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The use of a quartz crystal microbalance with dissipation for the measurement of protein–protein interactions: a qualitative and quantitative analysis of the interactions between molecular chaperones

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Biotechnology research and innovation depends on the ability to understand the molecular mechanisms of biological processes such as protein–protein and protein–ligand interactions. Surface plasmon resonance (SPR) spectroscopy is now well established as a quantitative technique for monitoring biomolecular interactions. In this study, we examined the recently developed quartz crystal microbalance with dissipation (QCM-D) method as an alternative to SPR spectroscopy to investigate protein–protein interactions, in particular, for chaperone–co-chaperone interactions. In mammalian cells, the Hsp70/Hsp90 organizing protein (Hop) is a co-chaperone required for the association of the molecular chaperones, heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90). The objective of this research was to characterize qualitatively and quantitatively the interaction of Hsp70 with Hop. A truncated version of Hop consisting of only the C-terminal region and lacking the Hsp70-binding domain (GST-C-Hop) was used as a non-Hsp70-binding control. Immobilized GST-Hop was found to bind Hsp70 successfully, displaying a QCM-D response consistent with formation of a complex that became slightly more flexible as the concentration of bound Hsp70 increased. GST-C-Hop did not bind to Hsp70, thereby validating the specificity of the GST-Hop interaction with Hsp70. The kinetics of the interaction was followed at different concentrations of Hsp70, and an apparent thermodynamic dissociation constant (K_D value) in the micromolar range was determined that correlated well with the value derived previously using SPR. This study represents a proof-of-principle that QCM-D can be applied to the analysis of chaperone–co-chaperone interactions. The economic and technical accessibility of QCM-D makes it a valuable tool for analyses of chaperone interactions, and protein–protein interactions in general.

Introduction

The harmonious functioning of a cell depends on the proper synthesis, folding and assembly of its protein machinery. Protein misfolding, denaturation and aggregation constantly challenge the cell under physiological conditions, and are particularly problematic when cells are stressed. Molecular chaperones facilitate the correct folding of other proteins under physiological and stress conditions. The main molecular chaperone families are heat shock proteins (Hsps), named according to their molecular size in kilodaltons.[1] Recently, it has become evident that a cohort of co-chaperone proteins mediates the regulation and specificity of action of the major molecular chaperones, Hsp70 and Hsp90. The Hsp70/Hsp90 organizing protein (Hop), which is able to associate directly with both Hsp70 and Hsp90, is one of the most extensively studied co-chaperones. Hop was first identified in yeast[2] and was named STI1, for stress-inducible protein 1. Homologues of Hop have now been identified in the human,[3] mouse,[4] rat,[5] insects,[6] plants[7,8] and parasites,[9–11] making up a protein family of co-chaperones generally referred to as STI1 or Hop. For convenience, the term Hop will be used here to refer generally to STI1 and Hop proteins.

In terms of binding kinetics, we have previously demonstrated and quantified the interaction between Hsp70 and Hop using surface plasmon resonance (SPR) spectroscopy.[12] In the study reported here, we evaluated the quartz crystal microbalance with dissipation technique (QCM-D)[13,14] as an alternative to SPR for studies of chaperone–co-chaperone interactions.

Aided by the possibility of measuring changes in interfacial refractive index upon biomolecule binding reactions at solid–liquid interfaces, SPR spectroscopy has emerged as an important technique for monitoring biomolecular interactions.[15] The SPR-based sensors rely on the excitation of surface plasmon polaritons (SPP), which are charge-density waves strongly coupled to optical modes at the interface between a flat and homogeneous gold or silver film and a dielectric medium. A change in the refractive index of the dielectric, for example due to biorecognition events near the metal surface, alters the conditions for SPR excitation, which in turn can be optically transduced and detected as a shift in resonance angle.[16,17] The possibility of recording changes in resonance angle with high sensitivity and temporal resolution, combined with the fact that there is, to a first approximation, a linear relationship between changes in interfacial refractive index and protein concentration, make SPR well suited to estimating mass uptake and binding kinetics.

