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A rational approach in probe design for nucleic acid-based biosensing

M.L. Ermini[a], S. Scarano[a], R. Bini[a], M. Banchelli[a,b], D. Berti[a,b], M. Mascini[a], M. Minunni[a,*]

[a] Dipartimento di Chimica "Ugo Schiff", Università degli Studi di Firenze, Via della Lastruccia 13, 50019, Sesto F.no (FI), Italy
[b] CSGI, Università degli Studi di Firenze, Via della Lastruccia 13, 50019, Sesto F.no (FI), Italy

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ABSTRACT

Development of nucleic acid-based sensing attracts the interest of many researchers in the field of both basic and applied research in chemistry. Major factors for the fabrication of a successful nucleic acid sensor include the design of probes for target sequence hybridization and their immobilization on the chip surface. Here we demonstrate that a rational choice of bioprobes has important impact on the sensor's analytical performances. Computational evaluations, by a simple and freely available program, successfully led to the design of the best probes for a given target, with direct application to nucleic acid-based sensing. We developed here an optimized and reproducible strategy for *in silico* probe design supported by optical transduction experiments. In particular Surface Plasmon Resonance imaging (SPRi), at the forefront of optical sensing, was used here as proof of principle. Five probes were selected, immobilized on gold chip surfaces by widely consolidated thiol chemistry and tested to validate the computational model. Using SPRi as the transducting component, real-time and label free analysis was performed, taking the *Homo sapiens* actin beta (ACTB) gene fragment as model system in nucleic acid detection. The experimental sensor behavior was further studied by evaluating the strength of the secondary structure of probes using melting experiments. Dedicated software was also used to evaluate probes' folding, to support our criteria. The SPRi experimental results fully validate the computational evaluations, revealing this approach highly promising as a useful tool to design biosensor probes with optimized performances.

1. Introduction

Nucleic acid sensing has always been an active field in chemical research. In particular, DNA sensing has been applied to a variety of analytical problems from clinical diagnostic to food and environmental analysis. Different transduction principles are evaluated, using both label and label-free approaches (D'Orazio, 2003; Scarano et al., 2010; Teles and Fonseca, 2008; Tothill, 2009). The aim of these approaches is the selective detection of target sequences at DNA or RNA level (Lucarelli et al., 2008). In the case of point mutation detection, with relevance in molecular clinical diagnostic, the sensors are designed to discriminate between sequences differing only by one base (Dell'Atti et al., 2006; Healey et al., 1997; Sato et al., 2003; Wilson et al., 2005). These devices have opened up new possibilities for cheap, fast, real time and eventually label free detection of analytes (Scarano et al., 2010 and references therein; Sendroiu et al., 2011; Halpern et al., 2011; Gifford et al., 2010; Chen et al., 2010). In DNA-based biosensor development, key steps are: the surface immobilization chemistry, which should prevent unspecific adsorption in order to lower background noise, and the probe design, responsible of the system selectivity. Different immobilization chemistries are now available for probe immobilization (Brockman et al., 1999; Caruso et al., 1997; Mannelli et al., 2005; Smith et al., 2001), despite much research in nucleic acid-based sensing being published, little work has been dedicated to strategies for probe design and the effect of its selectivity on the sensor analytical performances. Generally, the complementary sequence to the target analyte (a gene, a fragment or a short oligonucleotide) is first considered during the choice of probe. The probe length is generally set ranging from 15 to 20 bases, with one end usually linked to a functional group to be exploited for the immobilization chemistry. For example, biotinylated probes are used in surface functionalization involving streptavidin; thiolated probes (Kukanskis et al., 1999; Mannelli et al., 2005) are required for direct probe coupling to gold surfaces via Self Assembled Monolayer (SAM) formation (Allara and Nuzzo, 1983). Another criterion taken into account in probe selection is the C–G base content (three hydrogen bonds vs. two with A–T pairing); preferred composition varies from at least 40% to 60% (Powdrill, 2003) to stabilize the hybrid on the surface. Finally, to facilitate surface hybridization, it is

* Corresponding author. Tel.: +39 0554573314; fax: +39 0554573384.
E-mail address: maria.minunni@unifi.it (M. Minunni).

