This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

www.rsc.org/analyst
Transmission Line Model Analysis of Transcription Factors Binding to Oligoduplexes - Differentiation of the Effect of Single Nucleotide Modifications

Rogério M.M. Rodrigues, Jorge de-Carvalho, Silvia F. Henriques, Nuno P. Mira, Isabel Sá-Correia and Guilherme N. M. Ferreira

Received (in XXX, XXX) Xth XXXXXXXXXX 20XX, Accepted Xth XXXXXXXXXX 20XX
DOI: 10.1039/b000000x

Advanced impedance spectroscopy analysis based on the transmission line model (TLM) is explored as a novel QCM acoustic biosensing platform for the detection of single point mutations effect on the binding of transcription factor (TFs) to immobilized DNA oligoduplexes and the characterization of the protein-DNA mechanical properties.

The transcription factors (TFs) are proteins that probe the nucleotide sequence of a gene promoter region, bind specifically to its recognition element (RE) and control the gene transcription. Mutations in the RE can thus result in genetic-related diseases, such as cancer and immunological disorders, or in the incapacity of a cell to respond to certain stimuli, such as an environmental change. The study of the TFs binding to DNA is highly demanded for instance to characterize the binding kinetics, to identify the RE nucleic acid sequences and to assess the effects of mutations on these. Reliable analytical methods and tools are thus needed to test the TFs binding to DNA. To date the interaction of TFs and DNA has been studied by electrophoretic mobility shift assay, fluorescence spectroscopy, circular dichroism, atomic force microscopy (AFM), surface plasmon resonance (SPR), and quartz crystal microbalance (QCM). These biosensors based on the QCM and SPR technologies have become very popular mostly due to the suitability for detection in real time without the need for any labeling procedure. In particular, QCM can be used both to study binding kinetics to quantify association and dissociation constants but also to characterize and detect alterations of the mechanical properties of the immobilized biomolecules.

We have established a novel QCM bioanalytical method based on the transmission line model (TLM) to analyze the binding of TFs to immobilized DNA oligoduplexes. This method rely on the real-time monitoring of impedance spectra, which changes are related with the variations of the dynamic shear modulus and thickness of the biological films. To the best of our knowledge, the present work is the first use of this methodology to study the effect of DNA point mutations on the recognition mechanism of transcription factors. The biological model used is based on the interaction between the Saccharomyces cerevisiae transcription factor Haal with immobilized DNA strands. Gene and genomic transcription regulation in the yeast model provides an excellent platform to understand the TFs binding to DNA. Indeed, the YEASTRACT database provides to the public up-to-date information on 206,299 documented regulatory associations between TFs and target genes, including 326 DNA binding sites for 113 TFs. Among these DNA binding sites is the Haal-responsive element (HRE) which is a part of the promoter region, for instance, of the Haal-regulated TPO3 gene that is recognized in vitro and in vivo by Haal. Haal has an important role in yeast adaptation and tolerance to stress induced by acetic and propionic acids, with potential implications in the biotechnology and food-processing industries. Because the heterologous expression of the full-length Haal in Escherichia coli is difficult and leads to very low yield of purified protein, the interaction assays with immobilized DNA oligoduplexes were carried out with a peptide that comprises solely the DNA-binding domain of Haal (mapped to the 123 N-terminal residues). The interaction of this peptide with the HRE motif was proved to mimic the interaction observed with the full-length Haal TF. The Haal used for protein-DNA interaction assays was purified by affinity chromatography, as described before.

Four different 38 bp DNA oligoduplexes were used. The HRE contains the Haal wild-type RE, and the mutants contain a single point mutation on the RE in its 5-th nucleotide (a guanine) starting from the 5'-end (as underlined in the full oligonucleotide sequence, see notes). In the mutant HRE, this guanine was replaced by a cytosine, while in the mutant HRE it was replaced by an adenine. As control we designed HRE which consists in a random DNA sequence. 10 MHz QCMs from International Crystal Manufacturing Company were cleaned, activated with a mixture of 10% biotin-PEG disulfide (LCC Engineering & Trading) and 90% 11-hydroxy-1-undecanethiol (Dojindo) and saturated with streptavidin (Roche). The oligoduplexes were ordered with a biotin tag in the forward primer 5'-end (Sigma-Aldrich, see sequence details on notes) to mediate their immobilization in the streptavidin-coated surface of the QCM electrodes (Fig. 1).
Fig. 1 Schematic representation of the QCM methodology for real-time detection and assessment of transcription factors interaction mechanics. The biosensor consists in DNA oligoduplexes probes previously immobilized on sensor surface via SAM gold modification and biotin-streptavidin affinity coupling. These probes are oriented with a tilting angle (τ) away from the surface. The interaction of the studied transcription factor, Haa1, with DNA oligoduplexes containing the specific recognition element is known to promote a bending angle (β) in the DNA structure. We have designed other DNA oligoduplexes with single point mutations to evaluate their influence in the interaction mechanics.

