ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

The Effect of Salt and Phage Concentrations on the Binding Sensitivity of Magnetoelastic Biosensors for *Bacillus anthracis* Detection

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ABSTRACT: This article presents an investigation of the effect of salt and phage concentrations on the binding affinity of magnetoelastic (ME) biosensors. The sensors were fabricated by immobilizing filamentous phage on the ME platform surface for the detection of *Bacillus anthracis* spores. In response to the binding of spores to the phage on the ME biosensor, a corresponding decrease occurs in resonance frequency. Transmission electron microscopy (TEM) was used to verify the structure of phage under different combinations of salt/phage concentration. The chemistry of the phage solution alters phage bundling characteristics and, hence, influences both the sensitivity and detection limit of the ME biosensors. The frequency responses of the sensors were measured to determine the effects of salt concentration on the sensors' performance. Scanning electron microscopy (SEM) was used to confirm and quantify the binding of spores to the sensor surface. This showed that 420 mM salt at a phage concentration of 1×10^{11} vir/mL results in an optimal distribution of immobilized phages on the sensor surface, consequently promoting better binding of spores to the biosensor's surface. Additionally, the sensors immobilized with phage under this condition were exposed to *B. anthracis* spores in different concentrations ranging from 5×10^{1} to 5×10^{8} cfu/mL in a flowing system. The results showed that the sensitivity of this ME biosensor was 202 Hz/decade.

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KEYWORDS: magnetoelastic; biosensor; salt; phage; *B. anthracis*; binding sensitivity

Introduction

Bacillus anthracis (the cause of anthrax) has become a primary pathogenic species of interest due it use as a weapon in acts of bioterrorism (Inglesby et al., 1999) and possible use as a deliberate contaminate of the food supply (Edwards et al., 2006; Schmid and Kaufman, 2002). Unfortunately, the identification of *B. anthracis* is still a challenge because it is easily confused with other *Bacillus* species found in the environment (*Bacillus cereus*, *Bacillus thuringiensis*, etc.) (Harrell et al., 1995; Steichen et al., 2003). Most currently available techniques for detection require spore germination and outgrowth (Bell et al., 2002; Swartz, 2001) which are time-consuming, expensive, and lack sensitivity, especially for early detection. Clearly, the development of a direct, portable, rapid, specific, and sensitive detector for real-time detection is now an urgent need.

Recently, filamentous phage fd has been shown to be a viable alternative to antibodies as the biorecognition element of a biosensor for bacteria and spore detection (Petrenko and Vodyanoy, 2003; Smith and Petrenko, 1997; Turnbough, 2003). The combination of phage with an acoustic wave sensor platform has been demonstrated to be a simple and specific way for detecting *B. anthracis* spores (Wan et al., 2007). A magnetoelastic (ME) biosensor comprised of a ME particle as the sensing platform with phage fd immobilized on its surface for the detection of *Bacillus anthracis* was investigated in this research as another form of an acoustic wave biosensor.

The ME particle has a mechanical resonance frequency based on its physical characteristics, and can be made to vibrate at this frequency by applying an oscillating magnetic field. As spores bind to the phage-immobilized surface of the sensor, the additional spore mass results in a decrease in the magnetoelastic sensor's resonance frequency. This resonance frequency can be measured remotely and wirelessly.

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The ME platform has been successfully demonstrated for use in both chemical (Cai et al., 2000; Cai and Grimes, 2001) and biological sensing (Guntupalli et al., 2007; Huang et al., 2007; Ong et al., 2006).

Phages, which serve here as a bioprobe, are viruses that are genetically engineered and propagated in bacteria host cells. Filamentous phage is approximately 6 nm in diameter by 1 μm long, and has approximately 2,700 copies of coat proteins that encode the surface providing a multiplicity of binding sites for the target of interest (Petrenko et al., 1996). The phage used in this work was developed by V.A. Petrenko and his group and is described in Petrenko and Sorokulova (2004) and Petrenko and Vodyanoy (2003).