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Table 1
Probe and target sequences.

Probe 1: 101–120	5′-SH-$(CH_2)_6$-AGCGGAGCAGGAAAAGAGGA-3′
Probe 2: 5–24	5′-SH-$(CH_2)_6$-CCCTGTAAGGGAGCTTTCGG-3′
Probe 3: 138–157	5′-SH-$(CH_2)_6$-GTGAGGGGCAGGGAGGAGCT-3′
Probe 4: 150–169	5′-SH-$(CH_2)_6$-GAGCAGCCTGGGGTGAGGGG-3′
Probe 5: 145–164	5′-SH-$(CH_2)_6$-GCCTGGGGTGAGGGGCAGGG-3′
Target	5′-AGCTCCGAAAGCTCCCTTACAGGGCAAAGTTCCCAAGCACAGAAGAGAACCTGTTCACTTCTCCCCTGCTCGGCCCGCCCCCTGGCCAGGCACCTC-TACTTCCTCTTTTCCTGCTCCGCTGCTTGCTTTCTCTCTTCAGCTCCTCCCTGCCCCTCACCCCAGGCTGCTCGGCCACCTCCAACCTGCCACCTGAGGACA-CCCAGGCAGTCACTCATTCAACAGCGAGG-3′

important to avoid probe hybridization on regions that can assume conformations and may obscure the binding site of interest, i.e. by formation of secondary structures such as hairpins or loops.

Although very interesting works are present in literature related to DNA-based sensing and probe selection (Tomiuk and Hofmann, 2001; Kaderali and Schliep, 2002; Letowski et al., 2004; Lucarelli et al., 2008; Li and Stormo, 2001; Mulle et al., 2010; Tedeschi et al., 2005; Rahmann, 2003; Wang and Seed, 2003) there is a need for standardized strategies for rational probe design, that could be assessed for its validity before using the probes, avoiding the waste of money and time.

This work aims to develop an optimized and reproducible strategy for probe design for nucleic acid-based sensing. We here consider a computational assisted approach for probe design, based on a free available software. The *in silico* selection was validated by experiments conducted using optical transduction for DNA-sensing development. In particular Surface Plasmon Resonance imaging (SPRi) was used as proof of principle. In this case, the conventional SPR is coupled to a micro-array format through the introduction of a Charge-Coupled Device (CCD) as its detector system instead of a diode arrays. SPRi is at the forefront of optical sensing, allowing real-time and label free simultaneous measurements multi-analyte. In SPRi, probes are tethered on a chip gold surface in array format.

In this study, it was demonstrated that "smart" probe design for DNA-sensing significantly improves the sensor's analytical performances for DNA–DNA hybridization measurements that were coupled with a computational assisted approach (Wernersson et al., 2007). The idea we support is that it is possible to design a probe specific for target sequence using a predictive model, based on *in silico* computations, to rationalize chip design for DNA-sensing application to improve analytical sensor performances. The pool of probes sorted by the program, is scored on the base of key parameters.

As model system, we validated computational results obtained on actin beta (ACTB) gene fragment belonging to *Homo sapiens* by assaying a pool of selected probe sequences by SPRi. To better understand computational probe selection and experimental sensor behavior, melting experiments were performed by UV spectrophotometry. DNA self-complementariety of selected probes were evaluated in solution and then compared with the folding obtained with Mfold free software (Wernersson et al., 2007).

Our work demonstrated how rational design of nucleic probes with application DNA sensing can lead to improved analytical performances. This is, to the best of our knowledge, the first attempt in rational probe design with validation of probe selection, using *in silico* approach coupled to SPRi transduction. The theoretical model is confirmed by experimental testing, introducing new perspectives in the application of nucleic acid detection.