The QCM-D technique is based on the traditional QCM technology, in which mass added to the electrodes of a quartz crystal resonator is monitored by the change in resonance frequency. QCM equipment comprises specially cut quartz crystal sandwiched between electrodes such as gold and aluminium. The piezoelectric nature of the quartz means that it resonates at a particular frequency under the application of an a.c. voltage across the electrodes. For a certain amount of added mass, the frequency, *f,* decreases proportionally, as described by Sauerbrey.[18] The Sauerbrey relation is, however, valid only for rigid films, and is hence not sufficient for loosely structured (viscoelastic) adsorbents like water-rich protein films. The introduction of the QCM-D has allowed for the monitoring of an additional factor, *D,* the damping or dissipation. By measuring the damping (energy losses, or *D*) of an adsorbed film, the QCM-D quantifies dissipative losses, which, when combined with changes in *f,* can be used as a fingerprint to characterize structural variations in thin viscoelastic films,[19] or, when treated using theoretical viscoelastic representations, for corrections of

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the quantifications given by the Sauerbrey relation.[20] QCM-D thus has the advantage of providing information about whether the material added to the electrodes is rigid or viscoelastic, as well as data on structural changes that may occur during protein–protein interactions. The dissipation factor of a quartz oscillator is measured by recording how the oscillation decays after the oscillator has been excited into oscillation.[13] The QCM-D, commercially developed by Q-Sense AB in Gothenburg, Sweden, can thus be used to study the thin-film formation of proteins, cells and polymers in liquid including measurements of protein–protein interactions[21] and antibody-antigen reactions,[14] biomembrane formation on surfaces from vesicles in solution, and cell attachment experiments.[22,23]

The K_D of the binding of Hop to Hsp70 was calculated by SPR to be 2 μM, indicating a relatively low affinity association.[12] We have explored the interaction between Hsp70 and Hop using the QCM-D. The specific objectives were, first, to confirm the binding between the two molecules with that obtained using SPR, employing a truncated Hop as a control; second, to establish and compare binding affinities obtained using SPR; and third, to derive additional information reflected in changes in ΔD and Δf, originating from structural modifications during assembly of the Hop.Hsp70 complex.

Materials and methods

Protein synthesis and purification

Mouse Hop (also called mSTI1) was produced and purified as a recombinant glutathione S-transferase fusion protein (GST-Hop) according to published procedures.[12] A truncated version of Hop consisting of only the C-terminal region (the last 334 amino acids)[12] and lacking the N-terminal Hsp70-binding domain (GST-C-Hop) was used as a non-Hsp70-binding control. Bovine brain Hsp70 (constitutive form of Hsp70; also called Hsc70) was kindly donated by M.E. Cheetham (Institute of Ophthalmology, University College London, U.K.). These proteins were dissolved in 10 mM Tris-HCl at pH 8.0.

Preparation of quartz crystal surface

A Q-Sense Axial Flow Chamber was used for real-time simultaneous measurement of frequency and dissipation changes. Both flow mode and batch mode were used, with 0.89-mm-ID tubes. All measurements were performed at room temperature (22°C).

Au-coated sensor crystals (Q-Sense AB) were immersed in a 5:1:1 mix of deionized (Milli-Q) water, NH_3 (25%) and H_2O_2 (30%) for 5 min at 70°C, rinsed thoroughly in deionized (Milli-Q) water, and exposed in a UV/ozone chamber for 15 min, and then rinsed in deionized (Milli-Q) water again.

Immobilization reagents and protocols

Biotinylated albumin (Sigma-Aldrich Chemie GmbH, Germany) was dissolved in 0.05 M Tris-HCl at pH 8.0, 0.138 M NaCl (in some measurements), and 0.01 M phosphate-buffered saline (PBS buffer), pH 7.4 (in other measurements). Streptavidin (Sigma-Aldrich Chemie) and monoclonal anti-*sj*GST antibody (Sigma-Aldrich, Inc., U.S.A.) were diluted in the same buffers. N-Hydroxy-succinimide (NHS) conjugated biotin (Molecular Biosciences, U.S.A.) was dissolved in DMSO and diluted in Tris-HCl or PBS buffer. All aqueous media used were prepared in deionized (Milli-Q) water, and both water and buffers were degassed in a bath sonicator before measurements.