Work from our group (Lakshmanan et al., 2007; Wan et al., 2007) has proven the capability of ME biosensors in the detection of *S. typhimurium* bacteria and *B. anthracis* spores. However, during these experiments, we found that simply using a higher phage concentration does not necessarily lead to a higher binding sensitivity of the sensor. This is because the overall performance of the ME biosensor directly depends on the phage-antigen interactions. Phage is a rodlike polyelectrolyte with four net negative charges for each protein coat of a single phage (Wen and Tang, 2006). Various experiments have shown that phages will self-assemble to form bundles due to electrostatic interactions (Angelini et al., 2003; Nguyen et al., 2000). This bundling leads to a reduction in exposed protein binding sites and reduces the overall binding affinity of the biosensor surface. So, it is critical to understand the experimental factors that control the process of immobilization, the concentration of salt in the phage solution being one of these factors. The objective of this article is to present a study of the effect of salt concentration on the distribution of immobilized phage on ME sensor platforms for the detection of *Bacillus anthracis* Sterne strain spores. Also, towards a larger goal, this work will add to the general knowledge of substrate–ligand interactions in the area of phage display technology.

Materials and Methods

Principle of Operation

ME materials are ferromagnetic and amorphous (e.g., metallic glasses). When exposed to an external magnetic field, these materials undergo a change in dimensions. For a thin, ribbon-shaped platform of length L, a time-varying (AC) external magnetic field can be used to resonate this ME material. The ME platform will vibrate mainly along the longitudinal direction, its fundamental resonant frequency given by Equation (1) (Liang et al., 2007):

$$f = \sqrt{\frac{E}{\rho(1-\upsilon)}}\frac{1}{2L} \tag{1}$$

where L, E, ρ, and υ are the length of the sensor, Young's modulus of elasticity, density, and the Poisson's ratio of the sensor material, respectively. Liang recently demonstrated that plane-stress conditions dominate for thin, slender ribbons resonating mainly in the longitudinal direction, thus the term $E/(1-\upsilon)$ in Equation (1). The resonance frequency is dependent on the material properties as well as geometry of the sensor. For small, uniformly distributed changes in the non-magnetoelastic mass attached to the sensor surface, a shift in the resonance frequency will occur that is described by Eq. (2) (Stoyanov and Grimes, 2000):

$$\Delta f = -\frac{f}{2}\frac{\Delta m}{M} \tag{2}$$

where M is the initial mass of the ME material, Δm ($<<M$) the non-magnetoelastic mass attached on the ME material, f the fundamental resonant frequency, and Δf the shift in the resonant frequency of the sensor.

In our research, a DC biasing magnetic field provided by a bar magnet was used to amplify the resonance signal. The distance between the magnet and the coil was adjusted to maximize the amplitude of the resonance signal. A time-varying (AC) magnetic field, supplied by the network analyzer was used to resonate the sensors. Before spore loading, the frequency was recorded as f_0. When spores are captured by the specific phage immobilized onto the sensor's surface, there is an increase in mass of the ME biosensor, which in turn causes a corresponding decrease in the sensor's resonance frequency (f_{mass}). These measurements are performed remotely and wirelessly. Figure 1 illustrates the wireless nature of the individual sensor and the basic principle for detecting bound mass (spores).

Magnetoelastic Platform

Commercially available ME strips (2826MB Metglas™ film, $Fe_{40}Ni_{38}Mo_4B_{18}$, Honeywell) were polished and diced to final dimensions of $2.0 \times 0.4 \times 0.015$ mM. These platforms were cleaned with acetone to remove the adhesive used in the dicing process, and then annealed in a vacuum oven at 200°C for 2 h to remove residual stresses (Huang et al., 2007). Thin films of chromium followed by gold, were deposited onto both sides of the ME platforms using a Denton™ Vacuum Discovery-18 sputtering system (Moorestown, NJ). The gold layer is necessary for bioactivity, as well as corrosion resistance, while chromium serves as an interlayer between the gold and the Metglas™ material for improved adhesion.