2. Materials and methods

2.1. In silico design of DNA probes

Probes' design based on free software OligoWiz 2.0 (Wernersson et al., 2007). To obtain the specific probes, first the sequence of interest, i.e. target analyte, inserted. Then the list of possible probes to be immobilized on the chip surface is provided. The program identifies complementary sequences on the basis of five parameters before producing a final ranking of the identified probes defined as: Cross-hybridization, DeltaTm, Folding, Position and Low-complexity.

In this study, the 20-mer probes selection process was applied to a 227 mer fragment of the *Homo sapiens* beta actin (ACTB) on chromosome 7 (from position 896 to 1123 of the gene sequence). Scores were calculated by the program for each probe exploiting the nearest neighbor algorithm (Buser, 1983), taking into account the characteristics of neighboring nucleotides (identity and orientation) to evaluate the free energy involved in the stability of the base pairing. The score was expressed in a range between 0 (wretched probe) and 1 (excellent probe), based on the five parameters mentioned before.

The outcome of the program finally consists of a set of scored probes. From the ranked probes, we selected five sequences, one with the best scores, one the worst scored and three with intermediate score. The following weighting of the scores was applied in our evaluation: Cross-hyb: 0.0%; DeltaTm: 70.2%; Folding: 17.5%; Position: 0.0%; Low-complexity: 12.3%.

2.2. Buffers and reagents

Thiolated DNA probes were anchored on the biochip gold surface by diluting them to a concentration of 10 μM in Immobilization Solution (IS): 1 M KH_2PO_4 (Merck, Italy), pH 3.8. To passivate gold, aqueous solution of 1 μM 11-mercapto-1-undecanol (MU) and 1 μM 6-mercapto-1-hexanol (MCH) were used. Hybridization Solution (HS) for SPRi measurements used was as follows: 300 mM NaCl, 0.02 M Na_2HPO_4, 0.1 mM EDTA, pH 7.4, 0.05% (w/v) of TWEEN® 20 (Polyethylene glycol sorbitan monolaurate). 100 mM HCl solution was used to regenerate the biochip surface, after each measurement cycle. PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning, UK) was used for the micro-welled mask preparation. Unless otherwise stated, reagents were purchased from Sigma–Aldrich (Milan, Italy).

All solutions were prepared in MillQ water (Millipore Corporation, MA, USA) and filtered using vacuum filter cups (Millipore E express plus, 0.22 μm) and syringe filters (Puradisc, cellulose acetate, 0.2 μm from Whatman GmbH, Dassel, Germany).

5′-Thiol modified probes and complementary unmodified targets were purchased from Eurofins MWG Operon (Ebersberg, Germany). The relative sequences and positions on the target sequence are reported in Table 1.

2.3. SPRi instrumentation

For this work probes' hybridization efficiency was tested using a Surface Plasmon Resonance imaging instrument Lab+-SPRi (Genoptics-Horiba Scientific, Orsay, France) integrated with a microfluidic system (PEEK tubing, Restek corporation, 1/16 in. OD × 0.01 in. ID; Rheodyne valve, loop injection system, 50 μl loop volume) where flux is generated by a peristaltic pump (Miniplus 3, Gilson) using accurate tubing from Elkay, orange/black, 0.015 cm^3/min.

Under total internal reflection condition, a polarized light beam passes through the prism, hits the gold layer and it is reflected to a CCD camera. Under certain conditions depending on incidence angle, gold free electrons can absorb incident light photons, resulting in resonant oscillation and generating surface plasmon waves. Modifications occurring at the chip surface, i.e. hybridization between DNA probes and target sequences can induce changes of resonance conditions due to a change in refraction index of surface. The variation of intensity of reflected light at a fixed angle was monitored by a CCD camera. A dedicated software (SPRiview L3.1.2), provided by the producer, enabled monitoring in real-time, the biomolecular interactions both by SPR signals (sensorgrams) and digital images (i.e. changes in reflected light). This instrumentation set up was also reported previously (Scarano et al., 2010).

