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Characterisation of particle-surface interactions via anharmonic acoustic transduction

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Abstract

Most transduction methods for measuring particle-surface interactions are unable to differentiate the strength of interaction and largely reliant on extensive washing to reduce the ubiquitous non-specific background. Label-based methods, in particular, are limited in wide applicability due to their inherent operational complexity. On the other hand, label-free force-spectroscopic methods that can differentiate particle-surface interaction strength are skill-demanding and time-consuming. Here, we present a label-free anharmonic (nonlinear) acoustic transduction method employing the quartz crystal resonator that reads out ligand-receptor binding based on the interaction strength. We show that while stronger specific interactions are transduced more strongly, and in linear proportionality to the ligand concentration on microparticles, non-specific interactions are significantly attenuated. This allows ligand quantification with high specificity and sensitivity in realtime under flow without separate washing steps. Constructing an analytical model of a quartz resonator, we can relate the number and type (specific vs. non-specific) of ligand-receptor interactions with the change in characteristic nonlinearity coefficient of the resonator. The entirely-electronic and microfluidic-integrable transduction method could potentially allow a simple, fast and reliable way for characterising particle-surface interactions with economy of scale.

Keywords: quartz crystal microbalance; nonlinear acoustic transduction; label-free biosensor; realtime immunosensor; cell-surface profiling; immunophenotyping

1. Introduction

Characterising biomolecular interactions is pivotal to the development of novel therapeutic and diagnostic platforms. Although significant progress has been made in the development of analytical methods for these applications, there still lies some fundamental limitations^{1,2}. Labelled optical transduction often requires skill-demanding and time-consuming procedures involving multiple reagents and steps, which restrict its use to controlled environments. Moreover, biochemical sensor interfaces (i.e. receptor-ligand) are prone to nonspecific interactions and fouling caused by other sample constituents. Most transduction methods, labelled or label-free, lack the intrinsic ability to differentiate strength of interactions. As a result, extensive washing and assay development are employed to overcome the problem of non-specific binding^{3,4}. On the other hand, while force spectroscopic methods, such as atomic force microscopy and optical tweezers, allow label-free differentiation of particle-surface interaction strength, they are complex and slow, and require a laboratory infrastructure. Thus, there exists an unmet need for a label-free transduction method that is easy-to-use and scalable, and can characterise ligand-receptor interactions in realtime, providing reliable differentiation from non-specific interactions with minimal or no washing.

Quartz crystal resonators have been widely used for label-free quantification of biomolecular binding⁵. Quartz crystals are commonly driven close to their fundamental or higher modes of resonance. The thickness (mass) and softness (viscosity) are conventionally estimated from the shifts in resonance frequency and dissipation measured *at the driven mode*. This transduction method is referred to as the Quartz Crystal Microbalance (QCM)⁶. However, it should be noted that inertial (mass) loading, which causes decrease in resonance frequency, is dominant only in the case of thin and rigid adsorbent layer. When relatively large ($d > 1\mu\text{m}$) and more flexible adsorbates bind to the quartz crystal via small contact points, they form a coupled oscillator with the quartz crystal resonator. The binding of colloidal particles⁷ and biological particles, such as bacterial spores⁸, to the quartz crystal resonator via ligand-receptor bonds are examples of such coupled resonators. The mass coupling is weak for these particles. On the other hand, the drive frequency, which is typically in the MHz range, is significantly higher than the resonance frequency of the particle-bonds system. As a result, governed by the principle of vibration, the particle is unable to follow the thickness-shear oscillation of the resonator and remains almost stationary in space. The resulting stretch in the bonds contributes to a restoring force, which increases the effective stiffness of the resonator. Thus, elastic loading is dominant over mass loading, and causes an increase in resonance frequency that is proportional to the increase in stiffness of the particle-resonator contact⁹. Several strategies have been employed to study this phenomenon, including subjecting the particle-QCM contact to a range of centrifugation forces¹⁰, ionic strengths¹¹ and dehydration levels¹². In this study, we functionalised polystyrene microparticles with different concentrations of human immunoglobulin-E (IgE, ligand) and the quartz crystal resonator with anti-IgE aptamer (receptor), and investigated the particle-resonator interactions for the various ligand concentrations comparatively using changes in resonance frequency, dissipation and a novel acoustic parameter, the third Fourier harmonic current, which we explain below.

In our earlier work, the quartz crystal was *driven close to its fundamental resonance mode* and the electrical current was measured *exactly at three times the drive frequency (3f)*, referred to as the third Fourier harmonic^{5,8}. It should be noted that the third Fourier harmonic is a result of nonlinear response of an oscillator and may be present even in an oscillator with a single-mode of resonance, such as a spring-mass oscillatory system. Hence, the third Fourier harmonic is conceptually different from the third overtone resonance (a higher resonance mode), although they may be in the vicinity of each other depending on the crystal curvature. The third Fourier harmonic current is usually low as the quartz crystal is largely a linear oscillator at modest amplitudes. It was shown through modelling and experiments that when streptavidin-coated microparticles bound to the quartz resonator, the microparticle-resonator interaction forces distorted the pure sinusoidal oscillation,

contributing to a considerable change in the third Fourier harmonic ($3f$) current. The quantitative change in the $3f$ current was proportional to the number of bound microparticles, and interestingly, significantly smaller for physisorbed microparticles, where the particle-resonator interaction was weaker. The transduction method based on the measurement of the change in the third Fourier harmonic current was referred to as the Anharmonic Detection Technique (ADT)^{5,8}.