B. Anthracis Spores

B. anthracis Sterne strain spore suspension (5×10^8 spores/mL) was obtained from the Department of Biological Sciences at Auburn University. *B. anthracis* Sterne lacking

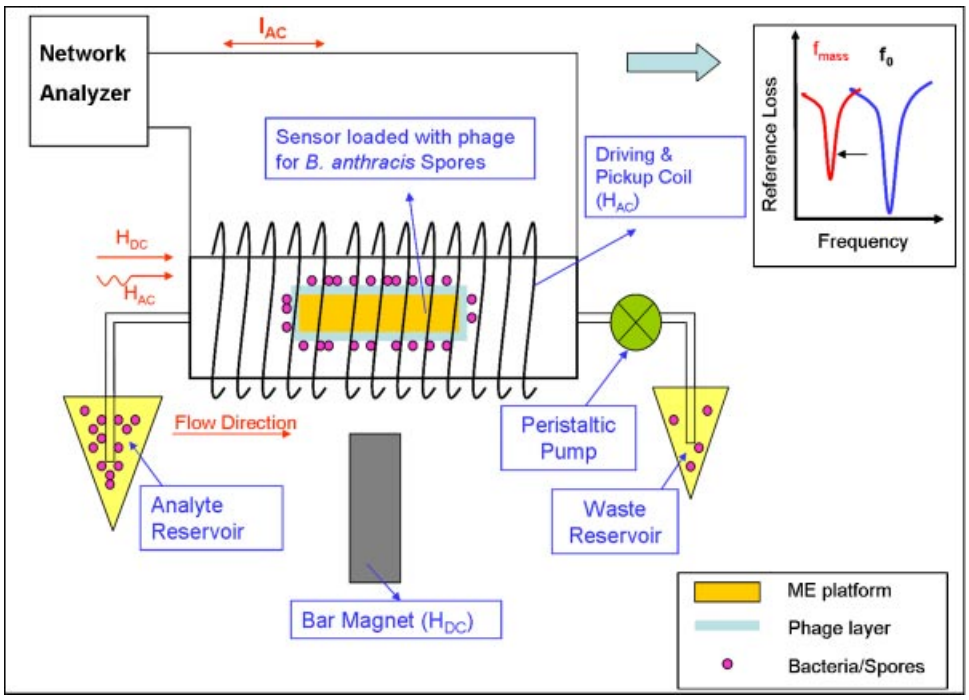

Figure 1. Schematic drawing illustrating the basic working principle of ME biosensors for detecting spores or cells and the flowing system for dynamic tests. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

one plasmid encoding capsule production is used as a vaccine strain for immunization against anthrax. All suspensions of spores were prepared and tested on the same day. Test solutions were stored at 4°C and equilibrated to room temperature prior to testing.

Phage Suspensions of Varying Salt Concentrations

Affinity selected filamentous phage used for this work was provided by Dr. Petrenko of the College of Veterinary Medicine at Auburn University. The clone JRB7 (1.05×10^{13} vir/mL) was derived from the landscape f8/8 phage library and has been demonstrated to be highly specific and selective to *B. anthracis* spores (Petrenko et al., 1996). All phage suspensions were diluted in 1× TBS (25 mM Tris, 3 mM KCl, and 140 mM NaCl at a pH of 7.4). First, 20× TBS solution (USB Corporation, Cleveland, OH) was diluted with filtered water into 1× TBS. Dry NaCl was then added to 1× TBS solution separately to obtain five different salt concentrations: 140, 280, 420, 560, and 840 mM. Finally, JRB7 phage suspension was diluted with 1× TBS (of differing salt concentrations) to obtain the phage concentrations of 1.05×10^{12} vir/mL, 1.05×10^{11} vir/mL, and 1.05×10^{10} vir/mL in five different concentrations of salt. This set of conditions was chosen to investigate the effect of the phage/salt ratio on the distribution of phage immobilized on the sensor, which in turn effects the binding affinities of the ME biosensors.