The assays were run at a constant flow rate of 100 μL min⁻¹ using Tris buffer pH 8.0 (10 mM Tris, 100 mM KCl, 0.005% Tween20) as running buffer and the binding of Haa1

<table>
<thead>
<tr>
<th>Film</th>
<th>Density (g cm⁻³)</th>
<th>G*film (kPa)</th>
<th>hfilm (nm)</th>
<th>Tilting (°)</th>
<th>Bending (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRE_{wt}</td>
<td>1180 ± 10</td>
<td>87.9 ± 3.8</td>
<td>11.6 ± 0.2</td>
<td>63.5 ± 1.8</td>
<td>NA</td>
</tr>
<tr>
<td>HRE_{ml}</td>
<td>1160 ± 36</td>
<td>95.9 ± 3.3</td>
<td>10.6 ± 1.4</td>
<td>55.9 ± 10.2</td>
<td>NA</td>
</tr>
<tr>
<td>HRE_{neg}</td>
<td>1157 ± 31</td>
<td>86.5 ± 8.6</td>
<td>11.2 ± 0.1</td>
<td>59.9 ± 1.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 1 - Summary of the extracted mechanical parameters using the mathematical algorithm based on TLM for characterization of the different tested biomolecular films.

The TLM calculated mechanical properties of the QCM biosensors with immobilized HRE_{wt}, HRE_{ml}, HRE_{neg} or HRE_{neg} are summarized in Table 1. The magnitude of the dynamical shear modulus (G*_{film}) as well as the h_{film} are similar for all these DNA oligoduplexes, which was expected given the identical contour length of the immobilized DNA oligoduplexes (12.92 nm). Therefore, these results demonstrate the good method reproducibility and robustness. We determined that at equilibrium, the 38 bp DNA oligoduplexes adsorb in average with a h_{film} of 10.8±0.8 nm and tilting angle of 57.2±6.0°, presenting a |G*_{film}| of 91.4±4.9 kPa.

The density of the immobilized biomolecules layers is a required input parameter to the model. We used the weighted density of the films at the QCM sensor surface (Table 1), estimated from the densities of water (d=1000 g dm⁻³), of the proteins (d=1350 g dm⁻³), and of the DNA (d=1700 g dm⁻³), from structural considerations and stoichiometry determination of the number of immobilized molecules at equilibrium, as detailed in the electronic supporting information.

The binding of the TFs to the specific DNA RE was proven to significantly change the mechanical properties of the immobilized DNA film which becomes more ordered and compact when complexed with the TF peptide. As previously published, we can monitor these alterations of the film mechanical properties when the Haa1 DBD peptide binds to the
immobilized DNA oligoduplexes. These alterations are expected to be significantly different for the HREm1 and HREm2, but the single point mutated sequences HREm and HREm2 introduced a new detection threshold challenge of mechanics differentiation. Indeed, it was previously shown by SPR measurements that the equilibrium dissociation constant of the Haa1-HRE complex is higher for HREm (KD=10000 nM) than for HREm2 (KD=6780.0±865 nM), HREm1 (KD=38.5±4.5 nM) and HREm (KD=2.0±0.2 nM). Here, the QCM data analysis of the binding of Haa1DBD peptide to the wild type (HREm), mutated (HREm1 and HREm2), and negative control (HREm) oligoduplexes with the mathematical algorithm based on the TLM clearly shows that these different kinetics are associated with significant differences in the layers mechanical properties.