In this study, we employed the ADT transduction method to investigate how the change in the $3f$ current varied for different particle-resonator interactions when the number of resonator-bound particles was similar. A range of specific interactions (IgE-antiIgE) were modelled using various concentrations of IgE functionalised on polystyrene microparticles and anti-IgE aptamer functionalised on the quartz crystal resonator. Non-specific interactions were modelled using goat immunoglobulin-G (IgG) functionalised on the microparticles and the same anti-IgE aptamer on the resonator. We observed that when similar number of microparticles was bound specifically to the resonator, the change in the $3f$ current was greater for stronger specific interactions, and linearly proportional to the ligand (IgE) concentration on the microparticles. We also noted that when similar number of microparticles was bound non-specifically to the resonator, functionalised with IgG, in the absence of adequate washing, the change in the $3f$ current was significantly lower. Interestingly, this differentiation between the various interaction strengths was much more pronounced from the change in the $3f$ current than from the change in the resonance frequency. In particular, the relative 'attenuation' in the shift of $3f$ current for non-specific interactions with respect to the specific interactions was more than one order of magnitude greater than similar attenuation in the change in resonance frequency measured from the same assay. The quantitative correlation of the change in $3f$ current with the particle-bound ligand concentration and its feature of differentiation of specific and non-specific interactions make it suitable to be developed as a reliable, fast and easy-to-use method for characterisation of particle-surface interactions. This may be applied to relative quantification of affinity of interaction for various ligand-receptor pairs, and relative quantification of ligand concentration on a particle for a given receptor, e.g. cellular immunophenotyping. It needs to be noted that in order to cause a nonlinear distortion of the acoustic response of the quartz crystal resonator, the particle needs to be flexibly attached to the resonator, such as in the case of colloidal particles or bacteria, which have a cell wall that offers the cells some rigidity of form. Here we employed polystyrene microparticles so that we could controllably functionalise a range of concentrations of the ligand (IgE) on them and investigate how their binding to the quartz crystal resonator correlated quantitatively with the change in the acoustic signal.

It may be noted that, in this paper we referred to the quartz crystal resonator as QCR to treat the 'transducer' with distinction from QCM, which generally refers to the 'transduction method' based on the shift in resonance frequency of a QCR.

Below, we present an analytical model by describing the quartz resonator using the one-dimensional Duffing equation. Analysing the experimental data using this model, we discuss how we can directly relate the type (specific vs. non-specific) and strength of ligand-receptor interaction (quantified by the protein concentration on the microparticles) with the change in the nonlinearity coefficient of the resonator, which underpins the change in $3f$ current.

2. Theoretical Modelling

To understand the theory underpinning the specificity and quantitative nature of the $3f$ current measurement, we modelled the QCR analytically as a nonlinear oscillator. It is well established that at relatively high drive amplitude, the thickness-shear mode the quartz crystal oscillator (QCR) behaves as a nonlinear oscillator¹³. Modelled as an infinite slab of

parallel planes, the oscillation of a QCR can be described by the one-dimensional Duffing equation as

$$\ddot{x} + 2\lambda\dot{x} + \frac{k_q}{m_q}(x - \delta_0 \cos(\omega t)) + \beta_q x^3 = 0 \quad (1)$$

where 2λ is the mass-proportional damping coefficient, primarily due to the liquid-QCR interface, k_q , m_q and β_q are the stiffness, mass and nonlinearity coefficient of the QCR respectively, and $\delta_0 \cos(\omega t)$ models the oscillatory drive of amplitude δ_0 and frequency $\omega = 2\pi f$. Only odd orders of x are considered in Eq.1 due to symmetricity of motion of QCR. 5th and higher orders are neglected.

When microparticles bind to the QCR through protein-receptor interactions, they form a spring-mass system coupled to the QCR. If k_b and N_b are respectively the stiffness of each bond and the number of bonds per microparticle of mass m_p , then $\sqrt{(k_b N_b)/m_p} \ll \omega$, i.e. the resonance frequency of the microparticle spring-mass system is much smaller compared to the drive frequency, which in our experiment was close to 14.24 MHz. As a result, the microparticle remains almost stationary in the laboratory reference frame with weak mass coupling with the QCR while the latter oscillates. This stretches the bonds, applying elastic loading on the resonator, which increases the effective stiffness of the QCR. The elastic loading also distorts the pure sinusoidal oscillation, consequently modifying the nonlinearity coefficient of the resonator. We model this change in nonlinearity coefficient due to the adsorbate (microparticles) using β_{ad} . Neglecting the coupled mass due to the microparticles and accounting for the changed stiffness and nonlinearity coefficient, the modified differential equation takes the following form.

$$\ddot{x} + 2\lambda'\dot{x} + \frac{k_q + k_b N_b N_p}{m_q}(x - \delta_0 \cos(\omega t)) + (\beta_q + \beta_{ad})x^3 = 0 \quad (2)$$

