Understanding the factors affecting the analytical performance of sandwich-hybridization genosensors on gold electrodes

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Abstract

Hybridization-based electrochemical DNA sensors have become a methodological mainstay in a wide variety of fields. Among the possible assay formats, sandwich hybridization is the most powerful one. It is so called because the target sequence to be measured is hybridized between two complementary sequences. The analytical performance of this class of sensors is influenced by many construction and operational parameters, which needs to be optimized. In this work, we comparatively analyze the analytical performance of different sandwich hybridization sensors, constructed on gold surfaces modified by chemisorption. We find that the structures of the designed probes as well as the topography of the gold support and the nature of the monolayer are key parameters affecting the sensitivity and reproducibility of the genosensors. Other important operational parameters are also described. The results of this study could serve as a guide in the design of genosensors for future applications.

Dedicated to Prof. Joseph Wang, on the occasion of his 70th anniversary

1. Introduction

Electrochemical nucleic acid sensors, often referred as genosensors, have become a mainstay to detect DNA or RNA targets of interest in clinical diagnostics, environmental monitoring and food quality control [1]. In these sensors, the biorecognition process involves the hybridization reaction between the target and an oligonucleotide immobilized onto the sensor recognition layer. A variety of designs have been investigated to transduce this interaction, including reagentless sensors and methods using covalently or non-covalently bound labels [2], among them, sandwich hybridization assays have garnered significant attention [3]. In a sandwich hybridization approach target sequences are bound between two separate probes: the capture probe, which is immobilized on a solid and conductive support acting as a catching reagent, and the signaling probe that incorporates a label to make possible the detection (Figure 1). The advantages of the sandwich design are multifold. First, the approach is quite convenient and eliminates the requirement of labeling the molecular target, facilitating the sensor fabrication. Second, because the sandwich assay requires the simultaneous binding of capture and signaling probes it is extremely selective. Additionally, as the signaling probe is usually coupled to an enzyme or amplified detection mechanism it achieves excellent sensitivity of detection.

Despite their excellent performance, key design aspects governing the response of sandwich hybridization sensors have not been discussed. The signaling mechanism of these sensors is linked to both solution-phase hybridization between the target nucleic acids and randomly distributed signaling probes in solution, and solid-phase hybridization of the preformed signaling probe-target duplex to surface-tethered capture probe. These two steps are distinct from a thermodynamic and kinetic point of view [4-7], and to the best of our knowledge, the extent to which they affect sandwich genosensor analytical performance has not been established. Over many years of work in this area, we have designed and characterized genosensors for a wide range of applications using a sandwich approach and monolayers of oligonucleotides immobilized on gold electrodes in combination with enzyme-reported electrochemical detection [8-23]. In this work, we analyze the different construction and operational factors affecting the analytical performance of these genosensors to provide a rational path toward their design and optimization. Specifically, we analyze the influence of the structure of the designed probes, as well as the effect of the topography of the gold transducer, the nature of the monolayer including the capture probe, and the key operating conditions that improve the genosensors' analytical performance.

2. Design of the probes

We have considered target DNA sequences longer than those usually involved in DNA chips (around twenty nucleotides in length), with lengths in the range between 52 and 134 mer. The reason of this selection is to combine genosensing technology with a previous molecular amplification step (PCR or an isothermal variant) and thus be able to accomplish the extremely low sensitivity that most of the real world applications demand.

To investigate the effects of the design of probes on the behavior of sandwich genosensors, we compared the analytical performance of genosensors for a wide range of DNA targets in combination with different signaling and capture probes immobilized on gold surfaces as summarized in Table 1. A sandwich hybridization genosensor typically involves two stages (Figure 1). In the first step, the sample is incubated with the signaling probe (in a large excess, typically larger than 30-fold the upper limit of the target concentration range), and the solution hybridization (homogeneous hybridization) takes place, where target, T, and signaling probe, SP, strands combine to form a duplex, T-SP, with a free energy change ΔG_1. Hybridization thermodynamics in solution is well understood, and the change in the free energy of the homogeneous hybridization reaction can be easily obtained from the nucleotide sequences based on the nearest-neighbor model. With that aim we employed the tools provided by Mfold webserver [24]. The folding of the single strands into secondary structures, which is accounted by ΔG_{self}, competes with hybridization, thus slowing the homogeneous process. To complete the assay, the T-SP mixture is incubated with the surface-bound capture probe, CP. In the heterogeneous hybridization, taking place at the sensor interface, the T-SP strands must penetrate into the layer of immobilized CP strands to hybridize. ΔG_{tot} accounts for the overall energy change if this reaction occurred in the bulk solution. Although deviations from expectations based on solution-hybridization might be anticipated in surface hybridization, these values are considered in order to establish a comparison between the different systems. The thermodynamic characteristics of targets and designed probes are summarized in Table 1. For the sake of clarity and in order to facilitate the comparison between the designs, we include in Figure 2 radar charts showing the information regarding the free energy changes associated to their self-hybridization, as well as the homogeneous and heterogeneous recognition reactions.

In sandwich formats the two probes, the capture and the labeled-signaling one, typically hybridize with consecutive regions of the target, usually each ends forming a perfect duplex. In real samples, amplified specific fragments can be quite long (>100 pb) so the generation of a full dsDNA would require extremely long probes. Though now synthesis of long sequences is

amenable and they are commercially available at relatively high cost, the probes do not need to cover the entire amplicon. In general, ssDNA overhangs at both ends are not deleterious for the electrochemical signal provided that the duplex formed is continuous, that is, no flexible ssDNA regions are in between the capture and signaling probes [8]. These regions can act as a fringe placing the tag close to the surface instead of towards the solution to facilitate the labeling.