2.4. Probes immobilization on SPRi biochip surface

DNA probes were immobilized in array format on SF-10 glass prisms coated with gold (50 nm-thick) (SPRi-Biochips™, Genoptics-Horiba Scientific, Orsay, France) using a dedicated PDMS mask for precise deposition of receptors, coupled to manual spotting procedure (Scarano et al., 2010). The PDMS film acted as micro-welled support on the gold surface. Each micro-well on chip was filled with 0.8 μl of thiolated probe solution (10 μM in IS). The selected probes were deposited on chip surface by exploiting the affinity between sulphur and gold, creating at least three spots for each probe. Two spots were used as reference surfaces (IS only).

The chip was placed into a moist chamber and vacuumed for 20 min and then left into the chamber for 12 h. During this time, the probes were anchored to the gold, forming a self-assembled monolayer (SAM). Before use, the chip surface was subjected to a passivation step, dipping the prism in the thiols mixture for 24 h. The surface was then washed with water and inserted in instrument's flow cell.

2.5. SPRi measurements

During each measurement, biochip surface was kept under a constant flow of HS (6 μl/min, 11 μl flow cell internal volume 11 μl). Calibration curves with synthetic oligonucleotides were obtained by injecting 100 μl of synthetic fully complementary targets at different known concentrations in the valve. The injection loop was 50 μl, thus volume in excess was used to wash off air bubbles or impurities from the loop assuring its complete filling. The target reaches the flow cell about 4 min after valve opening, leading to observed hybridization reaction (target-probe pairing) that lasted for about 10 min. Then the surface was washed by the HS flow, removing eventual excess of analyte from surface. Sensorgrams corresponding to hybridization binding were recorded in real time, plotting the reflectivity variation percent ($\Delta R\%$) against time. SPR signal was obtained by subtracting intensity of reflected light before injection, from the steady signal after the washing step. The differential image of the chip surface is generated by the software which recorded the reflected light variation during the experiment (from sample injection to washing step). Surface regeneration is achieved by dissociating the double-stranded DNA (dsDNA) complex on the surface with the regeneration solution (24 μl/min for 1 min), allowing probes to be available for subsequent analysis.

SPR signals were sampled on selected Regions of Interest (ROIs) of the array. For each immobilized spot, 3 circular ROIs with a radius of 70 μm were defined. Calibration curves were obtained with synthetic complementary oligonucleotides for the immobilized from 2 to 500 nM range. Each solution was prepared by diluting stock solutions of synthetic targets in HS. All measurements were performed at room temperature.

Table 2
Scores for each considered probe.

Probe	Total score	DeltaTm	Folding	Low complexity
1	0.936	0.963	1.000	0.692
2	0.936	0.952	1.000	0.692
3	0.725	0.674	1.000	0.621
4	0.500	0.338	1.000	0.709
5	0.255	0.000	0.996	653

2.6. Thermal denaturation/renaturation spectrophotometric measurements

Possible secondary structures on DNA probes sequences were investigated by melting experiments conducted on UV–VIS spectrophotometer Cary® 100 Bio (Varian, Inc.), interfaced with a computer by a dedicated software (Cary Win UV). Data analysis and fitting was performed using QtiPlot 0.9.7.13.

4 μM HS solution of each probe was subjected to four scans in temperature in a range between 25 °C and 80 °C (data interval 0.5 °C, scanning rate 1 °C/min) and relative melting profiles acquired (260 nm). Possible self-pairing structures were estimated by Mfold software (Zuker, 2003) simulating experimental conditions (25° C; 0.34 M Na^+).

3. Results and discussion

In this study a computational approach for probe selection in DNA-based sensing was performed using OligoWiz (Wernersson et al., 2007) and then experimentally validated. After identifying a pool of probes with different scores, among them, five different probes were selected (corresponding parameters summarized in Table 2). These five thiolated probes were immobilized on gold chip in an array format and relative interactions with complementary sequences were analyzed, using SPRi transduction.