Here, N_p is the number of bound microparticles, and λ' is the modified damping coefficient. If $\beta = \beta_q + \beta_{ad}$ and $\omega_0 = \sqrt{(k_q + k_b N_b N_p)/m_q}$ denotes the increased new resonance frequency due to the elastic loading, the differential equation in Eq.2 can be expressed as

$$\ddot{x} + 2\lambda'\dot{x} + \omega_0^2 x + \beta x^3 = A \cos(\omega t) \quad (3)$$

where $A = \delta_0 \omega_0^2$ denotes the amplitude of the drive acceleration. The periodical solution of Eq.3 can be presented as the sum of odd Fourier harmonics. For small drive amplitudes, we consider two most significant harmonics ω and 3ω as given by

$$x = a_{1f} \cos(\omega t + \varphi_1) + a_{3f} \cos(3\omega t + \varphi_3) \quad (4)$$

where a_{1f} and a_{3f} are the amplitude of oscillation, and φ_1 and φ_3 are angles of phase shifts at the first harmonic ($\omega = 2\pi f$) and the third harmonic (3ω) respectively. For small oscillation amplitude and in proximity of resonance, a_{1f} and a_{3f} are related as

$$a_{3f} = \frac{\beta}{32\omega_0^2} a_{1f}^3 \quad (5)$$

and the amplitude of first harmonic oscillation is given by the following equation¹⁴.

$$a_{1f} \sqrt{(\omega - \omega_0 - \frac{3\beta}{8\omega_0} a_{1f}^2)^2 + \lambda'^2} = \frac{A}{2\omega_0} \quad (6)$$

It may be noted from Eq.6 that the shift in resonance frequency due to drive level is,

$$\Delta\omega_0 = \frac{3\beta}{8\omega_0} a_{1f}^2 \quad (7)$$

Eq.6 leads to third order polynomial for a_{1f} . However, for small oscillation amplitude approximation, it can be solved iteratively. For the first iteration, i.e. at resonance, the first term under the radical is zero. Hence, in the first iteration,

$$a_{1f} \approx \frac{A}{2\lambda'\omega_0} \quad (8)$$

Substituting this solution under the root for the second iteration we have,

$$a_{1f} \sqrt{\left(\omega - \omega_0 - \frac{3\beta A^2}{32\lambda'^2 \omega_0^3}\right)^2 + \lambda'^2} = \frac{A}{2\omega_0} \quad (9)$$

Substituting back $A = \delta_0 \omega_0^2$ we have,

$$\Rightarrow a_{1f} = \frac{\delta_0 \omega_0}{2 \sqrt{\left(\omega - \omega_0 - \frac{3\beta \delta_0^2 \omega_0^2}{32\lambda'^2}\right)^2 + \lambda'^2}} \quad (10)$$

Using Eq.5 and Eq.10, the amplitude of third harmonic oscillation is given by,

$$a_{3f} = \frac{\beta \delta_0^3 \omega_0}{256 \left(\sqrt{\left(\omega - \omega_0 - \frac{3\beta \delta_0^2 \omega_0^2}{32\lambda'^2}\right)^2 + \lambda'^2} \right)^3} \quad (11)$$

In Eq.1, we have treated the QCR as a single mode system. However, a QCR in reality has higher modes of resonance. Oscillation with amplitude a_{3f} and frequency 3ω rad/s is enhanced due to its proximity to the third overtone resonance⁸. For our QCR, the third overtone resonance is not located exactly at $3\omega_0$ but slightly shifted upwards typically by $\epsilon \approx 75000$ rad/s (~ 12 kHz). The amplitude of third harmonic oscillation after enhancement due to proximity to the third overtone resonance in our QCR is given by

$$a_{3ftrue} = \frac{9a_{3f}\omega_0^2}{\sqrt{((3\omega_0 + \epsilon)^2 - 9\omega^2)^2 + 36\lambda_3'^2 \omega^2}} \quad (12)$$

where $2\lambda_3'$ is the mass-proportional damping coefficient at the third overtone resonance.

The amplitudes of the piezoelectric current at first harmonic (I_{1f}) and third harmonic (I_{3f}) that we measure in the experiments are proportional to a_{1f} and a_{3ftrue} respectively, with the

constant of proportionality given by $\chi = 0.8/\rho_w$, where ρ_w is the characteristic impedance of QCR¹⁵. Using Eq.10 and Eq.12, we get,

$$I_{1f} = \frac{\chi \delta_0 \omega_0}{2 \sqrt{\left(\omega - \omega_0 - \frac{3\beta \delta_0^2 \omega_0}{32\lambda'^2}\right)^2 + \lambda'^2}} \quad (13)$$

$$I_{3f} = \frac{\chi \beta \delta_0^3 \omega_0}{256 \left(\sqrt{\left(\omega - \omega_0 - \frac{3\beta \delta_0^2 \omega_0}{32\lambda'^2}\right)^2 + \lambda'^2} \right)^3} \frac{9\omega_0^2}{\sqrt{((3\omega_0 + \epsilon)^2 - 9\omega^2)^2 + 36\lambda_3'^2 \omega^2}} \quad (14)$$

Although the above analytical solution assumes small oscillation amplitude and proximity to resonance, we have confirmed using numerical modelling with realistic resonator parameters that even for relatively high amplitude and over a few resonance bandwidths, the deviation from this analytical expression is only a small fraction of ~1%. This modelling work will be presented in our future publication, which will demonstrate the expansion of Eq.14 into complex domain and fitting to experimental data for amplitude (I_{3f}) and phase (ϕ_3).