We have reported that ssDNA overhangs near the electrode surface reduces the sensitivity of the genosensor, but this is true only in certain cases, when a highly repetitive target sequence is used. In that case, redesigning the capture probe to form a perfect and continuous helix restores the magnitude of the analytical signal [17]. However, other sandwich designs also having overhangs did not corroborate that behavior [14, 20]. Gibbs free energy of the CP-ssDNA overhang hybridization showed very high values -23.9 kcal/mol for the repetitive target while the other design exhibited low ones (-3.1 [14] and -6.5 kcal/mol [20]). This fact can explain the special behavior for the repetitive target; a strong interaction of the overhang with adjacent capture probes is possible, which diminishes the number of probes available for incoming targets (Figure 3)

Selectivity of the detection improves intensely with shared stem-loop structured capture probes. This type of CPs are designed to hybridized with a region of the target by both the loop and part of the stem in order to oblige to open it to form the duplex. Therefore, the Gibbs free energy of the self-hybridized probe (ΔG_{self}) must be surpassed by the Gibbs free energy of the duplex formed (ΔG_{tot}). Though this is always much higher (in absolute value) the penalty of opening the hairpin makes the hybridization of mismatched sequences more difficult resulting in very low currents and great discrimination power [8,10]. In fact, this method can even exceed the discrimination ability of real-time PCR as was achieved with a genosensor for the detection of wheat gluten. The hairpin capture probe specific of a wheat sequence encoding the 33-mer immunodominant peptide did not recognized homologous sequences in celiac-disease-triggering species such as barley and rye, while the linear probe can recognize them with less sensitivity [18].

The background current has three main causes: unspecific adsorption of enzyme conjugate, hybridization of mismatches sequences or hybridization between the anchored capture probe and a tagged-signaling probe. The magnitude of the latter, computed by the ΔG_{blank} values included in Figure 2, must be minimized during the design of the sandwich approach. As a rule of thumb, duplexes with Gibbs free energy values less than -12 kcal/mol are not enough stable to survive washing and 2 h-heterogeneous hybridization steps. However, significant and

persistent blank current was observed with a system containing 50 mer and 75 mer CP and SP, respectively with ΔG_{blank}=-15.8 kcal/mol, which precluded the development of a sensitive genosensor. Importantly, the design of a hairpin structured CP resulted beneficial [18]. Even though the ΔG_{blank}=-14.8 kcal/mol is high, the need to open the hairpin structure avoided high blank currents. A similar behavior explains the low background current obtained with a system with ΔG_{blank}=-15.7 kcal/mol [16]. In this case, the capture probe was designed as a linear one (ΔG_{self}=-5.79 kcal/mol) but a close inspection of the secondary structure probe reveals a mostly self-hybridized probe with some small bulges. This structure seemed to be more difficult to disrupt than authentic non-structured linear probes.

Kinetics of the hybridization step is slower on a surface than in solution. 1-h hybridization reactions are typically used in genosensors though longer times can help to increase the signal-to-blank ratio (S/B) for certain applications as obtained by DoE optimization [16]. Hybridization reaction rate depends on the length and the secondary structure of the target. We have shown that shifting from 52 mer to 95 mer synthetic target oligonucleotide required lengthening the hybridization step to 120 min to obtain matching calibration plots, that is, identical sensitivities [9]. This also held true for highly structured targets [17].

3. Influence of the topography of the gold support

The nature of the conductive surface on which the sensing phase is constructed is the first fabrication parameter to be considered. We constructed the genosensor on gold surfaces by obtaining self-assembled monolayers of thiolated-CP. The density and orientation of immobilized CP are key factors, and they can be dramatically influenced by the gold substrate topography. To evaluate this effect we used the following substrates, spanning a range of topographies: bulk polycrystalline gold, thick-films obtained by screen-printing technology, evaporated gold thin-films, and three-dimensional (3D)-nanostructured gold nanowire ensembles.

The early-developed genosensors were prepared on commercially available gold electrodes, obtained by sealing gold wires into a plastic support [8,9]. In this way, planar polycrystalline bulk surfaces are obtained. These electrodes are no disposable and must be regenerated by chemical (piranha solution) and mechanical (polishing with alumina pastes) procedures to obtain a new bare electrode surface after measurements. These procedures are tedious and time consuming, hindering the adaptation of these surfaces to the mass production of genosensors.

Advances in microelectronic technology have made possible to fabricate gold surfaces that can be mass-produced at a reasonable price to use these surfaces as disposable electrodes.

We evaluated surfaces obtained by two standard methods, namely thin- and thick- film processes. Both consisted on layers of gold deposited on insulating surfaces such as glass, polymers or ceramic materials, but given rise to different gold thicknesses and topography.

Thin films were obtained by vacuum thermal evaporation on polyester substrates previously irradiated with UV (365 nm) for 5 days to enhance polymer unsaturation grade, which favours gold film adherence [20]. This process provides films that are highly reproducible, with a Au layer thickness of 65-80 nm. We also employed thin-films fabricated by gold sputtering on BK7-glass surfaces, with a chromium adherent layer and 50 nm gold films, which were commercially supplied as SPR discs [14]. Both types of gold plates were cut into pieces of about 30×10 mm to prepare homemade disposable electrodes. A copper strip was coupled to each fragment to provide the electrical contact, and an adhesive tape with a hole of 2 mm in diameter allowed defining the electrode surface (0.0314 cm^2 geometric area). Finally, lateral sides were wrapped with Teflon to protect them from the solution.

