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Profiling of Molecular Interactions in Real Time using Acoustic Detection

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Abstract

Acoustic sensors that exploit resonating quartz crystals to directly detect the binding of an analyte to a receptor are finding increasing utility in the quantification of clinically-relevant analytes. We have developed a novel acoustic detection technology, which we term Resonant Acoustic Profiling (RAP™). This technology builds on the fundamental basics of the “quartz crystal microbalance” or “QCM” with several key additional features including two- or four-channel automated sample delivery, in-line referencing and microfluidic sensor ‘cassettes’ that are pre-coated with easy-to-use surface chemistries. Example applications are described for the quantification of myoglobin concentration and its interaction kinetics, and for the ranking of enzyme-cofactor specificities.

Keywords

QCM; kinetics; microfluidics; affinity; label-free; binding

1. Introduction

The piezoelectric effect, which forms the basis of acoustic sensing, was first discovered by the Curie brothers in 1880 (Curie and Curie 1880). Today, acoustic sensors are generally based on quartz crystal resonators that are found in electronic devices such as watches, computers and televisions, with over a billion units mass-produced each year. As molecules interact with immobilised receptors on the surface of a quartz crystal, there is a concomitant modulation of the acoustic resonance of the crystal. This, in turn, results in a modulated electrical signal that can be analyzed using standard digital signal processing techniques. The signal indicates not only the presence of the material (Sauerbrey 1959), but also the specificity and affinity for a surface-bound receptor (Braunhut et al. 1999, Janshoff et al. 2000, Kanazawa 1997, Thompson and Hayward 1997). The number of publications that involve the use of QCM has increased rapidly since the mid 1980s and in particular since the 1990s (Fig. 1a). There has been a steady growth in the number of published papers describing detection of clinically relevant analytes (Fig. 1b) and, in the last five years, rapid growth in the number of publications on the analysis of pathogenic DNA (Caruso et al. 1998, Hook et al. 2001), viruses, (Sato et al. 1996, Susmel et al. 2000, Zhou et al. 2002) bacteria (Fung and Wong 2001, Otto et al. 1999, Pavey et al. 1999, Pavey et al. 2001, Spangler and Wilkinson 2000, Wong et al. 2002) and mammalian cells (Braunhut et al. 1999, Ebato et al. 1993, Ebersole et al. 1991, Fredriksson et al. 1998, Gryte et al. 1993, Richert et al. 2002, Rodahl et al. 1997, Shinohara 1999, Wegener et al. 2000). However,

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it is surprising that most of the data have been generated using “home built” QCM systems, often with insufficient attention to surface chemistry and assay reproducibility. A widely available and robust acoustic detection system that can generate reproducible data for the above classes of analytes could be of great benefit for life sciences research, clinical diagnostics, environmental sensing and homeland defence (bio-threat detection).

In response to this challenge we have developed a RAP system, the RAP $\diamond id^{TM}$ 4, that incorporates a number of key technical advances (Fig. 2a). We have produced higher frequency (16.5 MHz) resonators that possess high merit or ‘Q’ factors (a measure of the piezoelectric device's ability to convert electrical energy into kinetic energy). These resonators incorporate a parallel reference control, which enables analyte to be passed simultaneously over both ‘active’ and ‘control’ receptors (Fig. 2b). As quartz is a piezoelectric material, it is highly sensitive to flexural, shear and pressure changes (Janshoff et al. 2000). These artefacts are undesirable in a system designed to measure a specific response to an analyte. To address this potential problem, we have combined a proprietary stress-free mounting system for the quartz crystal with a micro-fluidic lateral flow device. The interface between the biosensor surface and the chemical or biological system to be studied is also a vital component of all surface-sensitive sensor systems. To generate data in this paper, we have employed a planar linker layer chemistry that enables robust immobilization of receptors, with retention of binding activity and minimal non-specific binding. This linker layer binds to the gold surface of the sensor by thiol-gold interactions, and enables covalent linkage via carbodiimide coupling to the receptor to be studied.

2. Experimental

Instrumentation and Sensors

RAP experiments were conducted using automated two-channel or four-channel RAP $\diamond id$ instruments (Akubio Ltd, Cambridge, UK). The instruments apply the principles of QCM, in that a high frequency (16.5 MHz) voltage is applied to a piezoelectric quartz crystal to induce the crystal to oscillate, and its resonant frequency is then monitored in real time. The instruments comprised a pair of oscillating crystal sensors mounted in parallel microfluidic flow cells, allowing sample to be flowed across two surfaces simultaneously. The four channel instruments use two pairs of sensors. As sample was flowed across ‘control’ and ‘active’ sensors, binding to the ‘active’ sensor was measured as a change in the resonant frequency, with the ‘control’ sensor acting as a subtractive reference.