Phage Immobilization and Spore Binding Measurement

Phage was immobilized on the sensor surface by incubating the ME particles in the phage suspensions (300 μL) for 1 h. The immobilization is based on physical adsorption (Nanduri et al., 2007), avoiding complex surface modification procedures. Following this step, the sensors were rinsed with distilled water three times to remove any loosely bound phage and any salts remaining from the buffer solution. In order to prevent nonspecific binding of spores on the gold surface, the phage-immobilized sensors were immersed into 1 mg/mL bovine serum albumin (BSA) for 1 h, followed by a distilled water rinse. BSA serves as a blocking agent to prevent nonspecific binding. And then the resonance frequency (f_0) was measured in air as already described. For the binding affinity experiments, sensors exposed to the different phage loading conditions and blocked by BSA were exposed to spore solution of a single concentration (5×10^8 cfu/mL in water) using the same technique of static immersion. Incubation time was again 1 h, followed by a single distilled water rinse. Finally, the sensors were tested again for resonance frequency to determine the frequency shift caused by the attached spores. Binding affinity was then determined by comparing the resonance frequency shift data and spore counts (based upon SEM images of the sensor surfaces) for each phage suspension. The phage suspension that promoted the highest binding affinity was chosen for dynamic sensitivity testing.

Dynamic Testing

In order to determine the sensitivity of ME sensors produced using the optimized phage loading conditions, dynamic (flowing) tests were performed using solutions of varying spore concentrations (5×10^1–5×10^8 cfu/mL). These dynamic tests were conducted by passing the successive concentrations of *B. anthracis* spore solutions through a detection chamber using a flowing system as shown in Figure 1. One sensor immobilized with phage and BSA was placed in the measurement chamber, which was filled with plain water. A peristaltic pump was used to achieve a flow rate of 100 μL/min, which was chosen to ensure laminar flow conditions over the sensors. Water was passed through the chamber for 10 min in order to ensure a stable baseline signal, and then each concentration of spore solution was introduced, also for a duration of 10 min, moving from the lowest to the highest concentration. Resonance frequency of the sensor was monitored in real-time by collecting data from the network analyzer with a personal computer.

Microscopic Analysis

A JEOL-7000F scanning electron microscope (SEM) was used to quantify the number of spores bound to the phage-immobilized ME biosensors. In preparation for SEM observations, the tested biosensors were washed with distilled water and then exposed to osmium tetroxide (OsO_4) vapor for 40 min, followed by coating with 60 nm thickness of Au to provide a conductive surface for SEM imaging. A PHILLIPS-301 transmission electron microscope (TEM) was used to verify the distribution of JRB7 phage under different salt and phage concentrations. The TEM samples were prepared on 400 mesh formvar/carbon coated nickel grids. The grid was floated on a drop (50 μL) of sample solution for 1 h. Upon removal from the drop, excess fluid was drained from the grid by touching its edge to filter paper. Then, the grid was washed gently with one drop of stain solution (phosphotungstic acid, PTA), and floated on another drop of stain solution for 3 min to obtain a negative stain of the sample. The grid was dried before examination.

Results and Discussion

The results of this study are shown in four parts. In the first section, the performance of the ME biosensors immobilized with a phage concentration of 10^{11} vir/mL but different concentrations of NaCl was measured and examined with SEM pictures. In the second section, TEM was used to determine the bundling characteristics of phage as both phage and salt concentrations were varied. In the third section, the spore counting results obtained from the SEM images under different phage immobilization conditions are summarized. In the last part, the dynamic response of a sensor immobilized with a phage concentration of 10^{11} vir/mL (420 mM NaCl) to increasing concentrations of spores in water is shown.