3.1. Setting of critical parameters

Cross-hybridization, DeltaTm, Folding, Position, and Low-complexity are the key parameters that OligoWiz combines to obtain the total score for each probe sequence. These parameters can be tuned based on experimental conditions of the analysis. This was done by defining a specific weight (expressed as percentage contribution) for each parameter considered. For instance, relative priority should be assigned to Cross-hybridization and Position parameter to reduce unspecific interactions between probe and possible interfering sequences present in genomic DNA samples. However, in our analysis we studied on purpose a model system, using only synthetics targets in order to reduce background effects and underlying the direct role of the specific probe characteristic, i.e. the bases sequence which influences significantly its secondary structure and thus its Tm.

This evaluation was strictly based on the aim of this work, which was to study a model using the simplest experimental conditions so that the analytical performances of the biosensor depend only on the specific characteristics of the probes. Cross-hybridization score has to considered in the analysis of samples containing possible interfering sequences, i.e. amplified or genomic DNA, that can affect the biosensor selectivity, absent in this case study. Since fully complementary targets were used for our purposes, 0% value for the Cross-hybridization score was imposed to correspond to the absence of any interfering contribution from complex matrix. For this reason, in the considered case study Cross-hybridization score different from 0% would not produce a realistic evaluation.

A higher priority, instead should be granted to the DeltaTm score, that appear to be very impactful for a good prediction in

this study. This parameter considers the distribution of the melting temperatures (Tm) for all possible probes; low DeltaTm score for a certain probe sequence indicates a high deviation of its Tm from the average Tm. In SPRi, DNA probes work simultaneously in an array format and, therefore, at the same temperature. Ideally, Tm relative to target-probes duplexes should be very similar, so that the affinities of base pairing recognition are comparable under the same experimental conditions. This would ensure high homogeneity during the hybridization process of all probe sequences. DeltaTm was weighted at 70.2% for all reported experiments.

This value was selected in order to make DeltaTm contribution higher than the remaining two parameters (low complexity and folding). In other words by assigning 0% to Position and Cross-hybridization, we increased the relative priority of DeltaTm to compensate.

The formation of secondary structures on the probe sequence is also an important parameter in probe design. OligoWiz assigns a poor Folding score to sequences subjected to a folding, strong enough to the extent that it interferes with target hybridization, by calculating the free energy potential of the strongest structures. Folding parameter was weighted at 17.5% for all reported experiments. The folding weight given was selected based on advice by the program developer, i.e. to keep low values for folding. The physico-chemical evaluation considered here only accounts for the energy involved in secondary structure intramolecular base pairing. However, as demonstrated in this paper, further evaluations involving loop position with respect to chip surface were also important in the estimation of the influence on the folding.

Finally, Low complexity parameter assigns high-scored values to probe sequences displaying a high degree of complexity in the primary nucleotide sequence, and thus its weight contributes to enhance the specificity of probe. Low complexity parameter was weighted at 12.3% for all reported experiments, as standardized by the program developer.

3.2. Computational design of probes

Once the probe length (20 mers) was defined, the program output indicated the calculated scores relative to the sequences in the different positions on the target DNA.

Suitable positions on target sequence for high-scored probes are displayed in red (Wernersson et al., 2007), whereas low-scored probes, with expected low performances in hybridizing target are colored in gray. Evaluating probes distribution along the target sequence, we can have an idea of the value of the different considered parameters, by walking on the target sequence (see Fig. S1). For example, Low complexity score in our case, oscillates between 0.9 and 0.6 shows that this parameter does not fluctuate dramatically. Thus, it has little influence over the total score. Cross-hybridization score varies, along whole sequence, between the maximum and minimum (1–0) value, identifying certain sequences, expected to be very selective (1–9, 112–115) and other probes with less specificity for the target. Folding score does not vary significantly along the sequence. It has about maximum values in all positions except few regions, where the value is still relatively high. This behavior could be due to the fact that intra-molecular paring involves fewer bases than complementary hybridization with target sequence.

