Direct and Quantitative Detection of Bacteriophage by "Hearing" Surface Detachment Using a Quartz Crystal Microbalance

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Direct and Quantitative Detection of Bacteriophage by "Hearing" Surface Detachment Using a Quartz Crystal Microbalance

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We show that it is possible to detect specifically adsorbed bacteriophage directly by breaking the interactions between proteins displayed on the phage coat and ligands immobilized on the surface of a quartz crystal microbalance (QCM). This is achieved through increasing the amplitude of oscillation of the QCM surface and sensitively detecting the acoustic emission produced when the bacteriophage detaches from the surface. There is no interference from nonspecifically adsorbed phage. The detection is quantitative over at least 5 orders of magnitude and is sensitive enough to detect as few as 20 phage. The method has potential as a sensitive and low-cost method for virus detection.

This paper describes a new method to detect the presence of submicrometer-sized particles attached to a surface by specific interactions. The method is based on oscillating the surface of a quartz crystal microbalance (QCM), on which a particle is attached, with increasing amplitude.[1] The surface undergoes transverse shear motion resulting from the converse piezoelectric effect[2] on application of an alternating voltage to the electrodes on the top and bottom of the QCM. As the amplitude of the applied voltage is increased, so the amplitude of oscillation of the QCM surface and the attached particle increases. In this way, the amplitude of the force exerted on the particle increases monotonically (this force is parallel to the QCM surface). At a particular amplitude, all of the interactions between the particle and surface are broken and the particle detaches. At this time, acoustic emission occurs, which enables detection of the presence of the particle. The same QCM acts as a sensitive microphone to convert the emitted acoustic power generated on detachment into an electrical signal of proportional power and so detects the rupture event. This approach contrasts with most QCM experiments in which mass changes on the surface of the QCM are detected by changes in the resonance frequency or phase when the QCM is driven at constant voltage.

Our working model of the underlying physics in this experiment is as follows: In air, the force exerted on a rigid particle attached to the surface is simply given by the product of the particle mass multiplied by the acceleration of the surface, neglecting any frictional forces. The particle detaches from the surface when the force exerted on it is large enough to break all of the interactions attaching it. At this time, the excess energy above that required to overcome the interactions is released. In particular, there are some mechanical stresses in the QCM and gold film caused by the inertial forces required to accelerate the particle. Elastic energy associated with those mechanical stresses will be released after particle detachment, resulting in the generation of an acoustic wave. Because this energy release is rapid, it excites other QCM normal modes, including excitation of the QCM at its third harmonic, and also higher odd harmonics. The QCM motion at its third harmonic is converted into an electrical signal at the same frequency that can be sensitively detected. In liquids, the process is similar. The differences are that in the liquid, a significant viscous drag is exerted on the moving particle, and depending on the particle size and rigidity, the entire particle may not be subjected to the acceleration at the surface.

In previous work[1] the detachment of 5-μm latex particles attached to the QCM surface by multiple nonspecific interactions, by streptavidin−biotin interactions or by covalent bonds was reported. The surface acceleration at which the detachment was detected depended on the mass of the particle and on the strength and number of interactions attaching the particle to the surface. The measurements were made in air or water. In this paper, this method is applied to the specific detection of bacteriophage to explore whether the method can be applied to detect these nanometer-sized particles and to determine the limits of sensitivity. The results are then compared to similar experiments using a conventional QCM.

EXPERIMENTAL METHOD

A schematic representation of the experiment is shown in Figure 1. The QCMs were made from polished quartz plates, AT cut at an angle of 35° (HyQ, Cambridge, U.K.). They were 8.25 mm in diameter with 14.3 MHz fundamental mode. A layer of chromium (200−300 Å) and then gold (1000−1200 Å) was deposited onto the surface. This procedure resulted in a QCM

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(1) Dultsev, F. N.; Ostanin, V. P.; Klenerman, D. *Langmuir* **2000**, *16*, 5036−5040.
(2) Ward, M. D.; Buttry, D. A. *Science* **1990**, *249*, 1000−1007.