Two immobilization protocols were examined in this study. The first involved the adsorption of biotinylated bovine serum albumin onto the Au surface, followed by streptavidin, biotin conjugated with NHS and the anti-*sj*GST antibody. Adsorption of either GST-Hop or GST-C-Hop followed before passing over Hsp70 to test its interaction with Hop. The second protocol involved adsorption of the anti-*sj*GST antibody directly onto the gold surface, followed by albumin, GST-Hop or GST-C-Hop and finally Hsp70. After each adsorption step, the surface was rinsed with PBS and allowed to stabilize before adsorption of the next layer. Volumes of between 150 μl and 300 μl of the immobilization reagents and proteins studied were added at each step; the flow rates employed are given in the figure legends.

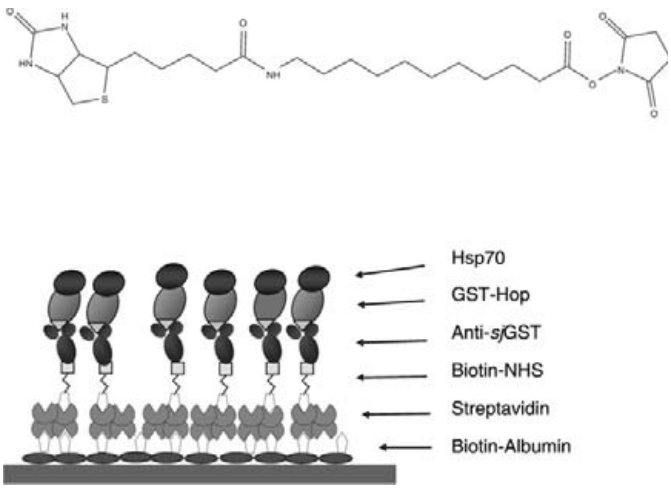

Fig. 1. **Top**: Biotin-NHS; **bottom**: schematic representation of surface build-up.

Results and discussion

One of the most challenging tasks in this study was the development of an effective strategy for the immobilizing of the GST-Hop on the quartz crystal. The approach adopted was an adaptation of the widely used biotin-albumin and streptavidin immobilization protocols,[24] which hinges on the strong affinity of biotin for avidin as well as the inertness of streptavidin to non-specific binding.

In this study, as schematically illustrated in Fig. 1, biotinylated bovine serum albumin was adsorbed onto the Au surface, followed by streptavidin. A biotin molecule conjugated with the reactive NHS group via a long alkane chain was then added as a linker to an anti-*sj*GST antibody. This antibody surface completed the platform for specific adsorption of the GST-Hop before introduction of Hsp70.

Figure 2 plots the changes in frequency and dissipation observed following immobilization of each successive layer onto the quartz crystal. Adsorption of biotinylated albumin resulted in a decrease in frequency of 11 Hz (1 in Fig. 2), whereas streptavidin (2 in Fig. 2) adsorption onto the biotin-albumin showed a large decrease in frequency (–41 Hz in the 3rd overtone), and a large increase in dissipation (2.5×10^{-6}). The expected f shift for a close-packed monolayer of a 60 000-Da

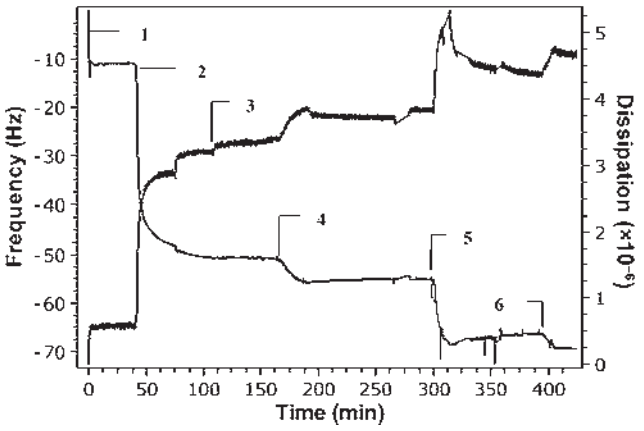

Fig. 2. Normalized frequency (thin line) and dissipation (thick line) shifts for 3rd overtone versus time using biotin-albumin and streptavidin immobilization for GST-Hop and Hsp70 study. The measurement started with: **1**, biotin-albumin coated surface (–11 Hz) at 0 min followed by the adsorption of (**2**) streptavidin (50 µg/ml); **3**, biotin-NHS (100 µg/ml) at 106 min followed by anti-*sj*GST (26 µg/ml) at 138 min (three dosages, not tagged on figure); **4**, anti-*sj*GST (260 µg/ml); **5**, GST-Hop (200 µg/ml); **6**, Hsp70 (50 µg/ml). PBS buffer rinse steps are not indicated in the figure.