For the considered beta-actin sequence (227 bases), only few regions are suitable for probe mapping, and only 10 possible sequences displayed show scores higher than 0.8. Among these high-scored probes, we selected two probe sequences with equal score of 0.936 to be tested experimentally for computational approach validation, mapping position 5–24 (Probe 2) and position 101–120 (Probe 1).

Three more probes, namely Probes 3, 4 and 5, with decreasing scores from 0.725 down to 0.255, were also selected and are reported in Table 1. Table 2 summarized the total scores and the values resulting from the software calculations for the parameters of the selected probes.

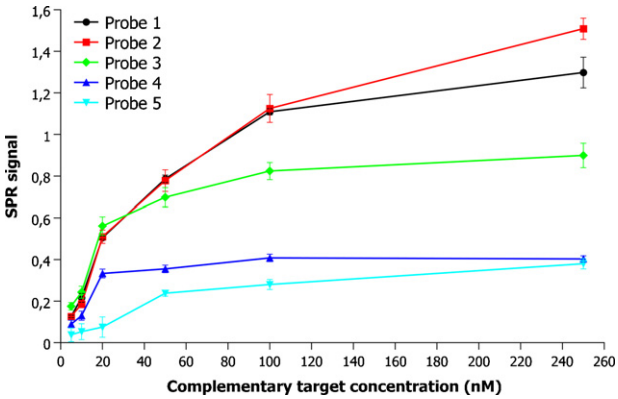

Fig. 1. SPRi calibration for the selected five probes. The reflectivity different percentage is reported for each probe vs. the target concentration in a range between 2 μM and 250 μM.

The selected five probes were then experimentally tested by SPRi to evaluate the validity of computational approach used.

3.3. Validation of DNA probes by SPRi transduction

The five probes selected were immobilized on the same chip surface and tested for their ability to bind to the complementary sequences.

Thiolated probes were coupled to gold surface in an array format, and remaining gold surface was then passivated using mixed thiols at different length (see Section 2). Complementary targets were injected separately in concentration range from 2 nM to 250 nM and binding signals were recorded as percent variation of intensity of reflected light $\Delta R\%$. In Fig. 1 calibration curves obtained for the five selected probes were shown. In agreement with OligoWiz calculations, calibration curves display different behaviors, demonstrating that probes follow the *in silico* predictions. Calculated scores results thus correlated with SPRi responses obtained from calibration curves. In particular, best-scored probes (Probes 1 and 2) show highest signals for all the tested concentrations; Probe 3 (score 0.725) gave intermediate signals between high and low scored probes and Probes 5 and 4 displayed the worst results. This behavior is clearly shown in Fig. 2 where scores compared with SPRi responses recorded at 100 nM target concentration, within the dynamic range of the biosensor.

3.4. Melting experiments

The strength of possible secondary structures on probes was experimentally evaluated by UV melting experiments (Eryazici et al., 2010). Folding and unfolding behavior of each probe sequence in solution was studied as temperature function. The number of bases involved in secondary structures and their melting temperatures could give an idea about the strength of the conformation of a given probe at the given temperature on the chip. In DNA-based sensing, this information is strategic for estimating the potential intermolecular folding of the selected probe on the chip at given working temperature. The lower the melting temperature, the higher is the number of unpaired bases at the working temperature. Also the hyperchromic effect for each probe considered, i.e. the variation of the intensity of absorbed light. The solution employed in melting experiments thus the same as applied in SPRi-based experiments. Melting profiles are reported in Fig. 4. Probe 1, one of the two best scored, does not show any melting curve,