2.1 Peak value of I_{3f}

For Anharmonic Detection Technique (ADT), transduction method presented here, we measure the peak or maximum amplitude of I_{3f} (i.e. I_{3fmax}) and monitor its relative shift over time as binding happens. To evaluate an analytical expression for I_{3fmax} , we assume that the peak value is reached for a drive frequency (ω) that is close to the fundamental resonance frequency, i.e. $\omega - \omega_0 - \frac{3\beta \delta_0^2 \omega_0}{32\lambda'^2} \rightarrow 0$ in Eq.14. Also, with first order approximation, the enhancement factor due to proximity to third overtone resonance reduces to $3\omega_0/(2\epsilon)$, noting that ϵ lies outside the half-resonance bandwidth of third overtone (λ_3') for our QCR. Further, substituting $D = 2\lambda'/\omega_0$, where D is the dissipation factor of the QCR, the maximum amplitude of third harmonic current is obtained from Eq.14 as

$$I_{3fmax} = \frac{3\chi \delta_0^3 \beta}{64\omega_0 \epsilon D^3} \quad (15)$$

In our experiments, we normalise this maximum 3f current by the corresponding 1f current, which at resonance is given by $I_{1f} = \chi \delta_0/D$. The normalised I_{3fmax} is given by

$$I'_{3fmax} = \frac{3\delta_0^2 \beta}{64\omega_0 \epsilon D^2} \quad (16)$$

2.2 Relative change in I'_{3fmax} due to binding of microparticles

When binding takes place on the QCR, there is a change in its nonlinearity coefficient (β) and dissipation factor (D). Since the relative change in ω_0 is negligible ($\sim 7 \times 10^{-6}$ in our results), we treat it as constant. The amplitude of the oscillatory drive (δ_0) is constant in our experiments. Since the shift in the third overtone frequency ($\Delta\omega_3$) and three times the shift in the fundamental resonance frequency ($3\Delta\omega_0$) are equal according to Sauerbrey equation, $\epsilon = \omega_3 - 3\omega_0$ also remains constant. Therefore, the *normalised 3f current varies only with the nonlinearity coefficient (directly) and the dissipation factor (inversely to the second order)*. Differentiating Eq.16, we get the following equation.

$$\begin{aligned}
& -\frac{dI'_{3fmax}}{I'_{3fmax}} = -\frac{d\beta}{\beta} + 2\frac{dD}{D} \\
& \text{or} \\
& -\frac{\Delta I'_{3fmax}}{I'_{3fmax}} = -\frac{\Delta\beta}{\beta} + 2\frac{\Delta D}{D}
\end{aligned} \tag{17}$$

Eq.17 gives the relationship between the relative decrease in the normalised $3f$ current, the relative decrease in the nonlinearity coefficient and the relative increase in the dissipation factor close to resonance due to binding of microparticles on the QCR.

3. Results

We functionalised polystyrene microparticles ($\phi=2.8 \mu\text{m}$, $\rho=1.6 \text{ g/cm}^3$) with 5 different concentrations of IgE using 0.016, 0.16, 0.8, 1.6 and $3.2 \mu\text{M}$ IgE solutions respectively. We did not measure the surface concentration of IgE on each microparticle. However, since the same number of microparticles (2×10^7) was used for all functionalisation reactions, we expect the number of IgE molecules on each microparticle was proportional to the concentration of IgE solution used in the respective cases. So we have used the concentration of IgE solution to present the results here. Assuming all IgE in the solution bound to the microparticles and uniformly across all microparticles, we can estimate that the abovementioned range of IgE solution concentrations would correspond to 0.016, 0.16, 0.8, 1.6 and 3.2 attomole (MW: 190 kDa) per microparticle respectively. However, in reality we expect the protein concentration per microparticle to be lower than this as full immobilization efficiency is less likely.

The IgE-functionalised microparticles were captured specifically via anti-IgE DNA aptamers functionalised on the QCR using streptavidin-biotin assay (**Figure 1a**). The binding was facilitated under continuous flow, without any washing steps, using a custom-microfabricated polycarbonate flowcell that was integrated with the QCR and a printed circuit board (**Figure 2a**). Using a nonlinear network analyser, also developed in this study (**Materials and methods**), the QCR was driven with a pure sinusoidal potential of 2.5 V at a range of frequencies around its fundamental resonance ($f_0 \approx 14.24 \text{ MHz}$), and recorded complex (in-phase and quadrature) current and voltage synchronously at the drive frequency ($1f$) and at three times the drive frequency (third Fourier harmonic or $3f$). We denote the currents measured at the drive frequency and the third Fourier harmonic frequency as I_{1f} and I_{3f} respectively. The resonance frequency (f_0) and dissipation (D) (inverse of quality factor) were estimated from the $1f$ impedance data using the Butterworth-Van Dyke (BVD) equivalent circuit of a QCR. The quality factor was estimated to be ~ 1900 before microparticle binding, as expected for a 14.3 MHz QCR with one side exposed to liquid. For controls, we used non-functionalised microparticles (referred to as blank) and microparticles functionalised with goat immunoglobulin-G (IgG) to model non-specific interactions with the anti-IgE functionalised QCR.