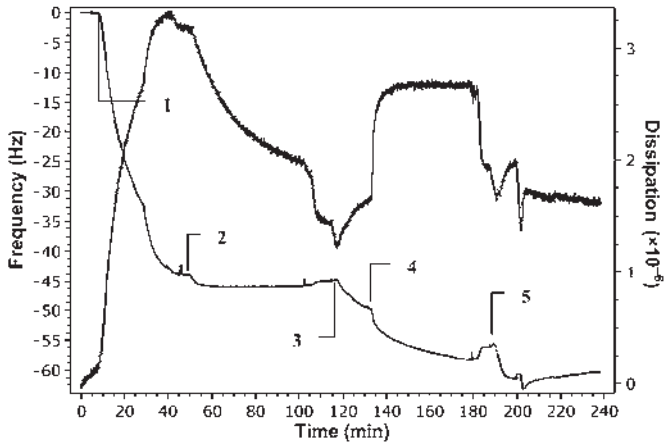

Fig. 3. Normalized frequency and dissipation shifts for 3rd overtone versus time following adsorption of anti-*sj*GST onto Au surface for GST-Hop and Hsp70 study. Steps: **1**, anti-*sj*GST (52 µg/ml, 150 µl again at 30 min); **2**, albumin (~100 µg/ml); **3**, GST-Hop (200 µg/ml); **4**, GST-Hop (400 µg/ml); **5**, Hsp70 (200 µg/ml). PBS buffer rinse steps are not indicated in the figure.

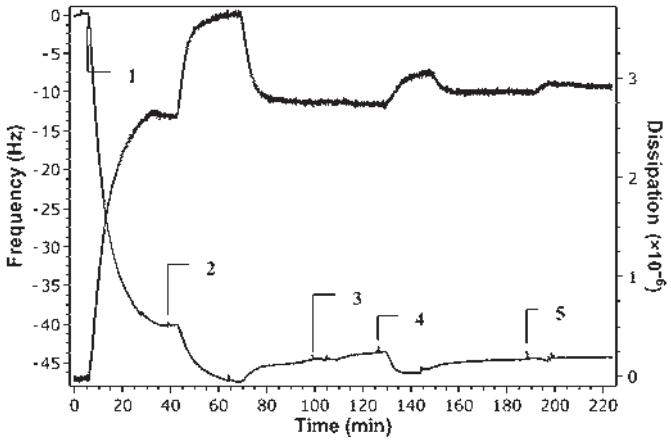

Fig. 4. Normalized frequency and dissipation shifts for 3rd overtone versus time following adsorption of anti-*sj*GST onto Au surface for GST-C-Hop and Hsp70 study. Steps: **1**, anti-GST (26 µg/ml, 150 µl again at 30 min); **2**, albumin (~100 µg/ml; rinsed with Tris-HCl buffer at 65 min); **3**, Hsp70 (50 µg/ml; **4**, GST-C-Hop (200 µg/ml); **5**, Hsp70 (50 µg/ml). PBS buffer rinse steps are not indicated in the figure.

protein (equal to the molecular weight of streptavidin) is around –25 to –30 Hz. This discrepancy is attributed to the amount of biotin per albumin molecule of the biotinylated albumin and the fact that the QCM-D senses water associated with adsorbed proteins.[20] The measurement was reproduced with a –47-Hz f shift for streptavidin. For comparison of the D shift, note that the ratio between ΔD and Δf is typically between 1×10^{-6} and 2×10^{-6} per 20-Hz protein.