Thick-films on the other hand, were obtained by the screen-printing technology using low temperature curing gold inks, which composition is proprietary of the manufacturer (Dropsens-220 BT SPE, Spain). The ink was deposited on a ceramic material, dried and cured resulting in 4 mm diameter circular working gold surfaces with thicknesses of tens of micrometers [10-13, 15-18]. The inks have three principal constituents, namely the conductive gold, a bonding agent and an organic vehicle, given rise to structures with greater variability than thin-film surfaces.

The fourth type of gold surface evaluated was prepared by electroless deposition of gold within the pores of a microporous polycarbonate (PC) membrane (porous size 50 nm and 6-14 μm thickness), followed by a controlled removal of the PC that surrounds the gold-filled micropores by chemical etching with 50:50 CH_2Cl_2:EtOH mixtures [19]. In this way, the gold nanowires are exposed creating a three-dimensional array of gold nanoelectrode ensembles (3D-GNEE), which exhibit a largely increased active area.

These four substrates were characterized by the electrochemical roughness factor (R_f), defined as the ratio of electrochemically active to geometric area. All the electrodes were subjected to an electrochemical cleaning process consisting on several potential cycles between 0 and 1.6 V at 100 mV s^{-1} in 0.1 M H_2SO_4 to obtain the ideal redox waves characteristic of bare gold electrodes. The resulting gold oxide layer was reduced through voltammetry in the same media and the electrochemically active area was calculated from the charge associated to this reduction peak, assuming that to achieve a monolayer coverage of adsorbed oxide a charge transfer of 386 μC cm^{-2} is necessary [25]. R_f values vary between 1.1,

for thin-films electrodes regardless they were prepared on PC or glass substrates and 3.4 for 3D-GNEE electrodes. The characteristic R_f values for the four evaluated surfaces, in order of increasing roughness are:

Thin-films (1.1) < bulk electrodes (1.4) < thick-films SPE (2.0) < 3D-GNEE (3.4)

Surface topography of thin- and thick-film gold electrodes was also evaluated using atomic force microscopy (AFM) in the contact mode, which provides a 3D-profile of the surface. Roughness is obtained from these images by calculating the RMS (root-mean square) of the absolute values of the height of the surface profile over the evaluated surface [26]. As expected, smooth surfaces were obtained in thin-films, with RMS values less than 5 nm, whereas SPE showed RMS factors as high as 245 nm [14]. These results are found to be in qualitative agreement with the electrochemical estimation of roughness. Notable, however, is the much higher roughness observed with this technique for SPE electrodes, which may be related to the presence of the binders that are insulators and in consequence not detected by the electrochemical method.

Smoother gold is assumed to result in more densely packed films with fewer defects. Using 20 nm thin films very dense packing was achieved (2-8 $\times$ 10^{13} molecules cm^{-2}). Even under those extreme conditions less dense domains were found and associated to preferential anchoring on top of gold grains. On edges and at the bottom a smaller DNA thickness suggested a lying down conformation, so fewer DNA strands are in those areas [27]. Consequently, the sensitivity (slope of the calibration curves current density vs. target concentration) of genosensors is expected to increase as the roughness of the gold surface decreases. Consistent with this, as it is shown in Table 2 summarizing the figures of merit of the evaluated sandwich genosensors, we observed that genosensors constructed on gold thin-films showed sensitivities in the range 30 to 50 $\mu A\ cm^{-2}\ nM^{-1}$, much higher than those obtained for genosensors prepared on SPE (in the range 0.6 to 19 $\mu A\ cm^{-2}\ nM^{-1}$). Some exceptions in this trend occur because the hybridization efficiency, and thus the sensitivity of the assay, is affected by the degree of homogeneity in terms of surface density of chemisorbed CP, which depends not only on the gold substrate but also on the incorporation of other blocking agents on the sensing layer (see the next section). We also found that genosensors constructed on bulk gold electrodes [8,9] showed very narrow linear ranges. In fact, in this case the response curve is characterized by a hyperbolic relationship between the signal and the target concentration (Langmuir isotherm), and only by representing the target concentration in a logarithmic scale can the linear range be obtained. Finally, we found a completely different behavior for the genosensors prepared on 3D-GNEE, which showed the highest roughness but

also the best sensitivity. In this case, the increase in electroactive area arises from nanostructuration to form accessible nanowires in a relatively controlled manner. Electrochemical treatment of the gold surface can produce extremely rough electrodes (R_f= 40) with a 10-fold CP strands loading but they must be maintained in water at 4 °C to preserve the nanopattern prior to their use. Interestingly after modification with the DNA SAM, the nanostructure is stable even at 40 °C temperature at which the hybridization was carried out [28].

It is also expected that higher surface uncontrolled roughness degrade sensor reproducibility, producing sensors with widely varying signals. Reproducibility ranging from 5 to 40% is observed (Table 2), but we find that genosensor reproducibility is only minimally affected by the topography of the gold support.