Figure 1. Principle of anharmonic acoustic detection. (a) Quartz crystal resonator is functionalized with a mixture of biotin- and methoxy-terminated thiolated PEG chains. Streptavidin is bound to the biotin-terminated thiols thereby facilitating the orientation of the biotinylated aptamer stem loop (receptor) away from the electrode (I,II). Microparticles displaying molecules of interest with high affinity toward the surface receptor are injected and allowed to come in contact with the resonator under continuous flow. The resonator is driven by a pure oscillating potential close to its fundamental resonance frequency (f) and the amplitude of third Fourier harmonic current (I_{3f}), is recorded together with fundamental frequency (f_0) and energy dissipation (D) (III, IV). (b, c) The magnitude of I_{3f} at resonance increases with the cubed power ($R^2=0.99$) of I_{1f} . Inset plots show upscaled magnitude of I_{3f} against drive frequency (b) and against current at fundamental frequency (I_{1f}) (c) when the quartz resonator is excited at 2.5V. Black plots correspond to experimental data while blue plots show fit obtained with Equation 13 and 14. (d) Sensitivity dependence of each acoustic parameter on the drive voltage (a-III).

3.1 Optimisation of drive amplitude

We applied frequency sweeps at a range of amplitude (0.5-15 V) before and after binding the microparticles. **Figure 1b** shows the representative characteristics of the 3f current (I_{3f}) against drive frequency. We recorded relative changes in the peak 3f current normalised by the respective 1f current ($-\Delta I'_{3fmax}/I'_{3fmax}$), changes in dissipation (ΔD), and changes in the resonance frequency (Δf_0) due to microparticle binding for the range of drive amplitudes applied (**Figure 1d**). Within the range of amplitudes explored, the largest changes in the peak normalised 3f current, dissipation and resonance frequency were found at amplitudes corresponding to 2.5 V, 0.5 V and 0.5 V respectively. Hence, these drive amplitudes were chosen for the frequency sweeps to measure the respective acoustic parameters. The 3f current was observed to decrease with binding, while the dissipation increased due to losses at the microparticle-QCR interface. The resonance frequency increased as elastic loading dominates over mass loading for microparticle binding to QCR⁷. Interestingly, the parametric plot of the 3f current against the 1f current, both being functions of f , showed a loop feature (**Figure 1c**, §1 Supporting Information).

Figure 2. Sample delivery and detection. (a) Microfluidic chip for controlled sample delivery to the quartz crystal resonator. The latter is housed between the microfluidic chip and a printed circuit board, providing a stable electrical connection with the network analyser. Magnitude of I_{3f} before (dashed) and after (solid) injection of IgE-coated microparticles, as a function of (b) frequency and (c) I_{1f} . Spectra correspond to scans taken every ten minutes, going from dark to light grey with increasing microparticle surface coverage on the QCR surface.

3.2 Baseline and binding measurements

Frequency sweeps of 0.5 V and 2.5 V were taken every 5 min under continuous flow over the 20 min baseline and 50 min association phase, where microparticles were continuously flowed over the resonator surface. **Figures 2b** and **2c** show the cumulative effect of binding of microparticles on I_{3f} - f and I_{3f} - I_{1f} graphs respectively. The time evolution of the relative decrease in the peak I_{3f} normalised by I_{1f} ($-\Delta I'_{3f\text{max}}/I'_{3f\text{max}}$), increase in dissipation (ΔD) and increase in resonance frequency (Δf_0) relative to the state at $t=0$ were analysed for baseline and microparticle flow stages (**Figure 3**, **Table S1**).

The stability of baseline is typically affected by the quality of surface functionalisation and changes in non-gravimetric parameters, such as temperature, viscosity and pressure. The challenge of establishing a stable baseline of a quartz crystal resonator before the binding data can be collected is well known. We did not install any temperature control in the microfluidic cartridge, but carried out careful washing before starting to record the baseline data. However, in some cases, such as the baseline before flowing the blank microparticles, we observed drifts for the resonance frequency (Δf_0) and dissipation (ΔD) shift graphs (**Figure 3b**, **3c**). Interestingly, such drifts were not observed for the $3f$ current shifts that were measured from the same assay simultaneously with the other two acoustic parameters (**Figure 3a**). It is plausible that loosely bound biomolecules were shaken off the surface during the $3f$ current measurement scans, which were at relatively higher amplitude than the other two acoustic parameters, giving better baseline stability. The baseline noise was also comparatively lower for $-\Delta I'_{3f\text{max}}/I'_{3f\text{max}}$ (**Table S1**).

As more number of microparticles were flowed continuously at a fixed concentration ($10^6/\text{mL}$), the binding curves followed a typical adsorption isotherm more closely for relative decrease in peak normalised I_{3f} than increases in dissipation or resonance frequency,

particularly for the top two concentrations as shown in **Figure 3a**. The change in signal in the first measurement taken 5 minutes after spiking of the IgE-functionalised microparticles was also observed more clearly (i.e. with greater signal-to-noise ratio) for the normalised $3f$ current ($-\Delta I'_{3fmax}/I'_{3fmax}$).

Figure 3. Relative response for I'_{3f} , f_0 and D . Time-dependent signal progression under continuous flow of running buffer (20 μl/min), showing baseline in the first twenty minutes followed by injection of microparticles (denoted by arrow). A low volume of concentrated microparticle solution in running buffer was spiked into the inlet reservoir and homogenized, resulting in a final concentration of 10^6 beads/mL. Exponential decay fit for the two highest concentrations of IgE (dashed lines) show expected response based on adsorption kinetics models. Data points represent averages calculated from replicate experiments (n=3).