The RAP instruments were fitted with a thermally-stable sensor mounting block providing temperature control between 10–40°C, and with microfluidic and electrical connections to the pair of piezoelectric sensors. Buffer flow was maintained with two syringe pumps under software control. Microfluidics comprised separate flow-paths to individual flow cells, as well as a common flow path split to address both flow cells simultaneously. Interchange between the different flow paths was by pneumatically-operated valves. Sensors comprised standard gold-coated quartz wafers, coated with a proprietary planar compact alkane chain linker layer terminated in carboxylic acid groups to provide a surface for protein immobilisation, mounted in an acrylic cassette (Akubio Ltd).

Sensor surface preparation

For the myoglobin binding assay, sensor surfaces were prepared before assessing antigen binding by immobilising rabbit anti-mouse Fc-specific immunoglobulin (RaM-Fc) onto the ‘active’ sensor surface and mouse immunoglobulin type G (Ms IgG) onto the ‘control’ sensor surface using conventional amine coupling chemistry. A four-channel RAP $\diamond id$ instrument was used for myoglobin assay. Sensor surfaces were activated with a 1:1 mixture of 400 mmol/L

1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 100 mmol/L N-hydroxysuccinimide (NHS; both from Perbio Science UK Ltd, Cramlington, UK), prepared in 0.22 µm-filtered deionised water, and mixed immediately prior to use (final concentrations; 200 mmol/L EDC and 50 mmol/L NHS). EDC-NHS was injected simultaneously across both sensor surfaces for 3 min. Affinity purified RaM-Fc and Ms IgG (Strattech Scientific Ltd/Jackson ImmunoResearch, Soham, UK) were prepared for immobilisation at 50 µg/mL in immobilisation buffer comprising 10 mmol/L sodium acetate, pH 4.5, and were injected simultaneously across separate sensor surfaces for 3 min. Non-reacted NHS esters were then capped with 1 mol/L ethanolamine (Sigma-Aldrich, Poole, UK) prepared in 0.22 µm filtered deionised water, pH 8.5. Running buffer between sample injections was Dulbecco's modified phosphate buffered saline (PBS; Sigma-Aldrich, Poole, UK), at a flow rate of 50 µL/min. Immobilisation of RAM Fc resulted in 483 Hz response and immobilisation of mouse IgG resulted in 447 Hz response.

Myoglobin binding assay

Mouse monoclonal anti-myoglobin (Spectral Diagnostics Inc, Toronto, Canada) was prepared at 5 µg/mL in HEPES-buffered saline comprising 10 mmol/L HEPES, 120 mmol/L NaCl, 3 mmol/L EDTA and 0.005% polyoxyethylenesorbitan monolaurate (Tween-20; Sigma-Aldrich, Poole, UK), and was captured onto the RaM-Fc surface by injection across both sensor surfaces for 3 min. A dissociation period of 5 min under HEPES-buffered saline flow allowed anti-myoglobin capture and dissociation to stabilise. The capture of mouse anti-myoglobin antibody on RAM-Fc surface gave 148 Hz response, and on mouse IgG surface less than 0.5 Hz non-specific binding was observed (data not shown). Recombinant human myoglobin (Spectral Diagnostics Inc, Toronto, Canada) was prepared at 100 nM in HEPES-buffered saline, and then diluted further in approximately semi-log dilutions to 1 nM. Myoglobin samples were injected for 5 min, followed by a 5 min dissociation period. Sensor surfaces were then regenerated for further binding cycles by injection of 100 mmol/L HCl for 60 seconds and 20 mmol/L NaOH for 30 seconds. Regeneration conditions were optimised to provide greater than 95% regeneration efficiency. Myoglobin binding curves were control subtracted, superimposed and normalised to the start of the sample injection. Initial binding rates were calculated from a linear fit model applied to the initial portion of each binding curve. Initial binding rate slopes were plotted against myoglobin concentration to generate a concentration-response calibration curve. Myoglobin concentrations in unknown samples were quantified by interpolation from this calibration curve.