4. Sensing phase construction

To obtain a highly sensitive genosensor, the density and orientation of immobilized capture probes are key factors. In all genosensing schemes evaluated, hybridization experiments were carried out by using monolayers of single-stranded oligodeoxyribonucleotide probes, linear (21-71 mer) or with a stem-loop structure (32-43 mer), immobilized onto gold supports. These capture probes are anchored at one end and hang into solution, creating a "brush-type" structure by means of different immobilization approaches: binary, ternary, and pure self-assembled monolayers (SAMs), which are discussed below and are schematically represented in Figure 4.

Attachment of thiol-modified DNA probes onto gold surfaces by chemisorption has become a standard immobilization procedure in DNA-based biosensors field. DNA forms a peculiar SAM due to its very large size and highly anionic nature that induce large electrostatic and steric repulsion. The self-assembly process is then slow and requires shifting from initial lying down conformation to random coil bound through the thiol group [27]. Although, in order to cover the pinholes in the resulting DNA films, remove non-specifically adsorbed DNA strands, and attain a more convenient orientation of the chemisorbed DNA molecules for efficient hybridization, an alkylthiol is subsequently incorporated, typically 6-mercaptohexanol (MCH) (Figure 4A) [29]. Nevertheless, such binary configuration widely used during the last two decades has been put into question since remaining bare gold regions have been reported, with the concomitant contribution to the background signal [30].

The incorporation of a dithiol by co-immobilization with the thiol-modified capture probe, followed by chemisorption of MCH in a subsequent step (Figure 4D), i.e. using a two-step

ternary SAM, provided higher signal-to-blank ratios as a consequence of a more efficient passivation of the free gold surface, probably related to a lying flat positioning of 1,6-hexanedithiol covering cavities that are strong unspecific adsorption points, without sacrificing the electron transfer through the sensing layer [12,31].

Alternatively, the use of p-aminothiophenol as a backfilling agent, instead of MCH in a traditional binary SAM (Figure 4B), gives rise to lower background signals that, depending on the type of gold substrate, ranged from 16-fold to 6-fold lower, for screen-printed electrodes and thin films, respectively. Likewise, the electron transfer through these thioaromatic mixed SAMs is significantly increased as revealed by faradaic impedance spectroscopy and cyclic voltammetry studies, by using the outer-sphere process of $[Fe(CN)_6]^{4-/3-}$ [13]. These properties could be attributed to its rigid aromatic structure as well as the stronger Π-Π intermolecular interactions that improve packing efficiency and electric conductivity.

On the other hand, two-component SAMs produced by conventional backfilling method are currently known for their behavior as 2D liquids, where the thiolated components exhibit lateral mobility on the gold surface, leading to the formation of DNA islands [32], despite the anionic character of DNA, that render difficult the heterogeneous hybridization event (high local crowding) and reduce the sensing layer stability. In this regard, the use of an aromatic thiol as a diluent has proven to be beneficial as well. Particularly, the incorporation of thiol-tethered capture probes into p-aminothiophenol monolayers previously subjected to potential cycling at acidic pH, i.e. the so-called inserting method with electrochemical rearrangement (Figure 4C), provides a homogeneous distribution of DNA in the binary sensing architecture with about 5 fold higher DNA surface coverage, Γ_{DNA}, than that achieved with conventional backfilling methods, that is $(5.0\pm0.4) \times10^{12}$ $versus$ $(1.1\pm0.3) \times10^{12}$ molecules cm^{-2} [13]. This is reflected in an enhanced detectability of 6 pM, more than 30-fold than that obtained with the backfilling method using p-aminothiophenol as a diluent (Table 2).

These excellent results are comparable with those obtained with a pure SAM of p-mercaptobenzoic acid, which serves as a scaffold to covalently attach amine-functionalized capture probes (Figure 4E). In addition to the improved blocking ability of the aromatic thiol and the evident absence of DNA clusters when using this configuration, p-mercaptobenzoic acid acts as a rigid spacer maximizing the accessibility of the target in solution to the capture probe, thus favoring the hybridization efficiency. This was evinced by comparing pure and binary thioaromatic SAMs interrogated with bacterial ribosomal RNA (~1500 mer target); better sensitivity (8.28 $versus$ 0.40 $\mu A\ cm^{-2}\ nM^{-1}$) along with a detection limit ten times lower was accomplished with the pure thioaromatic monolayer [13].

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Similar limit of detection (1 pM) has been previously described for a sandwich assay and the same peroxidase-based signal transduction, but using as sensing layer a DNA tetrahedron structure, whit three thiol groups at three of the vertices and a pendant DNA acting as CP in the other. This scaffold allows an excellent control on the orientation and nanoscale distance between the capture probes (~4 nm) thus improving hybridization efficiency and detectability [33]. The good performance observed with this architecture presumably arises from the high rigidity of the tetrahedral structure and its favorable orientation, avoiding entanglement between probes and aggregations, as well as its high resistance to fouling. This indirectly confirms the probe homogeneity achieved with the aromatic thiol monolayers in a much simpler approach.

In light of these results, it is apparent that the surface density and distribution of DNA probes play a key role in genosensor performance, establishing a balance between surface DNA coverage and solution-phase target capture efficiency. The optimum Γ_{DNA} values recorded for each immobilization approach are in the range 1-5 $\times 10^{12}$ molecules cm^{-2}, which has been classified as medium surface density [5]; the tetrahedral DNA scaffold, for example, accommodates 4.8 $\times 10^{12}$ molecules cm^{-2} [33]. Therefore, packing constraints become weak provided that a homogeneous distribution of the DNA probe is achieved.

