaboration with the group of Prof. Plaxco at the University of California, Santa Barbara (UCSB), we have recently monitored the effects of the nature of the coadsorbate forming the self-assembled monolayer, the composition of the stem-loop probe and the density with which the DNA probes are packed on the sensor [13-15]. These efforts have shed light on the platform's signalling mechanism.

Specifically, we have demonstrated that the signalling of E-DNA sensors is a complex function of probe packing density [13]. Maximum signalling is in fact observed at intermediate packing densities and falls at lower and higher densities. This suggests, in turn, that E-DNA signalling arises due to binding-linked changes in the efficiency with which the terminal redox tag strikes the electrode (i.e., with collision dynamics) and not to the binding-induced conformational change per se.

This "collisional" model of E-DNA signaling is supported by other evidences. We recently proposed the use of a ss-DNA linear probe for E-DNA based sensing and demonstrated that binding-induced changes in DNA dynamics are sufficient to support E-DNA signaling thus giving further evidence to the "collisional" theory [16]. Moreover, because target binding no longer competes with the unfavorable energy of breaking the stem, the gain of linear probe sensors is improved relative to the equivalent stem-loop architecture. Linear-probe E-DNA sensors maintain all the positive features of their stem-loop counterparts being label free, reusable, sequence specific and selective enough to employ directly in complex sample matrices such as blood serum, thus rendering them well suited for clinical applications [16]. Recently Plaxco and coworkers have expanded on this subject and have demonstrated, by studying the probe length dependence of the E-DNA sensors, that the signaling of this platform is a reaction-limited process in which the overall rate is proportional to the *equilibrium* probability that the end of the oligonucleotide chain approaches the surface [17]. Studies of the ionic strength and viscosity dependencies of electron transfer further supported this "chain-flexibility" mechanism, and studies of the electron transfer rate of methylene blue attached to the hexanethiol monolayer suggest that heterogeneous electron transfer through the monolayer is rate limiting. Thus, the *flexibility* (i.e., the equilibrium statistical properties) of the oligonucleotide chain defines the rate with which an attached redox reporter transfers electrons to an underlying electrode, an observation in agreement with previous similar findings [18, 19] that may be of utility in the design of new biosensor architectures.

The disclosure that E-DNA signaling requires only that a target binds to an oligonucleotide probe and, in doing so, changes the efficiency with which the attached redox tag transfers electrons to the electrode could account for the platform's generalizability thus suggesting that the same sensing principle would also support the detection of DNA binding events that lead to the formation of bulky and/or rigid complexes.

## E-DNA sensor for DNA binding proteins detection

In order to test this hypothesis (i.e. that the signaling mechanism of E-DNA sensors is mostly based on change of the collisional efficiency of the probe), we have recently proposed the use of E-DNA-like sensors for the detection of targets other than oligonucleotide sequences. In a first attempt in this direction we have tested the use of these sensors for the measurement of DNA binding proteins [20]. These proteins are abundant and essential in cells, interacting with DNA in order to organize its packing, regulate transcription, and perform replication and repair.

For this study we have selected two double-strand binding proteins, the eukaryotic TATA-box Binding Protein (TBP) (a core component of the eukaryotic transcriptional machinery) and the prokaryotic M.Hhal methyltransferase (M.Hhal) (involved in the restriction-modification system of bacteria). We have fabricated sensors against the double-strand