Glucose dehydrogenase substrate selectivity and affinity

Glucose dehydrogenase (GDH; Sigma-Aldrich, Poole, UK) was supplied in Tris buffer, which is unsuitable for amine coupling. Consequently, a NAP5 column (GE Healthcare, Amersham, UK) was pre-equilibrated with 10 mL of immobilisation buffer comprising 10 mmol/L sodium acetate, pH 4.5. GDH was diluted to 100 µg/mL in immobilisation buffer and 200 µL added to the column. A further 200 µL immobilisation buffer was added to the column, and the column allowed to equilibrate. GDH was eluted by adding 2 mL immobilisation buffer and collecting 2 drop (~100 µL) fractions. GDH activity was found in a spectrophotometric GDH + glucose + NADP⁺ turnover assay to elute in fractions 5–9. Final GDH concentration from multiple purifications ranged between 17 – 22 µg/mL, determined using a standard bicinchoninic acid (BCA; Pierce-Perbio, Cramlington, UK) protein concentration determination assay. A two-channel RAP^{id} instrument was used for GDH assay. For the immobilization assay, 0.5 mol/L glucose (Sigma-Aldrich, Poole, UK) was added to the GDH purification buffer in order to occupy the enzyme active site during coupling. Bovine serum albumin (BSA; Sigma-Aldrich, Poole, UK) as a non-binding control was prepared at 50 µg/mL in 10 mmol/L sodium acetate buffer, pH 4.5, without glucose. Sensor surfaces were activated with EDC-NHS for 8 min. GDH or BSA were then injected simultaneously across separate sensor surfaces for 4 min.

Non-reacted NHS esters were capped with 1 mol/L ethanolamine, pH 8.5, for 8 min. Running buffer between sample injections was PBS + 0.005% Tween 20 at a flow rate of 50 μ L/min. The immobilisation of GDH resulted in 366 Hz response and immobilisation of BSA resulted in 404 Hz response (data not shown).

Nicotinamide adenine dinucleotide phosphate (NADP⁺), nicotinamide adenine dinucleotide (NAD⁺) and flavine adenine dinucleotide (FAD⁺; all from Sigma-Aldrich, Poole, UK) as GDH-binding co-factors were prepared at 2 mmol/L in running buffer, and diluted in 4 steps to 222 μ mol/L. Co-factors at each concentration were flowed across GDH 'active' and BSA 'control' surfaces for 1 min, allowed to dissociate and return to baseline level, and then injected a second time. After a 2 min cleaning period in running buffer, the next co-factor concentration was tested, in duplicate.

3. Results and discussion

Myoglobin

Myoglobin is a 17 kDa cytoplasmic protein found in muscle cells that binds and stores oxygen. Normal levels of myoglobin in blood are very low (< 100 ng/mL). Following muscle damage (e.g. cardiac ischaemia), myoglobin is released into the blood; it can rise to > 1000 ng/mL immediately after a myocardial infarction and can remain high (~ 500 ng/mL) for some time afterwards. Myoglobin detection in blood is therefore a good diagnostic indicator of preceding and ongoing cardiac disease and damage (Casey 2004). We used a mouse anti-myoglobin antibody, previously captured onto a rabbit anti-mouse-Fc specific antibody (RaM-Fc) surface to measure binding of varying concentrations of human recombinant myoglobin (Fig. 3a). A control, mouse IgG surface was used to determine the very low level of non-specific binding of myoglobin to the protein-coated surface. The level of non-specific binding for myoglobin binding to anti-myoglobin was less than 4 Hz (data not shown). The data presented in Fig. 3a are the results after subtracting non-specific binding.

Assuming that all of the anti-myoglobin Fv sites are available to bind myoglobin, myoglobin immobilisation/capture efficiency is calculated as 74% using equation 1.

$$dF_{\max} = \frac{Mwt_{\text{analyte}}}{Mwt_{\text{ligand}}} dF_{\text{immobilisation}} \times \text{stoichiometry} \quad (\text{Eq. 1})$$

Varying concentration of myoglobin in the sample would give varying response levels at equilibrium, which would enable generation of a standard curve for analyte concentration. However, it can be seen from Fig. 3a that at lower concentrations of myoglobin considerable contact time would be required to reach an equilibrium level. In addition, in a 'real world' diagnostic application the sensor is likely to be used only once, and the level of receptor activity may vary upon shipping and storage, which would result in variable equilibrium binding levels. For a simple bimolecular association, $A + B = AB$, the process was assumed to be pseudo-first order with no interaction between separate receptor molecules. The dissociation rate is derived from the equation:

$$dF_t = dF_{t_0} e^{-k_d(t-t_0)} \quad (\text{Eq. 2})$$

where dF_t is the response at time t , dF_{t_0} is the response at time t_0 and k_d is the dissociation rate constant. The association rate constant can be derived using the equation:

$$dF_t = \frac{k_a C \cdot dF_{\max} (1 - e^{-(k_a C + k_d)t})}{k_a C + k_d} \quad (\text{Eq. 3})$$

where $dF_{\max}$ is the maximum response (proportional to the amount of immobilised ligand, Eq 1), C is the concentration of analyte in solution, and k_a is the association rate constant.