For each tested strategy, the concentration of CP in the immobilization solution was optimized by evaluation of the hybridization efficiency through recording the signal-to blank ratio for a fixed target concentration. In most of the cases, exposition to 1-2 µM CP solution led to the best analytical performance. However, in the case of ternary monolayers and thioaromatic SAMs prepared by the inserting approach subµM (0.05-0.1 µM) CP concentrations are required, thus turning out to be more cost-effective strategies.

The workhorse of sulfur-based SAMs on gold substrates is their long-term stability, which becomes an impediment to get a marketable genosensor. Conventional binary SAMs, obtained through backfilling method, lost ≈ 50-65 % in signal over the course of 7-10 days storage in buffer solution both at room temperature [34] or 4°C [35]. Such a drop in genosensor response was also accompanied by a decrease in the DNA surface coverage, Γ_{DNA} [34]. These results are in line with a displacement of DNA probes by the blocking thiol used in the construction of mixed monolayers. Conversely, when using ternary biolayers stored at 4 °C under dry conditions, no change in sensitivity was observed after 30 days, and 71% of their original sensitivity is retained after 90 days [35]. This significantly improved stability seems to be related to the surface anchoring of the biolayer components. In particular, co-immobilization of DNA probes with 1,6-hexanedithiol (a diluent with two anchors) presumably provides a less

dynamic sensing phase due to an enhanced affinity of the diluent for the gold surface. Moreover, the third component MCH was incorporated the day of the measurement for 15 minutes; therefore, the risk of DNA displacement is much lower. Out of these general trends, mixed SAMs consisting of a shared stem-loop structured capture probe and MCH designed for the detection of an intricate target, with a repetitive and structured sequence, exhibited a damped decline in genosensor response (just 27.2% after 11 days), when stored in the refrigerator [18]. The complexity of this system has been translated into unusual behaviors regarding several genosensor performances, namely sensitivity and stability.

In order to preserve the recognition and hybridization properties of the sensing phase, the structural integrity of the attached DNA probe must be also guaranteed. For that, two main factors should be beard in mind: DNA-degrading enzymes (i.e. the ubiquitous deoxyribonucleases or DNases) as well as dehydration. The incorporation of EDTA in washing and storage buffers to "sequester" divalent cations that act as cofactors for DNases is very convenient to avoid enzymatic cleavage of DNA probes. Furthermore, from a commercial point of view, dry storage of sensing platforms is particularly appealing; however, the dehydration of DNA impacts on its tertiary structure and hence the molecular recognition is negatively affected. In this regard, the replacement of water molecules by saccharides molecules such as trehalose has proven to provide protection against dehydration in different biological structures [36]. Thus, Lai *et al.* [34] evaluated the effect of different sugars in combination with BSA during the washing step of conventional binary SAMs, before being stored in dry conditions. After a rehydration step in citrate buffer before testing the sensing architecture, a decrease in signal of 7-9% was recorded upon storing the genosensors for one month. It indicates substantially enhanced storage stability with respect to wet storage or in the absence of these preservatives. Recently, thioaromatic SAMs, pure and binary monolayers resulting from the inserting method with previous voltammetric treatment prepared by our group were subjected to a similar protocol using 2.5% (w/v) each of glucose and BSA, without observing any signal loss during the time assessed, 1 week and 30 days of dry storage [13]. These improved results could be attributed to a synergism between the type of sensing architecture that involves aromatic thiols and the presence of the stabilizing agents.

5. Other operating conditions

Undoubtedly success in electrochemical genosensing is related to reduce background currents to nA levels. In addition to construct more compact (pure) SAMs or dithiol-based SAMs to fill the Au pinholes left by DNA probes, the use of blocking agents is also recommended. In general, addition of 2.5% BSA in the hybridization buffer reduces the blank

current without altering the signal in the presence of target, thus, increasing the S/B ratio. Since thermal shock to denature the secondary structure of target or to dehybridize amplicons is usually needed, BSA must be added after this step (heating and cooling) in order not to denature the protein, which yields a viscous solution unsuitable for the experiment. Good results were also achieved without BSA in this step but an additional blocking step was required prior to the labeling with enzyme conjugates. The blocking buffer contains 5% BSA and also Tween20 to help removing unspecifically bound strands [8,16,18]. Attempts to suppress the surfactant were infructuous due to increased blank currents.

Composition of the hybridization buffer can be tuned to accelerate the kinetics and to emphasize the selectivity, ideally specificity, that is, total discrimination of a single-point mutation. Volume-occupying compounds such as dextran, polyethylene glycol, ficoll or formamide reduce the free volume of DNA strands, that is, increase the local concentration, facilitating the interaction [37,38]. All of them contain groups capable of forming hydrogen bonds and contribute to destabilize the helix structure by competition with the nucleotide bases, so only perfect duplexes remain stable [39]. In our experience the use of 25% formamide alone does not improve the S/B ratio but in combination with dextran sulfate the dramatic increase is obscured by the high density of the solution that enhances the irreproducibility. Combination with SDS also diminishes the precision. Thus, crowding agents are only adequate when maximization of selectivity is the priority and cannot be achieved with smarter approaches like hairpin-like capture probes [8,9].