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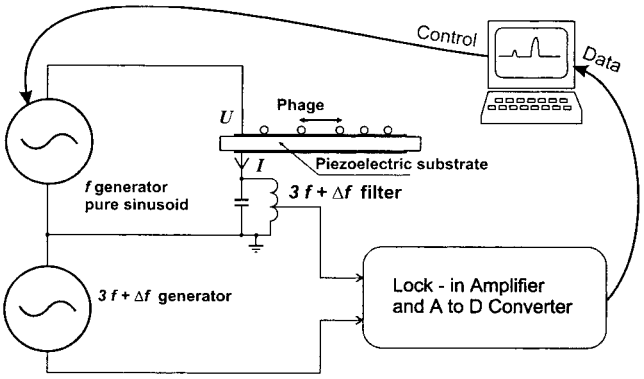

Figure 1. Schematic representation of the experiment.

surface flat enough to image the phage using an AFM. The first suitable mode for detection of acoustic emission is located near the QCM third harmonic. A signal generator, model DS345 (Stanford Research Systems) was used to drive the QCM at its fundamental. The main measuring device is a lock-in amplifier, SR 844 (Stanford Research Systems) operating near the third harmonic (third resonant mode near 42.96 MHz). The QCM operating at the third resonant mode sensitively detected the acoustic emission due to particle detachment. This electrical signal was fed into a lock-in amplifier. A second synthesizer was used to generate the reference signal for this lock-in. All devices were interfaced to a computer for control of the experiment and collection of the data. In all of the graphs, the sum of the squares of the *X* and *Y* outputs from the lock-in amplifier is used. This is proportional to the electrical power of the signal at the lock-in amplifier input.

A maltose-binding protein fusion to the amino terminus of indole glycerol phosphate synthase was displayed on the surface of an fd phage as a fusion to the amino terminus of the gene III encoded coat protein. For this purpose, an fd phage vector, pJB113, was constructed and encoded a genetic fusion between *MalE* (*Escherichia coli*), *trpC* (*E. coli*), and gene III. This phage vector carried a tetracycline resistance marker. The unmodified competitor phage was VCS M13 K07 helper phage (Stratagene) carrying a kanamycin resistance marker. Bacteriophage concentrations of 1×10^{12} cfu/mL were obtained by infecting a 3-mL mid-log-phase LB culture of *E. coli* strain TG1 with 10 μL of phage stock. After 2 h of shaking (250 rpm) at 37 °C, 1 mL of culture was inoculated into 100 mL of LB and shaken at 250 rpm, 37 °C, for 1 h. Tetracycline (10 μg/mL) or kanamycin (50 μg/mL) was added to the pJB113 or VCS cultures, respectively, and grown overnight at 30 °C, 250 rpm. Bacteria were pelleted (15 min, 4.1 krpm), and the phage were precipitated from the supernatant by addition of NaCl and PEG6000 to final concentrations of 0.5 M and 4% (v/v), respectively. After standing for 1 h on ice, the phage were recovered by centrifugation (30 min, 4.1 krpm), and the phage pellet was resuspended in 1 mL of H_2O and stored at 4 °C.

Soluble starch (500 mg, 0.01 mmol, 1 equiv) was dissolved in DMF (10 mL) and stirred for 5 min (partially soluble). To 11-mercaptododecanoic acid (11.5 mg, 0.05 mmol, 5 equiv) in DMF (0.5 mL) was added DIC (7.8 μL, 6.3 mg, 0.05 mmol, 5 equiv) and DMAP (cat.), and this solution was added to the starch solution. The reaction was left stirring overnight at room temperature. The product was then purified using a stirred cell with a 10 000 MW membrane cutoff by rinsing the solution with MilliQ water (6 times 50 mL) and concentrating, followed by lyophilization overnight. The surface was prepared using this modified starch containing a thiol group so that it could be chemically coupled to the surface using gold−thiol chemistry. The QCM was placed in a solution of the starch in methanol (1 μg/ml) for 12 h. The samples were then washed and dried under a stream of nitrogen. The phage were deposited onto the surface from solution and dried at room temperature for experiments in air. Different phage concentrations were made by dilution. To perform the experiments with the maltose blocking the maltose-binding protein, 100 mM maltose was added to the solution of phage. None of the QCMs were reused.