The result showed that the binding of the anti-*sj*GST (4 in Fig. 2), with a molecular weight of approximately 150 kDa, was not maximal: –4.8 Hz for the 3rd overtone is much lower than the approximately 80 Hz that would correspond to a complete monolayer of a 150-kDa molecule. The subsequent addition of GST-Hop (5 in Fig. 2) gave a frequency shift of –12 Hz, corresponding to a mass change of ~200 ng/cm^2. This shift was lower than the expected one of a complete GST-Hop layer (~ –100 Hz); however, the result agrees well with the binding of anti-*sj*GST in the preceding step. Although complete coverage of anti-*sj*GST and GST-Hop was not obtained, binding of Hsp70 (6 in Fig. 2) was clearly detectable: a saturated change in f and D of –3 Hz and 0.42×10^{-6}, respectively.

To improve the coupled amount of the anti-*sj*GST/GST-Hop complex, the immobilization concept described above was compared with a more direct immobilization strategy, consisting of the immobilization of the anti-*sj*GST antibody coupled directly to the surface, as shown in Fig. 3. The antibody addition (1 in Fig. 3) gave a saturated shift in f of –44 Hz, about 10 times larger than when adsorbed onto the biotin-NHS layer, suggesting that the biotin-NHS was not fully coupled to the streptavidin layer in the measurement above or that the NHS-coupling chemistry had not been optimized. To reduce any empty non-coated regions on the Au surface, which are likely to induce non-specific binding, albumin (2 in Fig. 3) was added in the next step. The resulting decrease in f signaled that there were indeed uncoated areas left on the substrate, and the associated reduction in D indicated a stiffening of the protein layer, as these water-filled gaps were between adsorbed antibodies and became occupied with albumin. However, although the coupled amount of anti-*sj*GST was higher when directly adsorbed on the gold substrate than when coupled via NHS chemistry, additions of GST-Hop (3 and 4 in Fig. 3) and Hsp70 (5 in Fig. 3) resulted in changes in f (and D) similar to the streptavidin measurements of –11 Hz and –4 Hz described above, respectively. This observation is attributed to reduced functionality of the antibody due to a combination of adsorption-induced denaturation and orientation effects. However, it is clear that for this particular system, spontaneous adsorption of the antibody competes with the NHS-based coupling.

Based on this observation, additional QCM-D studies with the truncated GST-Hop, GST-C-Hop, were performed using the simpler immobilization protocol (as in Fig. 3). As shown in Fig. 4, no decrease in the frequency occurred upon addition of the Hsp70 (5 in Fig. 4) to immobilized GST-C-Hop, being consistent with the expectations for this Hop construct that lacks the ability to bind to Hsp70.[12] In this particular study, Hsp70 was also added prior to addition of the GST-C-Hop, showing no change in frequency and no immobilization on the anti-GST albumin layer, proving the absence of non-specific binding of the Hsp70 to this layer.

Frequency and dissipation shifts and mass changes achieved for the different measurements are listed in Table 1. Even though the Sauerbrey equation might not necessarily hold true for viscoelastic films, the effects are expected to be minor for these systems.[20] However, changes in f also include coupled water, which means that the adsorbed molecular mass cannot be obtained. Still, numbers estimated from the Sauerbrey equation — $\Delta m = -C/n\ \Delta f$, with C = 17.7 ng/cm^2 Hz (at a 5 MHz fundamental frequency) and n = overtone number — can be used to compare the different steps. f and D measurements were taken in the 3rd, 5th and 7th overtones. Results for the 3rd overtone are shown here.

To evaluate the unique opportunity provided by combined f and D measurements, to obtain information about structural changes in the different immobilization steps, changes in D were compared with those in f. When plotting D versus f for each measurement, the time dependence (rate of binding) is avoided. A D versus f plot gives the change in damping for every new unit of mass adsorbed — in other words, the plots give an estimate of how new added mass affects the structure on the surface. If the adsorbing molecule forms a rigid layer, the $\Delta D/\Delta f$ ratio is low; if the molecule forms a relatively open structure or if it has low affinity for the layer beneath, the ratio is high. Structural changes are easily seen in a ΔD vs Δf plot, since a single-phased adsorption will appear as a straight line.[14]

The measurement protocol starting with the adsorption of anti-*sj*GST on Au (see Fig. 3) was used to evaluate that type of structural changes for the coupling of GST-Hop and the

interaction of Hsp70 with the GST-Hop modified surface. The results for the antibody displayed a perfect one-phased process, exhibiting a straight line in the ΔD vs Δf plot (1 in Fig. 5a), while the coupling of GST-Hop indicated that the film became denser with time (more packed), since the ΔD vs Δf slope decreased with coverage (2 in Fig. 5a). In contrast, addition of Hsp70 to immobilized GST-Hop appeared to turn slightly more flexible with increasing amounts of Hsp70 being coupled to the surface, as shown in Fig. 5(b).