There is no consensus in the role of ionic strength. Though a relatively high concentration of salts (about 0.3 M of Na ions typically obtained with $2\times$ buffers such as phosphate SSPE or citrate SSC) is compulsory to avoid repulsion between strands (in fact pure water is sometimes used for denaturation), higher concentrations achieved by a lower dilution of the hybridization buffer (up to $10\times$) do not improved the S/B ratio [18] nor the selectivity against mismatched sequences [39]. However, addition of 0.9 M of NaCl without altering other components resulted essential to obtain low limits of detection not only in genosensors but also in genoassays on magnetic microparticles [40]. The salt concentration increases the hybridization efficiency both on a surface and in solution. The optimum concentration is dependent on the immobilization method that controls the DNA surface density. For mixed SAMs with MCH an optimum value of 620 mM was found [41], which is far from the above mentioned concentration obtained by a DoE. DNA surface coverage and length of the capture probe that is longer in DoE case (32 vs 25 mer) can account for the discrepancy.

In sandwich formats an excess of signaling probe is commonly used especially when dealing with real samples where the target is a ds-fragment from genomic DNA. Using DoE an optimal value of 2 μM on screen-printed electrodes modified with mixed SAMs was found [16]. Though remarkably low limits of detection were obtained after DoE optimization of this and numerous parameters, a survey of our experiments shows that even at 100 nM low LDs can be derived and increasing concentration of signaling probe tend to increase the LDs. However, this fact cannot be attributed exclusively to this parameter. The synergistic contribution of substrate type and biolayer design should be also considered.

There are two types of conjugate frequently used in enzymatic genosensors depending on the signaling probe tag: Fab-fragment based and streptavidin-based conjugates. Multivalency of streptavidin causes cross-reaction between different labeled duplexes reducing the sensitivity. It is then preferable to use monovalent conjugates [42]. In addition to this, peroxidase (POD) and alkaline phosphatase (ALP) are widely employed but the POD turnover is much higher than ALP one, which means faster and higher responses. However, excellent results can be also obtained with ALP [8,16].

6. Conclusions and Outlook

This work presents a comparative evaluation of distinct sandwich genosensor designs in an effort to elucidate the main fabrication and operation factors that affect their analytical performance. Sandwich-hybridization in two steps, one of them in solution, between the target DNA and two probes, capture and signaling probes, is the most flexible sandwich configuration in terms of genosensor design that makes possible the detection of structured (relatively high ΔG_{self}) and long target sequences, circumventing their tagging. The formation of continuous ternary duplexes as a result of the recognition event is essential to get a sensitive sensing device; although, the effect of single-stranded tails near the surface should not be despised. In this regard, the Gibbs free energy for the interaction between this overhang and the capture probe must be controlled, and values lower than -7 kcal/mol are recommended. Probes design has also effects on the selectivity of the assays, and we find that when possible, the use of shared stem-loop capture probes is a smarter strategy to get improved selectivity. Comparison of the genosensors in terms of the topography of the gold transducer indicates that a decrease in the gold roughness leads to improved sensitivity, although in the case of 3D-surfaces the opposite trend is observed. This observation may be attributed to the role played by the nanowires in the control of the density of probe DNA, given rise to optimum recognition ability.

Another key aspect is the sensing layer architecture, which determines both the DNA surface coverage and the magnitude of non-specific interactions. In this regard, our group has been pioneer in exploring and applying the favorable properties characterizing aromatic thiols for the development of DNA sensing platforms to gain more control over monolayer formation. When the typical blocking MCH, chemisorbed after CP immobilization (backfilling approach), was substituted by p-ATP, optimal non-specific binding suppression was achieved. We have also observed that the sensitivity of genosensors is often defined not only by the effective CP concentration but also by the homogeneity of its distribution on the sensing layer. From this perspective, pure thioaromatic monolayers as scaffold to covalently immobilized the capture probe support maximum sensitivity, which is similar to that obtained by constructing the sensing layer by p-ATP chemisorption followed by its electrochemical rearrangement and subsequently inserting the thiolated-CP. These results are consistent with higher uniformity of the DNA layer with the inserting after electrochemical cycling method.

Finally, we advise to pay careful attention to the operational conditions under which the sensor is interrogated, especially the ionic strength of the hybridization media as well as the concentration of signaling probe exceeding in more than 30-fold the upper limit of the target concentration range and the valence of the enzyme conjugate used as label before the measuring step.

The past 10 years have witnessed great advances in hybridization sandwich genosensor. The description of the specific operational variables affecting its performance discussed in this Review provides us an opportunity to reflect the powerful of these approaches as promising candidates for applications in clinical diagnosis, food safety control and environmental monitoring. Advancements in biotechnology, synthetic chemistry and nanotechnology have allowed significant improvements in sensitivity and stability. However, there are still several issues that must be solved before these sensors may reach the market. Key areas of development include innovative sample pretreatment approaches, which could be integrated with the electrochemical sensors into automated platforms. This will be essential to achieve its widespread use for point-of-need applications. Most electrochemical genosensors are tested using as target synthetic oligonucleotides, but the analysis of genomic DNA sequences requires amplification of the desired target, usually by PCR. The development of robust isothermal amplification strategies, which allow to restrict the size of the genomic DNA and facilitate hybridization, is another field where greater emphasis should be placed. Integration of these amplification strategies in the automated platforms would allow a further step towards obtaining accurate results even in the absence of trained personnel. By focusing in this areas

and taking advantage of new advancements in nanotechnology and electronic, the final goal of fully automatic systems may be accomplished in the near future.