RESULTS AND DISCUSSION

The interaction between genetically modified M13 bacteriophage displaying a maltose-binding protein fused to the phage pIII coat protein and maltose was studied. The M13 phage is a long, thin, filamentous virus consisting of a flexible rod 900 nm long and 6 nm in diameter.[3] The genetically modified phage additionally display up to five maltose-binding proteins at one end of the virus as fusions to the phage pIII coat protein[4] and can be purified on amylose resin.[5]

The surface of the QCM was coated with a layer of soluble potato starch, which contains branched polymers of maltose. The starch was chemically modified with a mercaptodecanoic acid linker and then chemically attached to the gold surface via a sulfur−gold bond. Experiments were performed in both air and water. In the first experiment, an equal mixture of maltose-binding phage and unmodified phage (approximately 5×10^8 of each) were initially applied to the QCM surface, and a scan was acquired over 1000 s. The same scan rate of 10 mV/s was used in all experiments, because the loading rate can affect the force needed to break an interaction.[6] Figure 2a shows a plot of detected electrical power, which is proportional to the emitted acoustic power, versus driving voltage. For the unmodified, nonspecifically bound phage, an emission peak was detected at 1.2 V. The corresponding peaks for the maltose-binding phage were around 9 V. The greater magnitude of the peaks for the specifically adsorbed phage is due to their stronger interaction with the surface. Consequently, more acoustic energy is released per unit time when the phage detach from the surface. After detachment, the phage have no opportunity to reform the bonds to the surface, since no peaks were detected in a second scan (data not shown). The detached phage probably move into the solution above the QCM by Brownian motion and convection.

As shown in Figure 2b, when the experiment was repeated in air, an emission peak occurred at 7.5 V for the nonspecifically bound phage, and no peaks were observed from the specifically bound maltose-binding phage up to 10 V, the voltage limit in our experiment at present. A repeat scan of the same sample showed almost no peaks, which is consistent with the peaks' being due

(3) Berkowitz, S. A.; Day, L. A. *J. Mol. Biol.* **1976**, *102*, 531−547.
(4) McCafferty, J.; Griffiths, A. D.; Winter, G.; Chiswell, D. J. *Nature* **1990**, *348*, 552−554.
(5) JMB & RES, unpublished data.
(6) Merkel, R.; Nassoy, P.; Leung, A.; Ritchie, K.; Evans, E. *Nature* **1999**, *397*, 50−53.