A kinetic evaluation of the binding between Hsp70 and GST-Hop was also performed using the immobilization protocol shown in Fig. 3. The immobilized GST-Hop was exposed to a series of successively increasing concentrations of Hsp70. The total frequency shifts for the Hsp70 did not exceed 1 Hz, which is not ideal for kinetic evaluation. However, we could use the Q-Sense analysing software, QTools, which is based in part on first-order Langmuir kinetics. This yielded $K_A = 8 \times 10^{-5}$ M, corresponding to $K_D = 1.2\,\mu$M. This result is consistent with the reference value $K_D = 2\,\mu$M obtained previously for Hsp70 and GST-Hop using SPR.

Table 1. Adsorption rates and thickness changes for each step, calculated using the Sauerbrey equation.[18]

Measurement	Induced $\Delta f/3$, 3rd overtone	Induced ΔD, 3rd overtone	Mass (ng/cm^2)	Thickness (Sauerbrey) (nm)
Albumin–strept–biotin–NHS–antibody				
Biotin-albumin	–11	0.50	195	1.8
Streptavidin	–41	2.50	725	6.6
Biotin-NHS	0	0.25	0	0
Anti-*sj*GST	–5	0.60	88	0.8
GST-Hop	–12	0.40	212	1.9
Hsp70	–3	0.15	53	0.5
Antibody direct on Au surface				
Anti-*sj*GST	–44	3.10	780	7.1
Albumin	–1	1.50	18	0.2
GST-Hop	–11	1.95	195	1.8
Hsp70	–4	1.60	70	0.6
Antibody direct on Au surface for truncated Hop				
Anti-*sj*GST	–40	2.60	710	6.4
Albumin	–5	0.15	90	0.8
Hsp70	0	0	0	0
GST-C-Hop	–1	0	18	0.2
Hsp70	0	0	0	0
GST-Hop direct on Au surface				
GST-Hop	–40	2.90	710	6.4

Conclusions

Using the QCM-D, immobilized GST-Hop was shown to bind successfully to Hsp70, confirming the earlier studies performed with SPR.[12] The apparent thermodynamic dissociation constant determined using the QCM-D, $K_D = 1.2\,\mu$M, correlated well with the SPR value, namely $K_D = 2\,\mu$M, indicating a relatively low affinity association. These studies set the framework for further investigation of binding affinities of chaperones and co-chaperones. The data also point to the possibility of extracting not only affinity constants, but also information about structural changes during the interaction studies. In future work, we are interested in characterizing the association of Hsp90 with Hop, and the effect of Hsp90 binding on: (i) the affinity of Hop for Hsp70; and (ii) the number of Hsp70 binding sites. These *in vitro* studies will broaden our understanding of the mechanism by which Hop associates with Hsp90 and Hsp70, and therefore improve our appreciation of how Hop modulates the assembly of the Hsp90 chaperone complex *in vivo*. The Hsp90 chaperone complex is important in the folding and regulation of key signalling molecules, and is currently viewed as a target in the design of inhibitory drugs to regulate cell division signalling in cancer cells.[25] This and other research on the manner in which Hop associates with and modulates Hsp90 during the assembly of the Hsp90 chaperone complex is therefore important in the broader context of cell signalling networks and cancer biology.