3.3 Quantitative correlation

Microscopic images showed comparable coverage of the microparticles on the sensor surface for all IgE concentrations on them (**Supplementary Figure S1a and S1b**). Hence, the difference in the signals observed *at any given time* (after injection of microparticles) (**Figure 3**) could be attributed to the difference in the IgE concentrations on the microparticles. The relative decrease in the peak normalised $3f$ current at 50 min after the injection of microparticles was found to have a strong linear correlation ($R^2 = 0.997$) with the initial IgE concentrations used for their functionalisation, particularly for the highest four concentrations (3.2-0.16 μM solutions) (**Figure 4**). As explained earlier, as the same number of microparticles was used for all functionalisation reactions, we can therefore say that a linear correlation of the signal also exists with the number of IgE molecules on the microparticles. Interestingly, almost a direct linear proportionality with the IgE concentration was observed, as suggested by the low y-intercept value of the interpolation function. In comparison, the linear correlations between the increase in dissipation or the increase in resonance frequency and the IgE concentration were weaker. Hence, relative concentration of particle-bound proteins can be determined quantitatively using the relative decrease in the peak normalised $3f$ current. Absolute concentrations may be determined using a predetermined dose-response curve with a known protein concentration on the particle. The same particle concentration (number of particles/mL), flow rate and time of signal readout need to be used for all these comparisons, which constitute the protocol.

A dramatic increase in resonance frequency and dissipation was observed for the lowest IgE concentration (0.016 μM) and the blank (no IgE). We anticipate this was because non-specific interactions came into play and started dominating as the bare polystyrene surface got increasingly exposed at lower IgE concentrations. However, the $3f$ current was found to be negligibly affected by the growing non-specific interactions. This also means that the lowest concentration of microparticle-bound IgE that can be reliably readout is comparatively lower for $3f$ current measurement than the traditional acoustic parameters (resonance frequency and dissipation). It is possible that weaker electrostatic bonds of the non-specific interactions dissociated at the relatively higher amplitude used for the $3f$ current measurements.

Figure 4. Signal response for different IgE concentrations and the estimated nonlinear coefficient (β). Average and standard deviation ($n=3$) taken at the end of assay ($t=70\text{min}$) for all parameters. A strong linear correlation with the IgE concentration is observed for relative decrease in the 3f current (I'_{3fmax}). Linear interpolation for nonlinear coefficient (β) shows the extent of linear detection range (0.16 μM -3.2 μM). The lower detection range is limited for f_0 and D due to higher non-specific background at lower IgE concentrations.

3.4 Transduction specificity

For similar concentrations of IgE and IgG on the microparticles, corresponding to 1.6 μM of the respective protein solutions, the increases in resonance frequency and dissipation for non-specific binding of IgG-microparticles are found to be 2.9 and 9.3 times lower respectively than those for specific binding of IgE-microparticles, both measured at the end of the assay (**Figure 5a,b**). The relative 'attenuation' in the normalised peak 3f current for non-specific binding of IgG-microparticles is found to be even more remarkable (51.7) (**Figure 5c**) – more than one order of magnitude greater than similar attenuation in resonance frequency shift (2.9). This preferential transduction of specific interactions and decrease of non-specific background without any washing steps or assay optimisation is encouraging for realtime measurements, particularly as microscopic images show nearly similar levels of coverage for IgE- and IgG-microparticles, albeit with noticeable clustering for the latter (**Supplementary Figure S1a and S1c**). The results demonstrate an ability of the ADT transduction method, based on driving a quartz crystal resonator near one of its resonance modes (fundamental mode in this study) and measuring the current at three times the drive frequency (referred as the third Fourier harmonic current), to differentiate between specific and non-specific interactions. The third Fourier harmonic current should not be confused with the *third overtone mode resonance frequency*, which is commonly measured in quartz crystal microbalance by driving the QCR near the third overtone resonance.

Figure 5. Specificity comparison across all three measured parameters and the estimated nonlinear coefficient (β). Average and standard deviation (n=3) taken at the end of assay (t=70min) for all parameters. The relative decrease in β is found to be negative for non-specific binding i.e. IgG-antilgE and polystyrene-antilgE.

4. Discussion

The values of relative decrease in the nonlinearity coefficient ($-\Delta\beta/\beta$) (Table 1), which underpins the change in third harmonic current and is evaluated from Eq.17 using the experimentally observed relative changes in I'_{3fmax} and D , show a linear correlation with the IgE concentration (Figure 4). We noted earlier that as the concentration of IgE on the microparticles went down (corresponding to less than 0.16 μ M of initial IgE solution), dissipation (ΔD) reversed its trend and started going higher in value because of dominating non-specific interaction between the bare polystyrene and the resonator. We note the same trend for ΔD in Table 1. However, interestingly, we find $-\Delta\beta/\beta$ continues decreasing without any change in trend and changes in sign to grow in negative value for the lowest IgE concentration explored in this study and for blank microparticles (no IgE) (Table 1, Figure 4). As a consequence, $-\Delta I'_{3fmax}/I'_{3fmax}$ reduces to a small value for these cases governed by Eq.17. A negative value of $-\Delta\beta/\beta$ is also noted for non-specific IgG-QCR interactions (Table 1, Figure 5d) while ΔD is positive, which also reduces $-\Delta I'_{3fmax}/I'_{3fmax}$ to a small value according to Eq.17.

Table 1. Average values and standard deviations (n=3) taken at the end of assay (t=70min) for all three measured parameters and the estimated nonlinear coefficient (β).