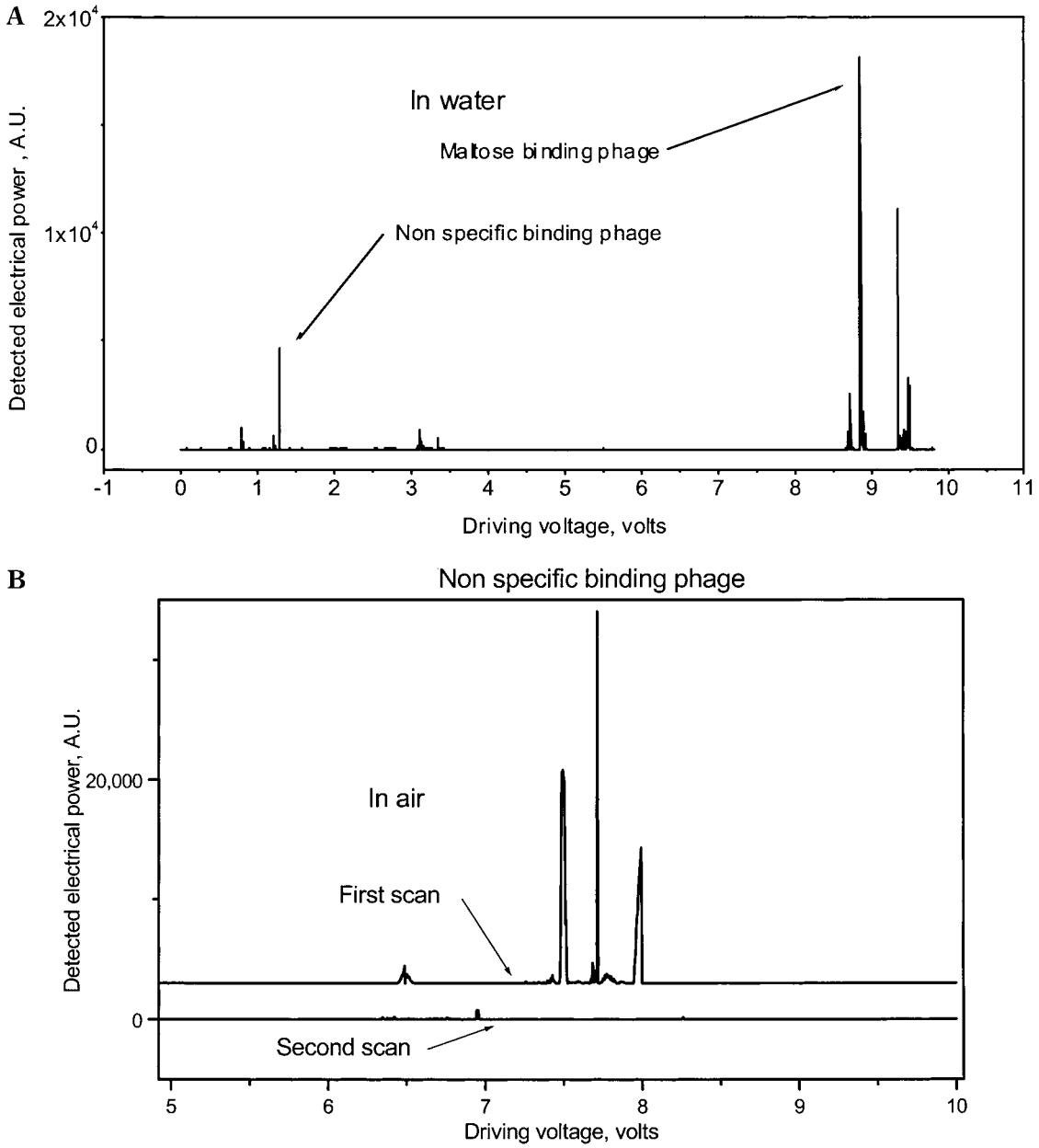

Figure 2. (a) Plot of detected electrical power versus driving voltage, in water, for ~5×10^8 nonspecific adsorbing phage and 5×10^8 maltose-binding phage on the starch-coated QCM (the starch was chemically bonded to the surface). The electrical power, produced as a result of acoustic emission when all of the interactions attaching the phage to the surface are broken, was measured as the driving voltage, and hence, force on the phage was linearly increased. The scan took 1000 s. There are clear emission peaks for both phage at well-separated voltages. (b) Plot of the detected electrical power versus voltage for 1×10^9 nonspecific binding phage on the starch-coated QCM in air. A second scan after the first scan indicated almost all of the phage have been removed from the surface of the QCM, since no emission peaks were detected.

to the detachment of the phage particles from the surface. A comparison of the results in air and water suggests that in water, the additional viscous frictional forces and also the increase in effective mass of the particle make it is easier to break the interactions between the phage and the surface. We observed a similar increase in the voltage needed for detachment in going from water to air in our previous studies of latex spheres.[1] In a further control experiment, the maltose-binding phage were preincubated with maltose. This blocks the maltose binding sites at one end of the phage. These phage were then deposited on the QCM and scanned in water. The scan showed only a weak peak at 1.2 V, which is consistent with nonspecific binding (data not shown). This experiment confirmed that the emission peaks observed in water at ~9 V were due to specific adsorption of the maltose-binding phage to the surface.

