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An integrated approach for the design and synthesis of oligonucleotide probes and their interfacing to a QCM-based RNA biosensor

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Abstract

The quantitative determination of specific cellular messenger-RNA is extremely important both in basic and applied research, especially in diagnostic and pharmacological fields. In order to perform a direct and easy quantification of transcripts on cell extracts, the feasibility of an analytical device able to selectively detect a defined target RNA in a complex mixture while avoiding labelling, retrotranscription and amplification steps, has been explored. In particular, several aspects necessary to obtain good selectivity in target recognition, stability, reusability and sensitivity of a gene specific biosensor were considered.

For the development of suitable probe-receptors, analysis of the nucleotide sequence of the target mRNA was carried out to localise the preferred binding regions. As criteria for optimisation, we selected accessibility and uniqueness. Oligonucleotide probes, designed to specifically bind these sequences, were synthesised by using particular monomers producing nuclease-resistant RNA strands with high affinity towards the target.

Quartz crystal microbalance (QCM) technology was selected to realise a microgravimetric sensor able to bind the RNA under investigation through a complementary oligonucleotide probe. Covalent immobilisation of bioreceptor molecules to the transducer sensitive surface ensured a stable integration between the two. The binding ability of immobilised probes was tested evaluating their annealing behaviour with both complementary oligonucleotides and full-length target mRNA. The conditions necessary for the regeneration of biosensor were also assessed.

Measurements of shift in QCM resonant frequency, performed by hybridisation experiments in liquido, demonstrate that a label-free RNA-biosensor with high specificity, reusability and the ability to give quantitative information, can be realised.

Keywords: Messenger RNA; QCM; Immobilisation; Silane; Oligonucleotide probe; RNA biosensor; Quartz crystal microbalance

1. Introduction

Gene expression analysis is a point of focus in many fields of biological research: typical applications are to obtain a molecular characterisation and to understand phenotypic differences between cells. When cells change their status during physiological remodelling or are influenced by physical, chemical or biological agents, they change their gene expression profile. Proteins are the active working components of the cellular machinery and mRNA, which carries out the instructions encoded in DNA, is a key molecule in mediating the flow of genetic information stored in DNA.

Recent works demonstrate the usefulness of gene expression analysis in characterisation of pathologic phenotypes related to various diseases (Ye et al., 2002) from cancer (Popescu, 2000), to coronary artery disease (Archacki et al., 2003), asthma (Yuyama et al., 2002) and diabetes (Shalev et al., 2002). Comparison of gene expression patterns by measurement of mRNA levels in normal and pathological cells could provide useful diagnostic "fingerprints" and help to identify aberrant functions that would be recognized as reasonable targets for therapeutic intervention.

Gene expression analysis can be performed at two different levels and for different purposes; a *panoramic* view of

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expression pattern referred to as *expression profiling*, and a *focused* one, devoted to analyse the fluctuation of specific diagnostically significant mRNAs. Microarrays, SAGE or also differential display, can be preferable in studies which plan to monitor a large number of genes differentially expressed in cell populations. More traditional techniques, such as Northern blotting and nuclease protection assay and real-time PCR can analyse only one or few genes at the same time, and are useful when it is necessary to focus attention on small fluctuations of their expression.

Affinity-based biosensors could represent an alternative tool able to quantify the expression of a set of selected genes by determining the amount of corresponding mRNAs in complex mixtures. The revolutionary innovation of this approach consists in avoiding labelling, retrotranscription and amplification steps that make conventional analyses laborious, time consuming and inaccurate.

Mass-sensitive biosensor systems have attracted considerable attention in recent years since many important physical and chemical processes can be followed by observing the associated mass changes. The quartz crystal microbalance (QCM) system is based on the principle that the resonant frequency shifts of a piezoelectric crystal are directly proportional to the adsorbed mass (Rickert et al., 1997; Muramatsu et al., 2002; Bizet et al., 2000). QCM is suitable for several applications, including biosensors (O'Sullivan and Guilbault, 1999; Lin et al., 2000; Mannelli et al., 2003).

The specific base-pairing of nucleic acids can be usefully adopted for RNA biosensing by QCM technology. Several aspects for the design of RNA biosensors have to be solved in order to assemble a device with adequate properties of selectivity in target recognition, stability, reusability and sensitivity. In particular, for the design of oligonucleotide probes to be used as the bioreceptor of a biosensor, not only the stability of the duplex but also the conformational accessibility (favourable for probe binding) as well as the absence of any recurrence in other known transcribed sequences (uniqueness) must be considered. For alternatively spliced RNA sequences, binding sites belonging to regions distinctive of each splicing variants must be selected.

With the aim to assemble a QCM-based RNA biosensor we focused our attention on the bioreceptor and its integration with the surface of the transducer, taking into account both aspects. As a model analytical substrate we choose the mRNA encoding for human O^6-methylguanine-DNA-methyl-transferase (MGMT), a suicide enzyme involved in DNA repair (Pegg, 1990). Following the computational selection of targets, complementary probe sequences were derived and synthesized. During the automated synthesis, some chemical modifications were introduced in order to confer resistance against nucleases to probes, increased binding strength and anchoring ability, useful for conjugation to the sensing element of the QCM.

2. Materials and methods

2.1. Materials

All monomers for automated synthesis of oligos were purchased from Glen Research as crystalline solids and were dissolved in anhydrous acetonitrile (CPG Corporation <10 ppm water) according to the specifications of the supplier. Reagents for the synthesis were purchased from ChemGenes Corporation. Controlled-Pore-Glass supports (glass pore size 1000 Å, synthesis scale 1 μmol) were purchased from CPG Corporation. The cross-linker Sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate and "Ellman's reagent" (5,5-dithio-bis-[2-nitrobenzoic acid]) were purchased from Pierce, mercapto-propyl-trimethoxysilane (MPTS) from Fluka and anhydrous toluene (<10 ppm water) from Biosolve.

Buffer solutions were prepared from DNase- and RNase-free Analytical Grade reagents and sterile deionised water.

In vitro transcription of MGMT RNA was performed according to the T7 AmpliScribe™ protocol (Epicentre). As a DNA template we employed plasmid pG3AT835 linearised with *Bam*HI (New England Biolabs). pG3AT835 was a generous gift of Prof. B. Kaina (Toxicology Institute University of Mainz, Germany), obtained inserting MGMT cDNA in a pGEM®7Zf vector cut with *Eco*RI.

2.2. Choice of probe sequence

To design high affinity selective RNA probes to the target we used a predictive computational method previously developed in our laboratory (Mercatanti et al., 2002). A comparative analysis of the calculated secondary structures of MGMT mRNA, ordered according to Boltzman's distribution, produced a probabilistic map of unpairing occurrence (%) nucleotide by nucleotide. A subsequent algorithm calculated the accessibility score parameter over a 25mer or 30mer window. Accessibility results were grouped in four classes according to their accessibility scores and best sequence regions were selected. Pairing energies ΔG° were analogously calculated using the Nearest Neighbour Nucleotide algorithm at standard conditions (25 °C, 1 atm) in order to select target sequences negatively exceeding a given energy threshold. The third selection level concerned the human genome data base interrogation in order to check any homology recurrence of selected sequences among the entire human known transcriptome. Target sequences sharing over 70% homologies with other unwanted RNAs were discarded. Finally, the intrinsic thermodynamic properties of deduced probe sequences to give self-structures were evaluated and those displaying the lowest ΔG° values were discarded.

2.3. Synthesis of chemically modified probes

We planned the synthesis of RNA probes by virtue of their more stable pairing to target (RNA/RNA duplexes) than DNA