	$-\Delta\beta/\beta$		$-\Delta I'_{3fmax}/I'_{3fmax}$		$\Delta D (\times 10^{-6})$		$\Delta f_0 (\text{Hz})$	
	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
<i>IgE (3.2uM)</i>	0.292	0.013	0.396	0.016	27.138	1.438	109.222	6.673
<i>IgE (1.6uM)</i>	0.116	0.010	0.188	0.014	18.725	1.190	91.935	4.554
<i>IgE (0.8uM)</i>	0.057	0.007	0.098	0.010	10.929	0.953	51.240	3.364
<i>IgE (0.16uM)</i>	0.010	0.003	0.033	0.003	6.057	0.192	45.339	0.968
<i>IgE (0.016uM)</i>	-0.013	0.001	0.041	0.002	13.990	0.560	94.126	4.489
<i>Blank</i>	-0.014	0.003	0.036	0.002	13.383	0.350	20.870	1.193
<i>IgG (1.6uM)</i>	-0.004	0.002	0.004	0.003	2.017	1.214	31.754	2.809
<i>IgE:IgG (1.6uM)</i>	-29.4		51.7		9.3		2.9	

These observations collectively lead to a novel finding that the nonlinearity coefficient (β) of the QCR decreases due to microparticle-resonator (ligand-receptor) interactions, the decrease being greater for stronger interaction and linearly correlating with the concentration of the ligand on the microparticle. The nonlinearity coefficient also depends on the type of interaction (specific or non-specific), i.e. the interaction strength, and the direction of its change is reversed (increases) when non-specific interactions start dominating. This characteristic dependence of the nonlinearity coefficient on the interaction strength underpins the quantitative correlation and transduction specificity of the $3f$ current measurement governed by Eq.17.

4.1 Comparative limits of resolution

The directionality in the change of β , which helps differentiating non-specific interactions, also allows resolving lower concentrations of microparticle-bound proteins compared to the widely used acoustic parameters of resonance frequency and dissipation. It may be noted that increased non-specific background from measurements of resonance frequency and dissipation shifts restricts their limits of resolving microparticle-bound IgE in this study to concentrations corresponding to 3.2 μM and 1.6 μM of initial IgE solution respectively. However, due to attenuated non-specific background analysed using I'_{3fmax} and β readings, the limit of resolution corresponds to 0.16 μM of initial IgE solution, which is one order of magnitude or more lower than the limit obtained with the traditional acoustic parameters - resonance frequency and dissipation. We did not measure the concentration of IgE on the beads directly. We can estimate 0.16 μM IgE solution corresponds to 0.16 attomole IgE per microparticle, which is equivalent to 9.6×10^4 IgE molecules with a molecular weight of 190 kDa, assuming full immobilization efficiency. This value lies within the physiological range (10^3 - 10^5 molecules) of common lymphocyte cell-surface antigens¹⁶. It is worth mentioning that the assay was not optimised here and that reference to IgE concentrations made throughout this document only serves as a term of comparison

between the three acoustic parameters. Moreover, no temperature regulation or flow optimisation of the microfluidic cartridge was carried out. So the abovementioned figures do not represent the absolute limit of resolution of particle-bound proteins (or particle-surface interactions), but reported here as a comparative limit of resolution of the three acoustic transduction parameters for the given set-up and protocol.

5. Conclusion

This study presents a label-free anharmonic acoustic transduction method, which reads out ligand-receptor binding in realtime based on the strength of interaction between bound microparticles and a quartz crystal resonator. The elastic loading due to binding of microparticles to the quartz resonator causes a nonlinear distortion of the sinusoidal oscillation, which modifies the nonlinearity coefficient of the resonator. Combining experiments with polystyrene microparticles, functionalised with human immunoglobulin-E, and theoretical modelling, we show that the change in the nonlinearity coefficient of the resonator is proportional to ligand concentration on the microparticles, which is a measure of the strength of microparticle-resonator interaction. We also show that the change in nonlinearity coefficient differentiates between specific and non-specific interactions by switching the sign in its magnitude. The change in the nonlinearity coefficient is evaluated by actuating the resonator close to its fundamental resonance mode and using the measured changes in third Fourier harmonic ($3f$) current and dissipation in an analytical equation (Eq.17) derived from the theoretical model. The $3f$ current is directly proportional to the nonlinearity coefficient (Eq.11, Eq.14). On the other hand, the current recorded at the drive frequency, which is near the fundamental resonance frequency and typically measured in a network analyser, does not depend on the nonlinearity coefficient (Eq.10, Eq.13).

The entirely-electronic and microfluidic-integrable transduction method would potentially allow a scalable, multiplexable, easy-to-use and fast way for characterising particle-surface interactions. Applications could include the relative quantification of microparticle-bound ligands or of their affinity of interaction with receptors functionalised on a quartz crystal resonator. Microparticles, such as colloidal particles or bacteria, which bind flexibly with the resonator through a dominant elastic coupling, would be suitable for this method. The intrinsic ability in the transduction method to differentiate specific and non-specific interactions beyond the efficacy of the receptor allows greater reliability of these characterisations.

6. Materials and methods

Reagents. All buffer reagents and biomolecules were procured from Sigma-Aldrich (UK) and prepared in ultrapure water (Milli-Q Integral system, Millipore, US), unless specified. Phosphate buffered saline solution (PBS) contained: 8.1 mM Na_2HPO_4 , 1.1 mM KH_2PO_4 , 1 mM MgCl_2 , 2.7 mM KCl, and 138 mM NaCl, pH7.4. Human immunoglobulin-E (ab65866) and goat immunoglobulin-G were purchased from Abcam (Cambridge, UK). The 37-mer IgE-binding aptamer (D17.4), with the sequence 5'-GGGGCACGTTTATCCGTCCTCCTAGTGGCGTGCCCC-3', was extended with 24 thymidine bases and biotinylated as at the 3' end as previously described¹⁷. Alkanethiol solutions, HS-(CH₂)₁₁-EG₆-Biotin and HS-(CH₂)₁₁-EG₃-OCH₃, were procured from Prochimia (Poland).