In Fig. 2A we represent the change over time of the |G*film| for the binding of Haa1DBD at 50 nM to the immobilized oligoduplexes. These transients reveal that the binding of Haa1DBD to its specific HREm and point-mutation HREm1 sequences (higher kinetic affinity) always results in stiffer films compared with binding to HREm2 and HREm (lower kinetic affinity). In fact, this is in accordance with previous observations of the Haa1DBD interaction with an immobilized random and nonspecific DNA sequence (HREm). The mechanism of TFs binding to its RE is known to be initiated by an electrostatic attraction to the DNA and TF’s sliding through the nucleotide sequence, probing the pattern of hydrogen bond donors and acceptors present laterally in the purine and pyrimidine aromatic nucleobases. The later are easily accessible to the TF in the major grooves of the DNA double helix or becomes exposed through a bending of the DNA structure at the minor groves. The initial sliding process was observed during the initial 100 s after starting the adsorption of the Haa1DBD peptide. As shown in Fig. 2 the lag variations of the |G*film| (Fig. 2A) and bending angle (Fig. 2B) occurred during this phase. After the initial sliding and probing process, it is commonly accepted that the TF mechanism of binding to DNA goes through the establishment of hydrogen bonds and water release from the hydration spheres of both interacting molecules. This is expected to lead to higher film viscosity, which is signaled by the QCM-TLM as an increase of |G*film| for the HREm1, HREm and HREm2.

(Fig. 2A). Another effect of the specific binding of the TF to the DNA REs is the forced bending of the DNA structure, particularly if the RE is located in a minor groove of the DNA structure. As such, the degree of DNA bending can be used to identify the specific binding of the TFs and possibly revealing also the location of the REs in the minor groove. As shown in Fig. 2B, the specific binding of the Haa1DBD to HREm resulted in a significant structural bending (>30º), suggesting thus the RE location at a minor groove. Consistent with this hypothesis, Ace1, a close homologue of Haa1, has been found to contact DNA through minor groove interactions. The estimated structural bending is however lower for the immobilized oligoduplexes containing a single nucleotide modification (Fig. 2B). It is conceivable that the nucleotide modification for HREm and HREm2 alters the pattern of available chemical groups to establish hydrogen bonds with the aminoacids of the Haa1DBD peptide thus interfering with its binding to the oligoduplexes. In the case of the negative control (HREm) the |G*film| decreases, while the film thickness increases and thus it is not possible to estimate a bending angle of the oligoduplex structure. This reveals that there is an established link between the mechanical properties of the Haa1DBD binding to immobilized oligoduplexes and the binding kinetics described elsewhere.

Conclusions

The complex impedance analysis of QCM is a reliable and efficient technique to infer about biological phenomena occurring at the sensor surface. When associated with the TLM, the QCM is suitable to characterize the mechanical properties changes associated to the binding of TFs to DNA. Single point mutations on the DNA recognition element can be detected. The model establishes a link between molecular interaction kinetics and mechanics, extending the application of QCM to more than mass variation sensing and qualitative assessment of viscoelasticity changes.

Acknowledgements

This work was supported by national Portuguese funding through FCT - Fundação para a Ciência e a Tecnologia, project ref. Pest-OE/EBB/LA00023/2013. The authors further acknowledge to FCT the financial support through the research projects PTDC/EBB/EQB/LA00177/2008 and PTDC/SFU-BEB/105189/2008 and the grants SFRH/BD/33720/2009, SFRH/BD/38136/2007 and SFRH/BD/78058/2011.

Notes and references

1. IBB-Institute for Biotechnology and Bioengineering, Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve, 8005-139 Faro, Portugal.
2. IBB-Institute for Biotechnology and Bioengineering, Center for Biological and Chemical Engineering, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Avenida Rovisco Pais, 1049-001 Lisboa, Portugal.
3. E-mail: gferrei@ualg.pt.
† The complementary oligoduplex sequences were 5'-[biotin]-TTT ACT GGA GCC CAA TC and 5'-GA TGT GGC TCC AGT AAA CCC CTC GCC AAG CAC AGA GAA-3' for HRE<sub>wt</sub>; 5'-[biotin]-TTT ACT GGA GCC CAA TC -3' and 5'- GA TGT GGC TCC AGT AAA CCC CTC GCC AAG CAC AGA GAA-3' for HRE<sub>mut</sub>; 5'-[biotin]-TTT ACT TGT CTC AGT AAA 65
Electronic Supplementary Information (ESI) available: experimental set-up details, estimation of the density of the layers, determination of tilting and bending angles, and experimental data processing See DOI: 10.1039/c000000x/