Sensor Response for Static Loading

Figure 2 shows the average frequency shift of each group of sensors immobilized using phage at a concentration of 10^{11} vir/mL, with varying concentrations of salt. Each data point represents the average of five individual sensors. As salt concentration is increased from 140 to 840 mM, the frequency shift of the sensors can be observed to increase until a maximum is achieved at a salt concentration of 420 mM. As salt concentration is increased further, the shift in resonance frequency of the sensors decreases. At the maximum studied concentration of 840 mM, the frequency shift of the sensors is similar to that of the lowest salt concentration. Since a higher frequency shift corresponds to a higher mass load on the sensor (as per Eq. 2), and since this mass load is due to spore binding, it is evident that the 420 mM salt concentration represents the optimum binding condition for 10^{11} vir/mL phage suspension.

Scanning electron microscopy images were used to verify the sensor response. Figure 2a–e depicts the results under five (140–840 mM) salt concentrations. As can be seen, the results are consistent with the sensor response curve, which shows that the maximum number of spores bound to the sensor occurs when phage is immobilized in a solution containing 420 mM NaCl. Moreover, the uniformity of the spore distribution under each condition may clearly be seen. For the lowest salt concentration (Fig. 2a), the spores tend to cluster, which is similar to the case of the highest salt concentration (Fig. 2e).

Effect of Salt Concentration on Phage Bundles

Recent studies (Guntupalli et al., 2007; Huang et al., 2007; Lakshmanan et al., 2007; Wan et al., 2007) have used phage that is suspended in 1× TBS solution (which contains 140 mM NaCl) in order to ensure stability of the phage over time. Another study (Overman et al., 2004) has also shown that salt concentrations lower than 100 mM will promote intervirion association and alignment of the filaments, resulting in bundles. Hence 140 mM NaCl was the lowest salt concentration investigated.

TEM was used to investigate the effect of salt concentration on phage distribution. Phage under different solution conditions may aggregate into bundles thereby reducing the number of protein sites available for binding. The TEM images of Figure 3 show the distribution of phage for different phage concentrations (10^{10}, 10^{11}, and 10^{12} vir/mL) at different salt concentrations (140, 420, and 840 mM). The phage filaments in solution cluster together when the phage concentration is high (10^{12} vir/mL). Upon increasing the amount of salt to 420 mM, the phage filaments tend to disaggregate, but a further increase in the salt content again resulted in formation of bundles. A similar trend was also