Acknowledgments

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Table 1. Description of the designs comparatively evaluated in this study. ΔG_{self} is the predicted free energy for the folding of each sequence.

No.	Analyte	Role	Sequence 5´→3'	Length	*ΔG_{self} / kcal mol^{-1}
1	Lp-1	Target	AAGTTATCTGTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGG	52	-6.21
		CP	HS-(CH$_2$)$_6$-CGGCCAGTATTATCTGACCGTCCCATGGCCGT	32	-6.56
		SP	GGTTAAGCCCAGGAATTTCACAGATAACTT-biotin	30	-0.66
2	Mon810	Target	TCGAAGGACGAAGGACTCTAACGTTTAACATCCTTTGCCATTGCCCAGCTATCTGTCACTTTATTGTGAAA	72	-7.06
		CP	TTAGAGTCCTTCGTCCTTCGA-(CH$_2$)$_6$ SH	21	-1.84
		SP	6FAM-TCTTCACAATAAAGTGACAGATAGCTGGGCAATGGCAAAGGATGTTAAACG	51	-4.26
3	Lp-2	Target	AAGGGTGCGTAGGTGGTTGATTAAGTTATCTGTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTTGACT	80	-6.21
		CP	HS-(CH$_2$)$_6$-AGTCAACCAGTATTATCTGACCGTCCCA H$_2$N-(CH$_2$)$_6$-AGTCAACCAGTATTATCTGACCGTCCCA	28	-1.75
		SP	GGTTAAGCCCAGGAATTTCACAGATAACTT-6FAM	30	-0.66
4	Sal-1	Target	ATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTTTACGGT	80	-13.87
		CP	GCGCGCGGCATCCGCATCAATAATACCGGCCTTCAAAT-(CH$_2$)$_6$-SH	38	-4.71
		SP	6FAM-ACCGTAAAGCTGGCTTTCCCTTTCCAGTACGCTTCGCCGTTC	42	-4.05
5	E. coli	Target	TGCAAACATGTTGGGCTATAACGTCTTCATTGATCAGGATTTTTCTGGTGATAATACCCGTTTAGGTATTGGTGGCGAATA	81	-7.41
		CP	ATGAAGACGTTATAGCCCAACATGTTTGCA-(CH$_2$)$_6$SH	30	-0.93
		SP	6FAM-TATTCGCCACCAATACCTAAACGGGTATTATCACCAGAAAAATCCTGATCA	51	-5.40
6	Sal-2	Target	GGTCTGCTGTACTCCACCTTCAGCCATTACGACGATATTCGTCCGGGTGAAGTGGGCCAGCGTCAGAACGGCGTACTGATCTCCAA	86	-13.29
		CP	HS-(CH$_2$)$_6$-CCGTTCTGACGCTGGCCCACTTCAC	25	-2.06
		SP	CCGGACGAATATCGTCGTAATGGCTGAAGGTGGAGTACA-6FAM	39	-4.64
7	Arah-2	Target	GCAGCAGTGGGAACTCCAAGGAGACAGAAGATGCCAGAGCCAGCTCGAGAGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGC	86	-18.91
		CP	HS-(CH$_2$)$_6$-GCATGAGATGTTGCTCGCAGGGCCTCAGGTTC	32	-4.31
		SP	GCCCTCTCGAGCTGGCTCTGGCATCTTCTGTCTCCTTGGAGTTCCCACTGCTGC-biotin	54	-5.79

No.	Analyte	Role	Sequence 5´→3'	Length	$^*\Delta G_{self}$ / kcal mol^{-1}
8	Lp-3	Target	AAGGGTGCGTAGGTGGTTGATTAAGTTATCTGTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTTGACTCGAGTATGGGAGAGG	95	-7.89
		CP	HS-(CH$_2$)$_6$-CGGCCAGTATTATCTGACCGTCCCATGGCCGT	32	-6.56
		SP	GGTTAAGCCCAGGAATTTCACAGATAACTT-biotin	30	-0.66
9	Gluten-1	Target	CTGCAGCTGCAACCATTTCCGCAGCCGCAACTACCATATCCGCAGCCGCAACTACCATATCCGCAGCCGCAACTACCATATCCGCAGCCGCAACCATTT	99	-10.47
		CP	HS-(CH$_2$)$_6$- AAATGGTTGCGGCTGCGGATATGGTAGTTGCGG	33	-1.60
		SP	CTGCGGATATGGTAGTTGCGGCTGCGGATATGGTAGTTGCGGCTGCGGAAATGGTTGCAGCTGCAG-6FAM	66	-11.58
10	Gluten-2	Target	CTGCAGCTGCAACCATTTCCGCAGCCGCAACTACCATATCCGCAGCCGCAACTACCATATCCGCAGCCGCAACTACCATATCCGCAGCCGCAACCATTT	99	-10.47
		CP	HS-(CH$_2$)$_6$-GTTCCGCAAATGGTTGCGGCTGCGGATATGGTAGTTGCGGAAC	43	-9.31
		SP	CTGCGGATATGGTAGTTGCGGCTGCGGATATGGTAGTTGCGGCTGCGGAAATGGTTGCAGCTGCAG-6FAM	66	-11.58
11	P-35S	Target	GTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTGACAAGCTGACTCTAGCAGA	105	-14.38
		CP	AGAGGAAGGGTCTTGCGAAGGATAGTG-(CH$_2$)$_6$-SH	27	-0.35
		SP	6FAM-CTAGAGTCAGCTTGTCAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATAT	53	-3.41
12	Tri a 18	Target	CTGTTGTAGCAAGTGGGGATCCTGTGGCATCGGCCCGGGCTATTGCGGTGCAGGCTGCCAGAGTGGCGGCTGCGATGGTGTCTTCGCCGAGGCCATCACCGCCAACTCCACTCTTCTCCAAGAAT	125	-21.93
		CP	HS-(CH$_2$)$_6$- ATTCTTGGAGAAGAGTGGAGTTGGCGGTGATGGCCTCGGCGAAGACACCA	50	-5.89
		SP	TCGCAGCCGCCACTCTGGCAGCCTGCACCGCAATAGCCCGGGCCGATGCCACAGGATCCCCACTTGCTACAACAG-6FAM	75	-11.32
13	Gluten-3	Target	CAGCTGCAACCATTTCCGCAGCCGCAACTACCATATCCGCAGCCGCAACTACCATATCCGCAGCCGCAACTACCATATCCGCAGCCGCAACCATTTCGACCACAACAACCATATCCACAATCGCAACCACAGTA	134	-9.52
		CP	HS(CH$_2$)$_6$- TACTGTGGTTGCGATTGTGGATATGGTTGTTGTGGTCGAAATGGTTGCGGCTGCGGATATGGTAGTTGCGG	71	-2.94
		SP	CTGCGGATATGGTAGTTGCGGCTGCGGATATGGTAGTTGCGGCTGCGGAAATGGTTGCAGCTG–6FAM	63	-8.34