In terms of collecting information about interactions of biological molecules, there are several advantages to SPR. First, no labelling of the sample is required and there is minimal loss of sample during the analytical process. Second, analysis can be performed on tissue culture media or bacterial broth with minimal purification of the analyte stream. However, the technique has some major disadvantages. One of these is the

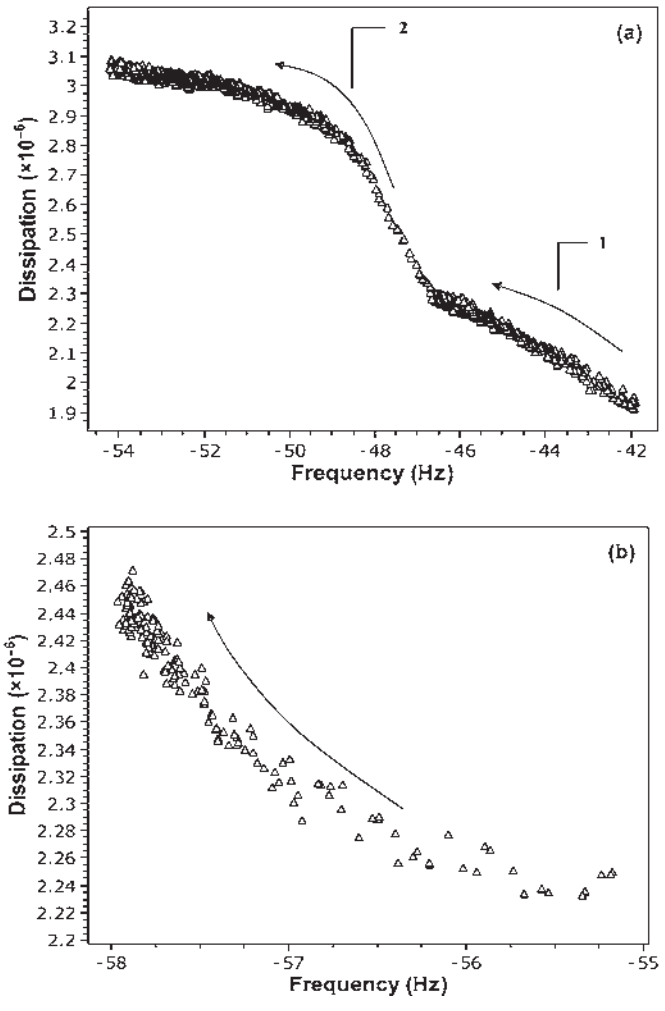

Fig. 5. **a**, *D* changes versus *f* changes for data shown in Fig. 3: detail of (2) GST-Hop binding to (1) anti-*sj*GST. **b**, *D* changes versus *f* changes for data shown in Fig. 3: detail of Hsp70 adsorption on GST-Hop.

effect of mass transport of molecules, which may give results that inaccurately depict the kinetics of the interactions. In some cases the immobilized ligand may be denatured due to steric effects or harsh regeneration conditions. Another major handicap of SPR generally is its inability to detect structural changes particularly in proteins during protein–protein interactions, because kinetic data are collected independently of the properties of the sample.

The QCM-D addresses some of these shortcomings by providing information on structural changes during protein–protein interactions, showing in these studies the flexible nature of the complex when Hsp70 bound to the immobilized GST-Hop. One of the limitations of the QCM-D technique for measuring the interactions between Hsp70 and GST-Hop, as in the studies reported here, is the lack of an effective immobilization protocol for GST-tagged proteins, whereas these procedures are established for SPR. While valuable data were obtained using the various immobilization strategies examined in this study, low-frequency shifts suggested weak coupling of, for example, the biotin-NHS layer to the streptavidin layer. This resulted in lower than expected frequency shifts for the GST-Hop. Furthermore, the anti-*sj*GST antibody gave an almost 10 times higher signal when adsorbed directly on the Au surface than to the biotin-NHS layer, indicating that this surface build-up was not optimal.

Effective immobilization strategies, however, creating inert, well-defined surfaces for studies on proteins with low non-specific binding, can be studied with histidine tags,[26] and are available for QCM-D measurements. Therefore, while this research serves as a platform for the development of immobilization strategies for GST-tagged proteins, similar research using His-tagged proteins will benefit from the optimized immobilization strategy for these proteins.

The QCM-D may become a powerful tool in the hands of scientists interested in protein–protein interactions of biological, medical and toxicological significance. In addition, the economic and technical accessibility of the equipment could contribute usefully to developing research capacity in these areas in South Africa.

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