The emission peaks in Figure 2 have a narrow line width. The full width at half-maximum is 5−40 mV. This suggests the majority of the interactions between the phage and the surface are broken simultaneously, due to positive feedback, when the driving voltage and, hence, force applied to the phage reaches a critical value. This results in the acoustic emission occurring in a short time period and, hence, makes the method very sensitive. In addition, the peak from the specifically adsorbed phages is well-separated from the nonspecific binding phage. This implies that nonspecific adsorption does not affect the measurement of specific adsorption.

To estimate the force that is necessary to detach the specifically adsorbed phage in water, we used the following scaling argument. In water, the peaks for the specifically adsorbed phage are at 9 V, and the peak for the nonspecifically adsorbed phage occurs at 1.2 V. The detachment force is proportional to voltage at which acoustic emission is detected. Thus, the force required to detach the specifically adsorbed phage is ~7.5 times greater than for the nonspecifically adsorbed phage. Solving the equations of motion for the phage in water is complex, so we first calculated the force required for detachment of the nonspecifically adsorbed phage in air. This is estimated to be ~20 pN,[7] based on the estimated amplitude of oscillation at detachment of 90 nm[1] and the mass of the phage.[3] If the force attaching the nonspecifically adsorbed phage to the surface is not significantly altered by the presence of water, then the force required to detach the nonspecifically adsorbed phage in water will be approximately the same as in air. Making this assumption, then the force required to detach the specifically adsorbed phage in water is estimated to be ~150 pN. This corresponds to ~30 pN per protein, assuming five maltose-binding proteins per phage. This force has not been reported previously but appears reasonable in comparison with the rupture force for the streptavidin−biotin interaction, 160 pN,[8] and a typical antigen−antibody interaction of ~50 pN.[9]

To determine the sensitivity of the detection method, a dilution experiment was performed with different numbers of phage present on the surface. The number of phage was confirmed by direct imaging of a dilute sample using AFM (data not shown). The measurements were performed in a random order on separately prepared QCMs. In the first set of experiments, the detachment of maltose-binding phage were measured in water. The integrals of the intensities of the emission peaks are plotted versus the number of maltose-binding phage on the surface in Figure 3a. This shows that the intensity is proportional to the number of phage over at least 5 orders of magnitude. The data have been fitted by the formula $a + (x/b)^c$ where a, b and c are constants and x is the number of phage on the surface. This formula adds together the noise power, which is constant, and the signal power, which is assumed to depend on the number of phage, raised to the power of c to give the total detected signal. In this formula, a represents the noise floor, b is a normalization factor and $(x/b)^c$ is the signal produced by the detachment of x phage from the surface. This simple formula fits the data quite well. It was found that c was 0.5 in water and 0.7 in air.

The fitted curve has been used to estimate the sensitivity of the method. Using some knowledge of the experimental peak shape, it is possible to construct a linear optimal numerical filter to recover the peak from the surrounding noise.[10] In this work, a simplified filter was used that was not fully optimal.[11] The width of the smoothing was chosen to be approximately equal to the experimental width of the peak. In our case, we chose 20 points (out of a total of 2000). After one looks at the smoothed signal curve, a decision can be made if a signal peak is present or absent. This can be done with some probability, say 99%. Assuming that the expected position of the peak is known with some uncertainty, then the signal level at the region of the expected peak center is compared with the noise floor power, Σ, of the baseline region nearby. This comparison allows us to estimate probability of detection. We used Monte Carlo methods to simulate the random process of detection (using MathCAD 2000 software) and concluded that if the height of the peak is above 1.7*Σ, then the peak can be considered present with a probability >99%.[12]