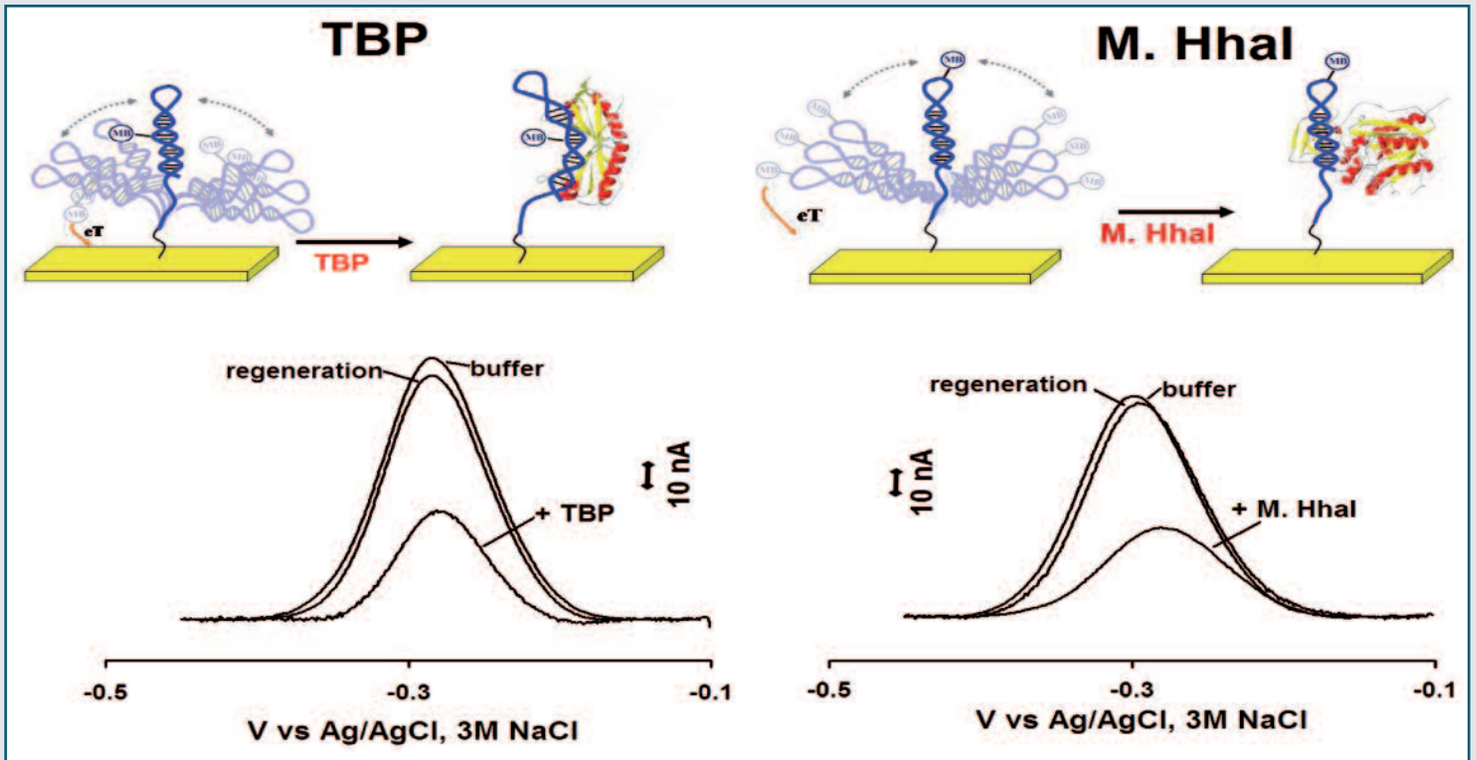


Fig. 2 - Schematics of E-DNA-like sensors for the detection of DNA binding proteins. (Top) The sensor is comprised of a DNA hairpin covalently attached to a gold electrode using thiol-gold self assembled monolayer chemistry and containing an internal methylene blue redox tag. (Bottom) In the absence of target relatively efficient collision between the label and the electrode produces a large faradic current. Upon target binding this faradic current is significantly reduced, presumably because the bulky structure of the protein reduces the collision rate [20]

binding proteins using short, stem-loop probe DNAs in which the relevant recognition sequences are contained within the double-stranded stem [20]. These probes were modified with a 3' thiol group, supporting strong chemisorption to an interrogating electrode, and a methylene blue redox tag pendant on a thymine base along the double-stranded stem (Fig. 2). In the absence of target both probes produce a large faradaic peak at the potential expected for the methylene blue redox tag (Fig. 2, bottom). This current is due to the collisions of the relatively dynamic ds-DNA probes. The relative dynamicity of the probe is ensured by the fact that this was designed as to have a short (5 bases) single stranded element at the proximity of the gold electrode thus ensuring enough freedom for the collision of the probe. In the presence of saturating TBP and M.Hhal the binding of these proteins leads to a bulk and rigid DNA-protein complex which in turns reduces the currents by 45 and 55% respectively. The two sensors support the ready detection of their target proteins at concentrations as low as 2 and 25 nM for TBP and M.Hhal respectively.

Again, as in the case of E-DNA sensors for hybridization detection, because all of the sensing components are strongly adsorbed to the electrode surface, this sensing architecture is readily regenerable; a short wash (30 s) in 8 M guanidine chloride is sufficient to regenerate 98% of the original signaling current of both sensors (Fig. 2, bottom), allowing multiple cycles of detection and regeneration. Of note, not only ds-DNA but also ss-DNA probes support this analytical approach.

