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

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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

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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.