Using the above method ,we estimate that ~800 phages on the QCM can be detected in water with 99% probability, and in air, 20 phage could be detected with 99% probability. If the QCM

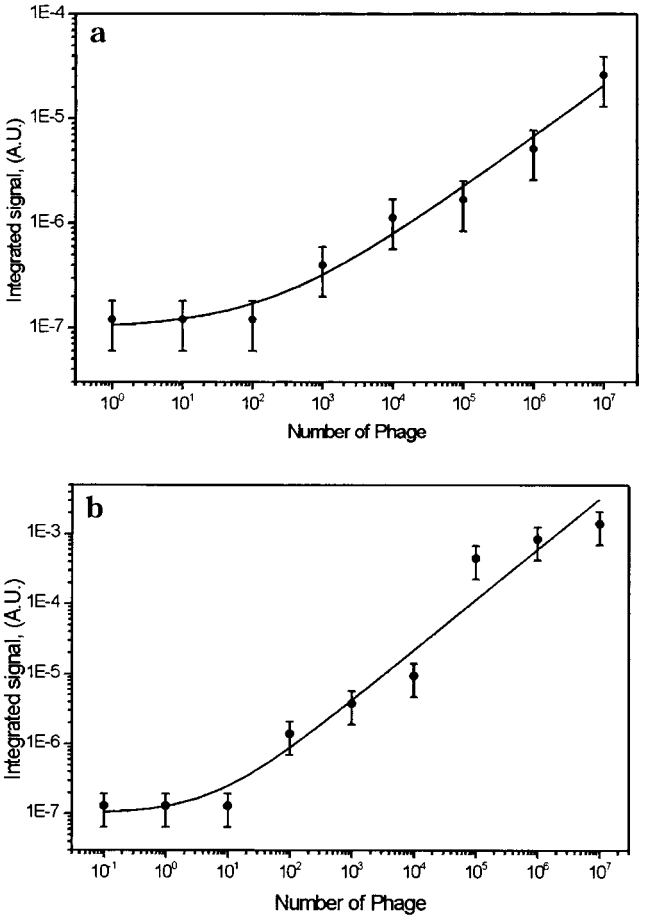

Figure 3. Integrated emission peaks versus the number of phage on the surface for (a) the maltose-binding phage in water, and b) the nonspecifically binding phage in air.

(7) The acceleration amplitude, a is estimated using the formula

$$a = \sqrt{8\pi f \cdot \frac{QP}{M}}$$

where P is electric power dissipated in the crystal, Q is the merit factor of the quartz, M is the effective mass of the crystal involved in motion (estimated as 10% of the full crystal mass), and f is the working resonance frequency.

(8) Florin, E.-L.; Moy, V. T.; Gaub, H. E. *Science* **1994**, *264*, 415−417.

(9) Fritz, J.; Anselmetti, D.; Jarchow, J.; Busquets, X. F. *J. Struct. Biol.* **1997**, *119*, 165−171.

(10) *Detection of Signals in Noise*; McDonough, R. N., Whalen, A. D., Eds.; Harcourt Publishers Ltd., Academic Press, Inc.: San Diego, 1995.

(11) We used a Gaussian kernel running average filter function **ksmooth-(vx,vy,b)** from the MathCad library.

(12) To evaluate performance of this filter, we simulated multiple instances of a real signal, then added a white noise signal to match the formula $a + (x/b)^c$. We assumed a 20% variation in peak position. The threshold value for phage detection was altered, and the number of times a phage was detected counted. The probability of phage detection at a particular threshold was determined in this way.

were used more conventionally, the frequency shift due to 100 phage particles would be only 2×10^{-5} Hz, or ~50 000 times below the smallest detectable frequency shift (1 Hz under ideal conditions in liquid).