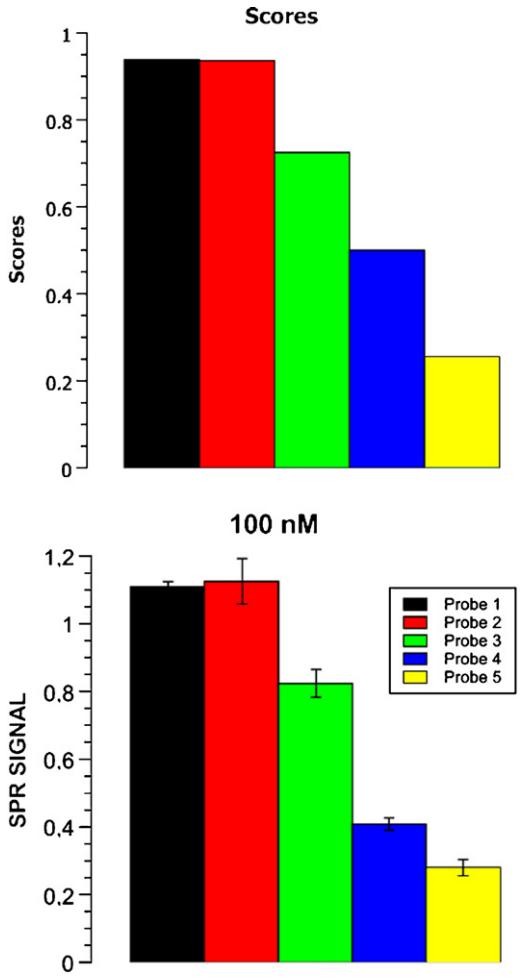

Fig. 2. SPR signals for the selected probes for the target concentration 100 nM (upward) and comparison with compared to calculated scores (down).

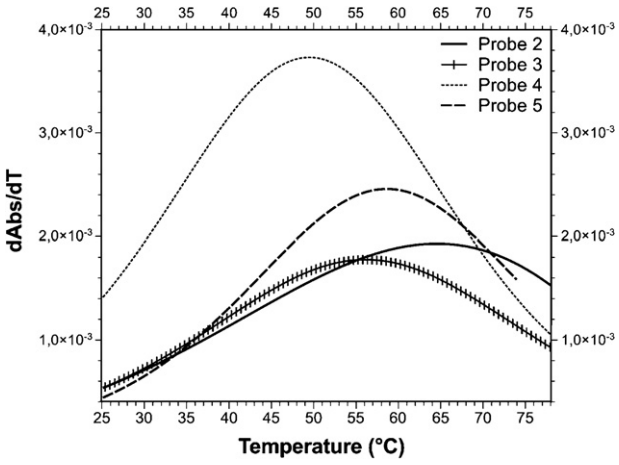

Fig. 4. Derivative curves of melting profiles are shown for each probe. Probe 1 is the only one that does not fold into a secondary structure, thus no melting curve was recorded.

meaning that, in the considered temperature range, its sequence remained unfolded. However, Probe 2, with the same score as Probe 1, displayed a melting curve characterized by an intense hyperchromic effect and the highest melting temperature (64 °C) among the considered probes. Probe 4 results the probe with lowest melting temperature (49 °C) but with hyperchromic effect similar to Probe 2. Probe 3 and Probe 5 curves profiles show intermediate melting temperature, 56 °C and 59 °C respectively. Considering the hyperchromic effect, Probe 3 and Probe 5 also showed intermediate values.

In Fig. 3 the estimated secondary structures and melting profiles for the selected probes were reported. In particular, estimated structure for Probe 2 showed four bases involved in the loop, at the 5′ end, in close proximity to sensing surface. This secondary structure results to be one of the most stable with respect to the others, as deduced from melting profiles (i.e. from hyperchromic effect) and from data obtained with Mfold software (Zuker, 2003). Free energy of Probe 2 loop is comparable to Probe 5, the worst scored probe.