The initial rate of binding (k_{obs}) is thus proportional to the concentration of analyte ($k_{\text{obs}} = C \cdot k_{\text{ass}} - k_{\text{diss}}$). To fit the initial association rates the first 10 to 15 s of association data was selected and then subjected to both a linear and a quadratic fit. The R^2 values for these two fits were then compared, and a sub-set of the fitted data was chosen for which the difference between the two fits was minimal. This results in selection of an optimal linear portion of the binding data, which is representative of the observed rate for a pseudo-first order association process. The linear term from the quadratic fit was then used to give the initial association rate, which could be correlated with analyte concentration by simultaneously applying the above process to binding curves generated using variant analyte concentration.

As the kinetic rate constants are invariant, analyte concentration could thus be accurately and rapidly determined by analyzing the initial rate of binding of myoglobin to the antibody, and then comparing the measured observed association rate with a data set calibrated to analyte concentration (Fig. 3b). This enabled the concentration of myoglobin in the sample to be determined in less than 6 minutes (Table 1).

Glucose dehydrogenase

GDH is a key initial enzyme in the energy production process that uses nucleotide cofactors to 'activate' monosaccharide sugars as a prelude to their subsequent breakdown into pyruvate to enter the Krebs cycle. NADP^+ (765 Da) is the preferred cofactor, NAD^+ (663 Da) will act as a lower activity cofactor, and FAD^+ (30 Da) will not bind, nor act as cofactor. GDH was coupled to the carboxylic acid terminating linker layer using standard carbodiimide amine coupling as described earlier. BSA was coupled in a similar manner to a second sensor surface to be used as a passive control. NADP^+ , NAD^+ and FAD^+ were then independently serially diluted then flowed across the GDH 'active' and BSA 'control' surfaces for 1 min. The complexes were then allowed to dissociate for 1 min until pre-binding baseline levels were attained (Fig. 4). From the magnitude of the binding response for the different co-factors, a rapid estimate of binding level could be made: $\text{NADP}^+ > \text{NAD}^+ > \text{FAD}^+$. This demonstrates that RAP can be applied to the accurate determination of enzyme-co-factor specificities in less than 10 minutes.

4. Conclusion

Rapid identification of predictive disease markers, pathogens and toxins causing an infection or other deleterious effect on human health brings many benefits, including rapid institution of specific therapy and appropriate infection control measures (Henderson 1999). Application of rapid methods in routine practice has, unfortunately, lagged behind their adoption in other areas of medicine, in part because of the need to grow organisms and isolate them in pure culture before they can be identified. While PCR methods can provide valuable early results in some situations, local facilities are not available to most laboratories and specimens are often transported to reference laboratories for such tests. Early data from the RAP $\diamond$ id system suggests that the technology could potentially form a core detection module for new clinical tests and for screening of drug candidates in drug discovery. The system can detect most classes of clinically-relevant analytes in real-time without enzyme, fluorescent, isotopic or chemiluminescent labels. The system is also entirely electronic and possesses modest requirements for power and data processing compared to analogous optical systems. Hence,

assays in the future could be carried out both in the laboratory and at the point of care or in the field.