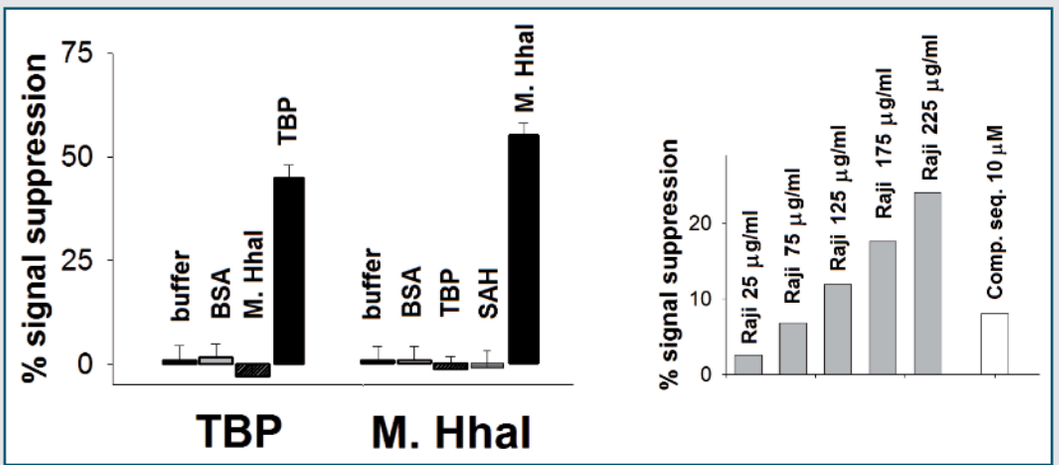


Fig. 3 - DNA binding protein sensors are as selective as the DNA probes from which they are fabricated. (Left) For example, no significant cross-reactivity is observed between the two sensors directed against double-strand binding proteins in the presence of 1.5 µM BSA and saturating concentrations of the non-targeted double-strand binding protein (80 nM M.Hhal or 10 nM TBP). (Right) Likewise our sensors are also effective in rejecting false positives arising due to interferents and perform well when challenged with realistically complex sample matrices such as crude Raji nuclear extracts [20]

Using single-stranded, poly-thymine probes (neither of our target proteins exhibits any significant sequence specificity) we have fabricated sensors for the detection of the single-strand binding proteins SSBP and eukaryotic Replication Protein A (RPA).

All three sensors are as selective as the DNA probes from which they are fabricated. For example, we do not detect any significant cross-reactivity between the two sensors directed against double-strand-binding proteins (Fig. 3 left). Likewise, because their signaling is linked to a binding-specific change in the probe DNA (and not simply to adsorption of target to the sensor surface), our sensors are effective in rejecting false positives arising due to the non-specific adsorption of interferents and can be employed directly in complex samples. For example, our single-strand sensor supports the selective detection of exogenous levels of RPA directly in crude Raji cell nuclear extracts (Fig. 3 right).

This study demonstrated that the measuring principle of E-DNA sensors could allow its use not only for DNA sequences detection but also for the measurement of DNA-protein interactions.