Instrumentation. A dedicated electronic instrument, described as nonlinear network analyser, was designed and built completely in-house and used for our experiments. The instrument is unique in terms of its capability of driving with a wide range of amplitude (0 up to 40 V) and frequency (RF 0.1 to 300 MHz) and recording the complex (in-phase and quadrature) current and voltage sensitively (noise $\sim 1\mu\text{V}/\text{Hz}^{1/2}$) and synchronously at 3 harmonics. We are unaware of any other nonlinear network analyser with this capability.

The quartz crystal oscillator piezo-material acts as an actuator and a microphone at the same time. The driving and pickup electrodes are the same, i.e. only two electrodes used, which is the same crystal design conventionally used as a Quartz Crystal Microbalance (QCM). The microvolt level odd harmonics signals are separated from powerful driving signal applied near fundamental resonance frequency by appropriate highly linear passive filtering network.

Microscope images of QCR under experimental conditions were obtained with a Nikon SMZ18 stereomicroscope using ambient light.

Functionalisation of microparticles. Superparamagnetic microparticles (Carboxylic Acid, M270, 2.8 μm diameter), acquired from ThermoFisher (UK), were repeatedly washed in 10mM 4-morpholinoethanesulfonic acid (MES) buffer (pH5.0) followed by surface activation with a 1:1 solution of 0.4M ethyl(dimethylaminopropyl) carbodiimide (EDC) and 0.1M n-hydroxysulfosuccinimide (NHS) for 20min. After being washed, microparticles (0.52 mg) were re-suspended in respective protein (IgE or IgG) solutions, at final concentrations of 40, 20, 10, 2, 0.2 and 0.02 μg per mg of microparticles (MPs) and incubated for one hour. Unreacted esters were quenched with 50mM ethanolamine-HCl (pH8.0). Particles were re-suspended in PBS supplemented with 0.1% bovine serum albumin (BSA) and 0.05% Tween-20 and final concentration measured with a Countess Automated Cell Counter (ThermoFisher, UK).

QCR aptamer assay. AT-cut quartz crystal resonators, with 14.24 MHz fundamental resonance frequency, were custom-made for this work from Laptech Precision Inc, Canada. The quartz crystal was cleaned in acetone and isopropanol sonication baths, followed by argon plasma treatment at 250W for 60s (Harrick Plasma, US). The cleaned crystal was left immersed for 24 h in 1mM ethanoic solution of 1:10 biotin to methoxy-terminated alkanethiols. The crystal was then placed in a fluidic cell and cleaned by flushing 200-proof ethanol followed by copious amounts of water. Streptavidin, diluted in PBS to 16 $\mu\text{g}/\text{mL}$, was injected until surface saturation was reached. DNA aptamers diluted (1 μM) in PBS (138mM NaCl, 5mM MgCl_2) and heated to 95°C for 5min before being added to the gold resonator surface. After several washes, PBS was flowed at 20 $\mu\text{L}/\text{min}$ for 30min until baseline was stable. Concentrated MP-PBS solution was added to the buffer and mixed, achieving a final concentration of 10^6 MPs/mL. No magnetic field was used to control the trajectory of the microparticles.

Fabrication of microfluidic chips. The quartz crystal resonators were integrated with ADT cartridges, consisting of a top polymer fluidic part, an UPS class VI EPDM O-ring (Trelleborg, Sweden), a middle polymer spacer part and a FR4/Cu/Au printed circuit board (PCB) (**Figure 2a**). The polymer parts were fabricated by precision milling of polycarbonate (PC) substrates using a Protomat S62 printed circuit board plotter (LPKF, Germany). The optically transparent top PC part with fluidic ports on the side allowed for microscopy of the resonator surface during experiments. The PCB was patterned using precision milling to form electrical contact pads for the resonator, electrical lines and contact points towards the sides of the PCB for external contacting to the network analyzer. Vertical pins fixated in the PCB ensured accurate alignment of the symmetrically designed cartridge parts upon assembly. The cartridge was assembled by clamping and the applied pressure on the crystal and the o-ring was precisely set and remained consistent by the use of the spacer, which ensured limited dampening and reproducible performance of the resonator as well as reliable liquid sealing of the flowcell. The flowcell was designed with a height of 240 μm to optimally reduce the effect of interference between the resonator and compressional acoustic waves in liquid that are generated out-of-plane by the shear wave oscillation of the crystal. The cartridge was connected via tubing, which was inserted into the fluidic inlet and outlet ports of the top polymer part, to a syringe pump procured from Harvard Apparatus (UK). When the flow cell was filled with aqueous liquid, the resonator featured a quality factor of 1900.

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Author Contributions

CSG carried out and analysed all the experiments in this work and drafted the paper; NS designed and fabricated the microfluidic flowcell and the printed circuit board under the supervision of WW; IE contributed to the analytical modelling; VPO designed and developed the nonlinear network analyser supported by DK and contributed to the development of the underlying Physics of the method along with SKG, who also defined and led the project.

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