References

- Braunhut SJ, Marx KA, Zhou T, Warren MC, Schulze HM. *Mol Biol Cell* 1999;10:64a–64a.
- Caruso F, Furlong DN, Niikura K, Okahata Y. *Colloids Surf, B* 1998;10(4):199–204.
- Casey PE. *AACN Clin Issues* 2004;15(4):547–57. [PubMed: 15586156]
- Curie P, Curie J. *C R Acad Sci* 1880;91:294.
- Ebato H, Okahata Y, Matsuda T. *Kobunshi Ronbunshu* 1993;50(6):463–469.
- Ebersole RC, Foss RP, Ward MD. *Nat Biotechnol* 1991;9(5):450–454.
- Fredriksson C, Kihlman S, Rodahl M, Kasemo B. *Langmuir* 1998;14(2):248–251.
- Fung YS, Wong YY. *Anal Chem* 2001;73(21):5302–5309. [PubMed: 11721933]
- Gryte DM, Ward MD, Hu WS. *Biotech Prog* 1993;9(1):105–108.
- Henderson DA. *Science* 1999;283(5406):1279–82. [PubMed: 10037590]
- Hook F, Ray A, Norden B, Kasemo B. *Langmuir* 2001;17(26):8305–8312.
- Janshoff A, Galla HJ, Steinem C. *Angew Chem Int Edit* 2000;39(22):4004–4032.
- Kanazawa KK. *Faraday Discuss* 1997;(107):77–90.
- Otto K, Elwing H, Hermansson M. *J Bacteriol* 1999;181(17):5210–5218. [PubMed: 10464189]
- Pavey KD, Ali Z, Olliff CJ, Paul F. *J Pharm Biomed Anal* 1999;20(1–2):241–5. [PubMed: 10704028]
- Pavey KD, Barnes LM, Hanlon GW, Olliff CJ, Ali Z, Paul F. *Lett Appl Microbiol* 2001;33(5):344–348. [PubMed: 11696093]
- Richert L, Lavalle P, Vautier D, Senger B, Stoltz JF, Schaaf P, Voegel JC, Picart C. *Biomacromolecules* 2002;3(6):1170–1178. [PubMed: 12425653]
- Rodahl M, Hook F, Fredriksson C, Keller CA, Krozer A, Brzezinski P, Voinova M, Kasemo B. *Faraday Discuss* 1997;(107):229–246. [PubMed: 9569776]
- Sato T, Serizawa T, Okahata Y. *BBA-Biomembranes* 1996;1285(1):14–20. [PubMed: 8948470]
- Sauerbrey G. *Z Phys* 1959;155:206–212.
- Shinohara H. *Electrochemistry* 1999;67(3):280–284.
- Spangler BD, Wilkinson EA. *Abstr Am Chem Soc* 2000;219:U115–U115.
- Susmel S, O'Sullivan CK, Guilbault GG. *Enzyme Microb Technol* 2000;27(9):639–645. [PubMed: 11064045]
- Thompson M, Hayward GL. *Proc IEEE Int Freq Control Symp* 1997:114–119.
- Wegener J, Seebach J, Janshoff A, Galla HJ. *Biophys J* 2000;78(6):2821–2833. [PubMed: 10827965]
- Wong YY, Ng SP, Ng MH, Si SH, Yao SZ, Fung YS. *Biosens Bioelectron* 2002;17(8):676–684. [PubMed: 12052353]
- Zhou XD, Liu LJ, Hu M, Wang LL, Hu JM. *J Pharm Biomed Anal* 2002;27(1–2):341–345. [PubMed: 11682242]

A)

B)

Fig 1.

A: Yearly cumulative number of publications found using an ISI Web of SCIENCE® search for the term "quartz crystal microbalance" in the title, keyword or abstract. **B:** As above, but cross referenced using the terms "DNA", "proteins OR immunoassay", "bacteria", "cell" or "virus".

A)

B)

Fig 2.**A:** The RAPid 4 system and **B:** microfluidic sensor cassette.

Fig 3.

A: Recombinant human myoglobin was prepared at 100 nM in PBS, and then diluted further in approximately semi-log dilutions to 1 nM. Data are overlaid, aligned responses for myoglobin samples at the different concentrations tested, injected for 5 min. Each sample injection was then followed by a 5 min dissociation period, then sensor surfaces were regenerated with a 1 min injection of 100 mmol/L HCl and a 30 second injection of 20 mmol/L NaOH (data not shown). **B:** Myoglobin concentration-response calibration curve, generated by plotting the initial binding rate for the set of standard concentrations shown in (A). Two unknown samples are also plotted on the standard curve to indicate the concentrations estimated in Table 1.

Fig 4.

Binding of preferred substrate, poor substrate, and non-preferred adenine-nucleotide co-factors to the metabolic enzyme GDH. **A:** $NADP^+$ (765 Da, preferred substrate) binding shows a clear equilibrium concentration-response relationship. **B:** NAD^+ (663 Da, metabolite) shows a lower equilibrium binding level, and compressed concentration-response relationship. **C:** FAD^+ (830 Da, non-preferred substrate) shows very low levels of equilibrium binding.

Table 1

Estimates of myoglobin concentration in unknown samples were calculated by linear interpolation from the calibration curve (Fig. 3b) with an accuracy of 8% to 12% over the concentration range tested.

Sample	Predicted Myoglobin Concentration (nM)	Actual Myoglobin Concentration (nM)	Error
Unknown 1	20.65	23.52	12%
Unknown 2	2.16	2.35	8%