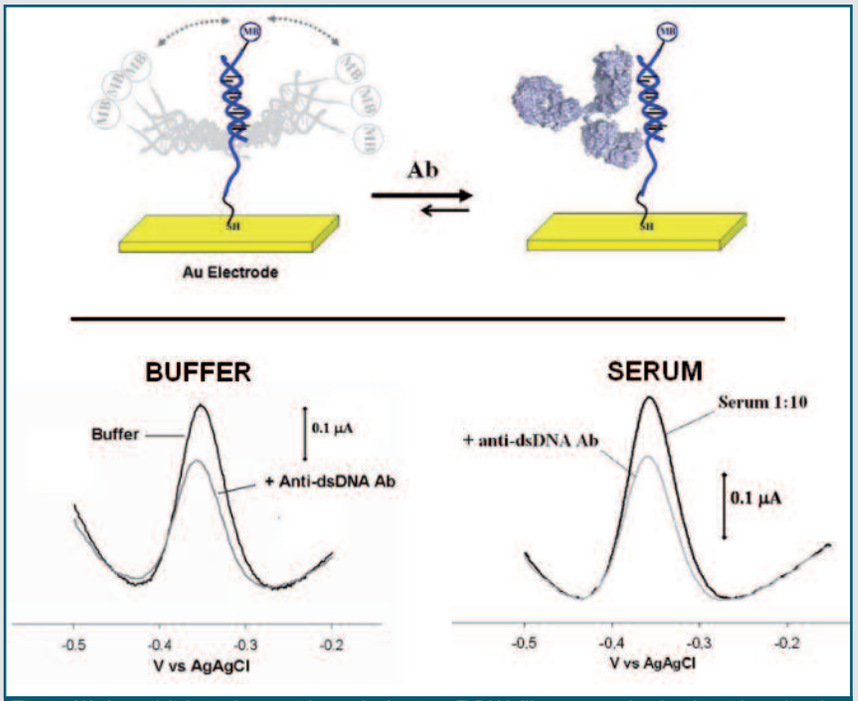


**Fig. 4 - We have fabricated reagentless, single-step, E-DNA-like sensors for the detection of anti-DNA antibodies by employing a ds-DNA probe covalently attached to a screen printed gold electrode containing a methylene blue redox tag at the 3' end. In the absence of target relatively efficient collision between the label and the electrode produces a large faradic current from the attached metheylene blue. (bottom, left). The binding of the antibody to the recognition probe reduces the efficiency with which the redox reporter approaches the electrode, significantly reducing this faradaic current. (Bottom) The sensors readily detect anti-DNA antibodies in both buffered saline and in more complex, clinically relevant samples, such as blood serum at nanomolar concentrations [21]**

## E-DNA sensor for anti-DNA antibodies detection

In an effort to broaden the fields of applicability of the E-DNA platform and to develop a quantitative molecular diagnostics suitable for point-of-care, we have recently proposed a reagentless, single-step electrochemical sensor for the detection of *anti*-DNA antibodies at low nanomolar concentrations [21]. Anti-DNA antibodies are important markers for the diagnosis of several autoimmune diseases such as systemic lupus erythematosus (SLE), a chronic autoimmune connective tissue disease. Both *anti*-ssDNA and *anti*-dsDNA antibodies are involved in disease development, and high levels of *anti*-DNA antibodies are associated with disease flares (acute exacerbation of the disease). Consequently, the quantitative monitoring of sera levels of *anti*-DNA antibodies provides key insights into the activity and progression of the disease.

We have fabricated *E*-DNA sensors for *anti*-DNA antibodies detection using a double-stranded probe recognized by the antibody (Fig. 4, left) [21]. In the absence of a target this double-stranded probe produces a significant current at the potential expected for the reduction of the methylene blue redox tag (Fig. 4, right). In the presence of *anti*-dsDNA antibodies this current is decreased significantly, presumably because antibody binding reduces the flexibility of the probe and thus reduces the efficiency with which the redox tag collides with, and thus exchanges electrons with, the electrode. Upon titration with *anti*-dsDNA antibodies the dose-response curve of the sensor exhibits an EC50 (the analyte concentration inducing 50% of the maximum signal decrease) of 19 nM, and a detection limit of ~10 nM (~1.5 μg ml$^{-1}$). Also in this case a brief wash in 8 M urea is sufficient to regenerate 99% of the sensors original signal, demonstrating that the observed signal decrease is not due to degradation of the sensor and allowing for ready re-use. The sensors developed are specific and selective. For example, we do not detect any significant signal change after incubation of our sensors with high concentrations of either non-specific antibodies or BSA and we did not observe any significant change in sensitivity when they are used in complex samples (i.e. serum). Finally, our sensors are rapid and convenient, rendering them well suited for point-of care applications. Our sensors are, for example, supported on inexpensive, screen-printed electrodes and require only a simple, hand-held potentiostat for data collection. They are also rapid, exhibiting an equilibration time constant of ~3 min and require neither wash steps nor the addition of exogenous reagents. These attributes compare quite favourably to those of existing methods for detecting *anti*-DNA antibodies. Given these attributes, it appears that this novel approach is significantly more convenient-and significantly better suited for point-of-care applications-than existing methods for the detection of this important class of diagnostic markers.

I believe that generalizability and flexibility of E-DNA are among the most appealing advantages of this platform. For example, the same sensing platform could be expanded to a wide range of other targets not only limited to those that bind to unmodified DNA or RNA.