It appeared that the location of these secondary structures occurring along the probe was important. In contrast to Probe 2, all the other three probes, had folding in region closer to 3′ end, which was more exposed to solution. This folding orientation may influence the hybrid formation at the sensor surface, between the probe and its complementary sequence in solution. This evaluation suggested that the relative position of the loops on probes should be considered in the computational evaluation. It could be useful to

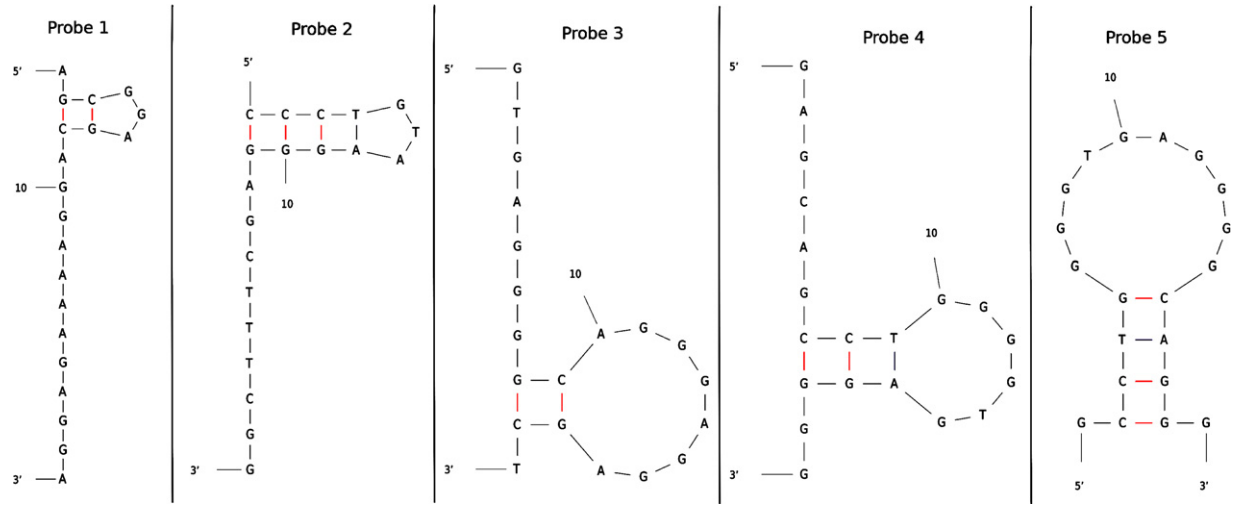

Fig. 3. Probes folding simulated with Mfold (Zuker, 2003) in order to mimic experimental conditions (DNA at 25 °C, [Na^+] = 0.34 M).

be able to manipulate this aspect by including a new factor among the parameters weighted in the program.

However, it was thus apparent that neither hyperchromic effect nor melting temperature of the loop alone is enough to predict the probe behavior. If we consider the probe solely on the basis of the intensity of the hyperchromic effect or melting temperature, we would not have insightful informations about the hybridization characteristics of probes on the sensor. This supported the idea that only a combination of suitable parameters, integrated using a rational design, can provide complete description of the phenomenon; in our case, we showed that a computational assisted tool such as OligoWiz (Wernersson et al., 2007), could take into account the different parameters involved for optimizing probe design in nucleic acid-based sensing.

Probe design is a delicate step in sensor development and to the best of our knowledge this is the first reporting about rationalization of probe selection in DNA-based sensing. This effort aimed to standardize the different steps in designing model probes for nucleic acid based sensing in order to go further in analytical system performances, in line with previously reported work on signal sampling and data management standardization (Scarano et al., 2010).

The approach has a non-restricted validity and can eventually be applied to different transduction principles. The reported method can also be used to select probes as secondary sequences, to hybridize the target analyte in a different region, as reported in electrochemical and optical sensing for signal detection or enhancements.

4. Conclusions

We described here a novel and rational approach in probe design by computational evaluation coupled with experimental validation for application to nucleic acid sensing. Optimized performances of the sensor achieved with best scored *in silico* probes, demonstrated the validity of the theoretical modelling, in affinity biosensor analysis. Our experimental results are in complete agreement with the assigned scores, validating the theoretical evaluation as an useful tool for selecting biosensor receptors. It has general validity and it can be extended, for example, to RNA analysis, like microRNA which is now of interest in cancer diagnostic or coupled to different transduction principles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.06.004.

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