$^*\Delta G_{self}$ was estimated at 25 °C and 0.3 M NaCl, except for Arah-2 calculated for 0.9 M NaCl

Table 2. Operational and fabrication conditions, and overall analytical performance of the genosensors compared in this study. No. design as summarize in Table 1.

No.	Conditions				Analytical performance				Ref
	Transducer	*SAM*	*[SP]/ μM*	*Enzyme-conjugate*	*Linear range*	*LOD/ pM*	*Sensitivity /μA cm^{-2} nM^{-1}*	*RSD*	
1	Bulk Au	MCH	2	Strp$_2$-ALP	0.2 nM-1 μM	340	3.9 (log)	10%	8
1	Bulk Au	MCH	2	Strp-POD	0.2 fM-2 nM	0.0002	0.18 (log)	13%	23
1	SPE	MCH	2	Strp$_2$-ALP	0.2-20 nM	400	11.5 (log)	10%	10
2	3D-GNEE	MCH/HDT	0.5	antiF-POD	0.25-5 nM	250	88	14%	19
2	SPE	MCH	0.5	antiF-POD	0.25 -10 nM	480	1.42	5%	19
3	SPE	MCH	0.1	antiF-POD	---	200	---	---	13
3	SPE	pMBA	0.1	antiF-POD	0.1-1 nM	40	19.9	18%	13
3	SPE	pATP	0.1	antiF-POD	0.05-1 nM	6	19.4	20%	13
3	Thin-film	p-ATP	0.25	antiF-POD	0.005-2 nM	1.8	33	39%	21
4	Thin-film	pATP	0.25	antiF-POD	0.005-5 nM	3	31.4	19%	14
5	SPE	MCH/HDT	0.25	antiF-POD	0.1-1 nM	60	90.5	7%	12
6	SPE	pATP	2	antiF-POD	5-300 M	4000	0.07	14%	15
6	Thin-film	pATP	2	antiF-POD	0.1-1 nM	120	47.8	13%	22
7	SPE	MCH	2	Strp$_2$-ALP	0.05-50 nM	10	23.9	7%	16
8	SPE	MCH	0.25	antiF-POD	0.5- 5 nM	300	1.8	41%	11
8	Bulk Au	MCH	2	Strp$_2$-ALP	0.2 – 20 nM	340	3.9 (log)	14%	9
9	SPE	MCH	1.25	antiF-POD	1-50 nM	300	1.11	3.5%	17
10	SPE	MCH	1.25	antiF-POD	5-50 nM	1000	0.57	4.3%	18
11	Thin-film	0.1	0.1	antiF-POD	0.1-3 nM	9	42	7%	20
13	SPE	MCH	1.25	antiF-POD	1-20 nM	200	6.45	21%	17

CAPTIONS FOR THE FIGURES

Figure 1. Schematic of the sandwich hybridization assay in two steps. (A) Homogeneous hybridization between target and signaling probe (B) Heterogenoeus hybridization on the sensor surface with the immobilized capture probe.

Figure 2. Radar charts summarizing the free energies (kcal/mol) of (left) folding of the different studied sequences, (right) hybridization between T and SP (ΔG_1), T and SP+CP (ΔG_{tot}), and CP and SP (ΔG_{blank}). The same order No. as in Table 1 is used.

Figure 3: Scheme showing the effect of an overhang of the target (blue sequence) after the formation of the ternary duplex when this overhang is partially complementary to the CP (red sequence).

Figure 4: Schematic illustration of the sensing layer architecture using binary (A-C), ternary (D) and pure (E) self-assembled monolayers. The blocking agents are: 6-mercaptohexanol (MCH) (A); *p*-aminothiophenol chemisorbed after the CP (B) or before the CP, which is adsorbed after a previous electrochemical cycling (C); 1,6-hexanedithiol co-immobilized with the CP and MCH (D) and *p*-mercaptobenzoic acid to which the CP-NH$_2$ is covalently immobilized (E).