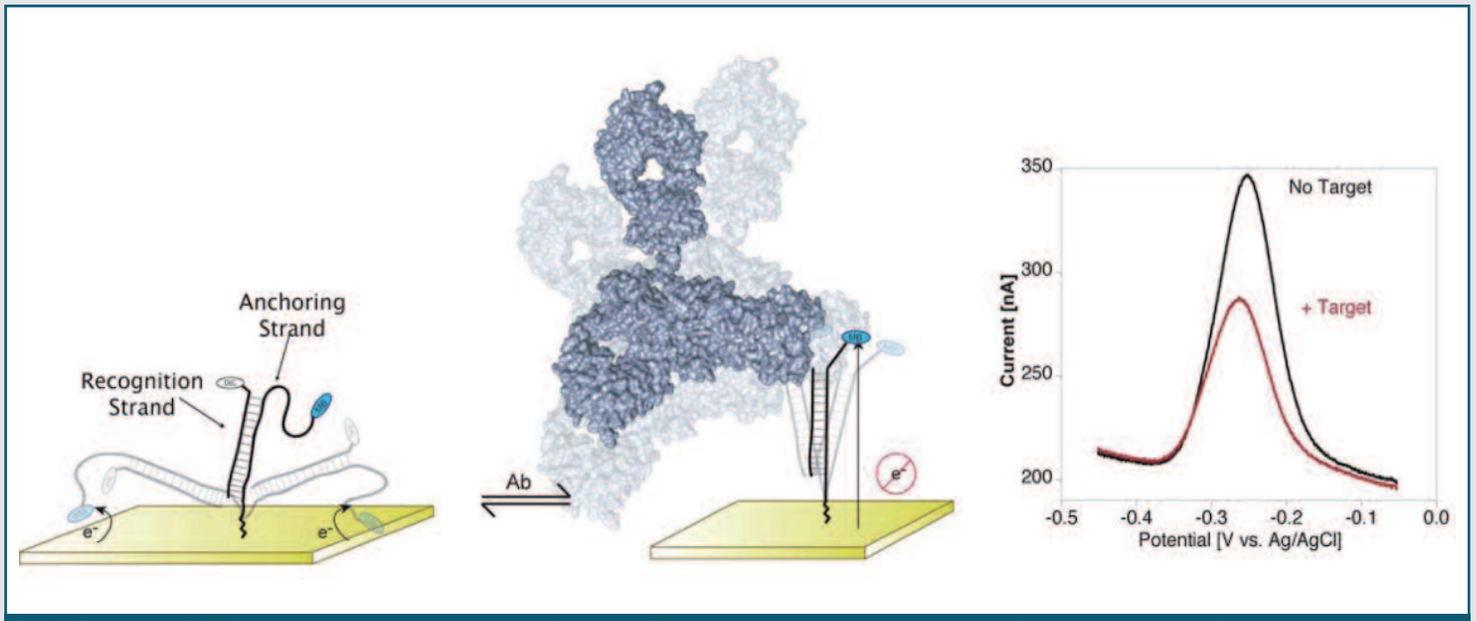


**Fig. 5 - The "scaffold" E-DNA sensors broaden the possible applications of E-DNA sensors to the detection of proteins/enzymes that bind small molecules by simply appending the small molecule to the DNA probe [22-23]. One of the two scaffold strands, the anchoring strand, is linked to the electrode using self-assembled monolayer chemistry via a 3' thiol group and is modified with a redox tag (methylene blue) at its 5' terminus. The second strand, the recognition strand, is complementary to a region of the anchoring strand and is covalently modified with the relevant small molecule recognition element at one of its termini. In the unbound state, the scaffold is dynamic enough to allow electron transfer between the redox label and the electrode. The binding of the macromolecular target to this recognition element inhibits redox label collisions with the electrode, thus significantly reducing the observed faradic current. Shown here are representative square wave voltammograms for the two states (shown here for detection of anti-digoxigenin antibody in 50% blood serum)**

## E-DNA sensor as a scaffold system for detection of small-molecules/macromolecules interaction

The "collisional" mechanism suggests in fact a ready means of detecting proteins/enzymes that bind small molecules by simply appending the small molecule to the DNA probe thus expanding widely the possible E-DNA applications. In collaboration with the group of Prof. Plaxco at UCSB I have recently demonstrated this interesting suggestion by conjugating a small molecule recognition element onto a relatively rigid, partially double-stranded DNA scaffold that is chemi-adsorbed to an interrogating gold electrode [22] (Fig. 5). One of the two scaffold strands, the anchoring strand, is linked to the electrode using self-assembled monolayer chemistry via a 3' thiol group and is modified with a redox tag (methylene blue) at its 5' terminus. The second strand, the recognition strand, is complementary to a region of the anchoring strand and is covalently modified with the relevant small molecule recognition element at one of its termini. As a preliminary test for the development of this technology we have employed the small molecules biotin and digoxigenin as recognition elements to fabricate sensors directed against streptavidin and anti-digoxigenein antibodies, respectively. In the absence of target, the modified, double-stranded scaffold is free to collide with the electrode surface, and thus produces a large faradaic current at the potential expected for methylene blue. In contrast we observed that upon target binding this current is reduced, signaling the presence of the target. This reduction presumably occurs because target binding reduces the efficiency with which the redox tag collides with the electrode, either due to the increase in steric bulk or hydrodynamic radius associated with the target.