In a conventional QCM, the reduction in resonance frequency on binding of a particle to the surface of a QCM is used to determine the number of particles present using the Sauerbrey equation.[13] Although this works well in air, it is more difficult in liquids because of problems in liquid loading. In general, the behavior is more complex, and full impedance analysis is needed to understand the changes on particle binding. These are often associated with changes in interfacial properties rather than mass changes.[14] The QCM has been used to study the herpes simplex virus;[15] however, in this case, the virus was dried on the surface, and the measurements made in air. A linear dependence of frequency shift with number of viruses was observed from 50 000 viruses up to 10^9. The QCM has also been used to screen phage libraries in solution.[16] In this case, the sensitivity limit was 17 Hz. Thus, the method presented in this paper appears significantly more sensitive than a conventional QCM for direct virus detection. Other methods to detect viruses using the QCM are based on detecting antibodies to the virus rather than detecting the virus directly.[17,18]

In contrast to a conventional QCM, the experiments described here measure the amount of acoustic emission generated on detachment of the phage. If there is no detachment, there is no emission. This has been demonstrated by the observation that the scan of a QCM with no particles shows no peaks and that a second scan, after particle detachment, also shows no peaks. The fact that it is not necessary to detect small changes on a large background contributes to the high sensitivity of the method. Like a conventional QCM, particle binding may alter the interfacial properties at the surface of the QCM and, consequently, alter the force exerted on the particles due to oscillation of the QCM. This will affect the voltage at which detachment is detected; however, the amount of acoustic energy emitted on detachment would be expected to depend on only the number of particles on the surface and the strength of the interaction attaching the particle to the surface, as has been found in this work on phages. Detachment of each particle will result in an acoustic emission, and the larger the number of particles, the greater the emission. If the interaction attaching the particle to the surface is stronger, then a larger acceleration will be required for detachment, and hence, there will be more excess energy released, giving rise to a greater acoustic emission. Because the distance between the QCM and the particle is very much less than a quarter of the acoustic wavelength, the coupling efficiency of the emitted acoustic energy to the QCM is not altered by changes at the QCM interface. Thus, it is anticipated that the method will be far less sensitive to changes in interfacial properties than conventional QCMs; however, this remains to be tested experimentally by performing experiments in different solutions.

The experiments described in this paper demonstrate the quantitative detection of phage particles in a novel, sensitive, and quantitative way. The approach has specific advantages over conventional QCM experiments and with further improvements, potentially has the sensitivity to compete with PCR[19] as a novel method for virus detection.

(13) Sauerbrey, G. Z. Z. *Phys.* **1959**, *155*, 206.

(14) Cavic, B. A.; Hayward, G. L.; Thompson, M. *Analyst* **1999**, *124*, 1405−1420.

(15) Konig, B.; Gratzel, M. *Anal. Chem.* **1994**, *66*, 341−344.

(16) Hengerer, A.; Kosslinger, C.; Decker, J.; Hauck, S.; Queitsch, I.; Wolf, H.; Dubel, S. *Biosensors* **1999**, *14*, 139−144.

(17) Abad, J. M.; Pariente, F.; Hernandez, L.; Lorenzo, E. *Anal. Chim. Acta* **1998**, *386*, 183−189.

(18) Sato, T.; Serizawa, T.; Okahat, Y. *Biochim. Acta* **1996**, *1285*, 14−20.

(19) In these experiments the number of phage on the QCM surface is detected, but when using PCR-based detection, the number of copies of viral DNA is detected in solution. If it is assumed that all of the virus particles in the solution sample will bind to the surface, which will occur if there is either a strong virus−surface interaction or the solution is allowed to evaporate, leaving the viruses on the surface, then direct comparison of sensitivity is possible. The sensitivity of the PCR-based method is ~100 copies of viral DNA/mL, which is ~1 order of magnitude higher than the detection sensitivity for the phage in water in these experiments.

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