This technology readily detects sub-nanomolar to low nanomolar concentrations of antibodies and other small-molecule-binding proteins in complex, contaminant-ridden samples such as blood and blood serum. Recently, for example, we have demonstrated the possible detection of both anti-TNT antibodies and, via a competition assay, parts-per-trillion TNT in crude soil extracts [23]. I believe this "E-DNA scaffold" approach may prove a general method for the detection of macromolecules that bind to low molecular weight targets for application in, for example, drug screening.

## Conclusions and future perspectives

I believe the studies I have summarized in this review represent a nice overview of E-DNA sensors, a novel electrochemical DNA platform, which is sensitive, convenient, specific, and, importantly, selective enough to deploy directly in whole blood, cellular lysates, soil extracts, and other realistically complex samples.

I have shown here that the approach requires only that target binding alter the efficiency with which the redox tag on the probe biomolecule strikes the electrode. This lends the approach great versatility and accounts for the wide range of targets for which such sensors have already been reported. As I demonstrated in this critical review the "collisional" mechanism makes possible the monitoring of theoretically any DNA-protein or DNA-antibody interactions which leads to the formation of bulky and/or rigid complexes. We have confirmed this suggestive hypothesis with a three-successes-out-of-three-attempts success rate in fabricating sensors targeting both sequence-specific,

double-strand binding proteins, non-specific, single-strand binding proteins and anti-DNA antibodies.

Moreover, by simply appending a recognition small molecule on the DNA probe we can in principle detect any target protein or macromolecules binding to such label.

This new sensing approach offers several significant advantages over other methods for monitoring protein-small molecule interactions or detecting proteins that bind to specific small molecule targets. For example, standard methods for protein detection, such as ELISAs and western blots, are time and capital intensive, and typically require the addition of reagents and multiple washing steps. Less cumbersome methods for monitoring protein-small molecule interactions, such as fluorescence polarization and plasmon resonance (SPR) and quartz crystal microbalance (QCM) approaches, often fail in complex samples because of background fluorescence or non-specific adsorption. In contrast, the approach I have demonstrated here is rapid and convenient, and functions even when challenged directly in clinically and environmentally relevant samples. Moreover, it appears that this new approach is general; in principle, given a target macromolecule large enough to alter the collision dynamics of the scaffold, the only limiting factor is the ability to effectively conjugate the relevant small recognition molecule to a relatively rigid and dynamic molecular scaffold [22]. Our future efforts will for this reason be directed to the use of the E-DNA platform for the detection of Transcription factors and other clinically relevant macromolecules. Moreover, the recent funding of the grant "FIRB - Futuro in Ricerca" will allow me to investigate the possible use of this platform for the detection of endocrine disruptors in different food and clinical matrices.

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Francesco Ricci is a researcher at the Chemistry Department of the University of Rome Tor Vergata since 2006. He won the "Young Researcher" award of the Italian Chemistry Society (Analytical Chemistry Division) in 2005 and the "PhD thesis" award of the Italian Chemistry Society (Electrochemistry Division) in 2007. In 2006, 2008 and 2009 he was a visiting scholar at the University of California, Santa Barbara working on different aspects of DNA sensors. In 2010 he was funded by the Italian Ministry of Education in the frame of the call "Futuro in Ricerca". His research activity is focused on the development of novel electrochemical sensors for clinical and environmental applications. He is author of more than 40 publications in peer reviewed high impact factor international journals (PNAS, JACS, Anal. Chem, Chem. Comm., Biosens. Bioelectron. etc.) (ca. 1000 total citations), 3 international patents and 6 chapters in books. His h-index (Scopus source) is currently 16.

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## RIASSUNTO

### *Sensori elettrochimici a DNA*

*In questa critical review verranno illustrate le caratteristiche principali di una nuova classe di sensori elettrochimici a DNA. Oltre a descrivere il principio di misura e le performance analitiche di questi sensori per la misura di sequenze oligonucleotidiche, verranno illustrate le più recenti applicazioni che dimostrano l'utilità di tale approccio per la rilevazione di importanti target clinici, quali i fattori di trascrizione e gli anticorpi anti-DNA.*