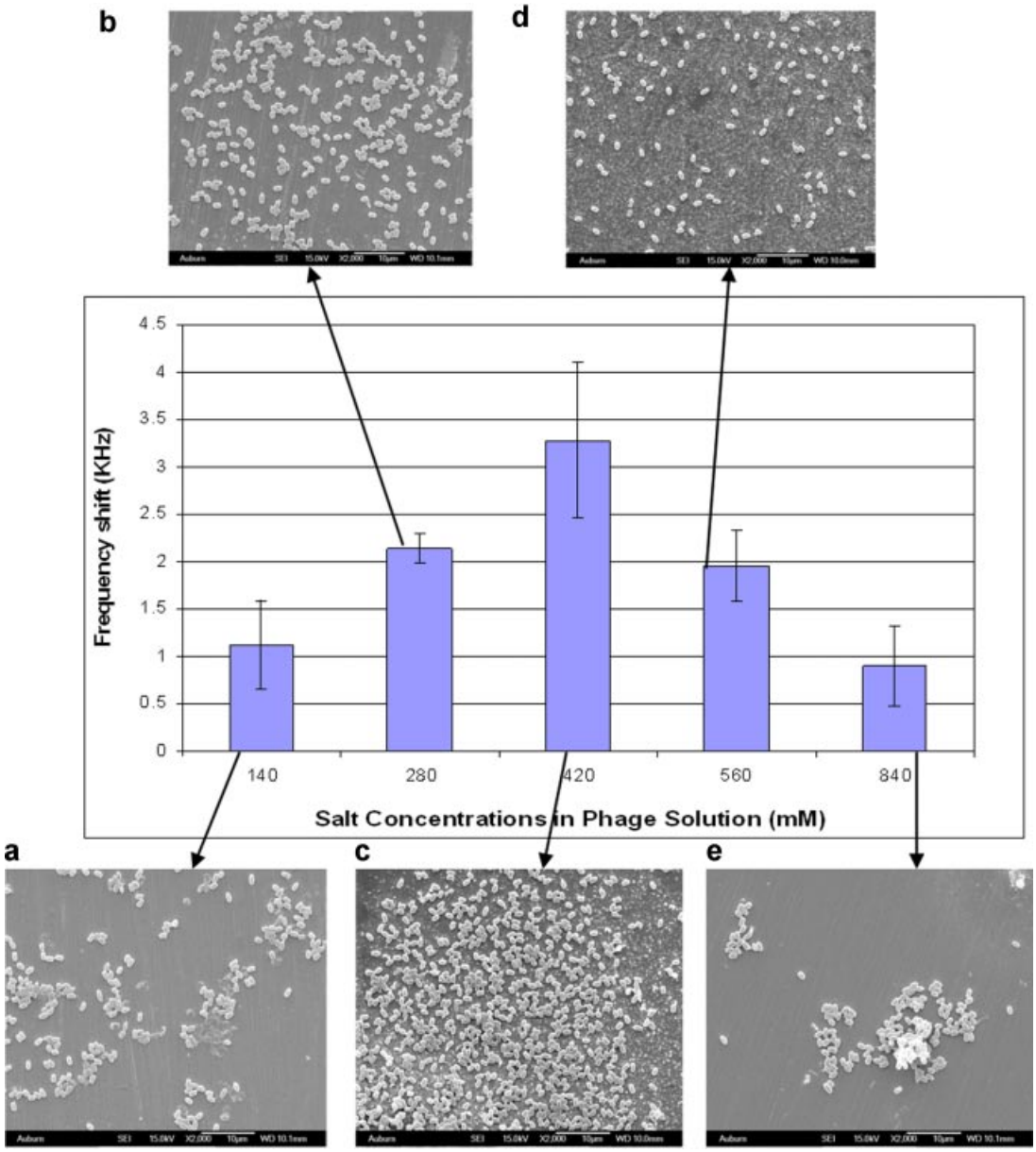

Figure 2. Frequency shift of sensors versus salt concentrations in 10^{11} vir/mL phage solution and SEM photographs of sensors immobilized with 10^{11} vir/mL phage in solutions with NaCl concentrations of: (**a**) 140 mM, (**b**) 280 mM, (**c**) 420 mM, (**d**) 560 mM (**e**) 840 mM. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

found for the phage concentration of 10^{11} vir/mL. For a phage concentration of 10^{10} vir/mL, the distribution of the phage filaments is very low, and there was no bundle formation regardless of salt concentration.

Similar results were obtained after repeating these conditions for several batches of ME biosensors. Based on these analyses, we expect that an optimum balance of phage/salt concentration is critical to the binding affinity of the ME biosensor. Salt induced aggregation of filamentous phage has been studied and documented by other researchers (Ha and Liu, 1998; Nguyen et al., 2000; Tang et al., 1997; Wen and Tang, 2006). In most of these reports, the effect of the presence of divalent counterions in wild-type fd phage suspensions was studied. Evidence from previous studies (Overman et al., 2004; Record et al., 1998; Tang et al., 2002) show that decreasing salt concentration in the phage suspension will decrease axial charge density of the filaments, promoting aggregation or "clustering." Likewise, as salt concentration is increased, axial charge density of the phage filaments increases, leading to less aggregation up to some optimum level and, hence, more even distribution of filaments in solution. Till now, the understanding of the exact molecular mechanism for this phenomenon remains unclear and needs to be studied further.

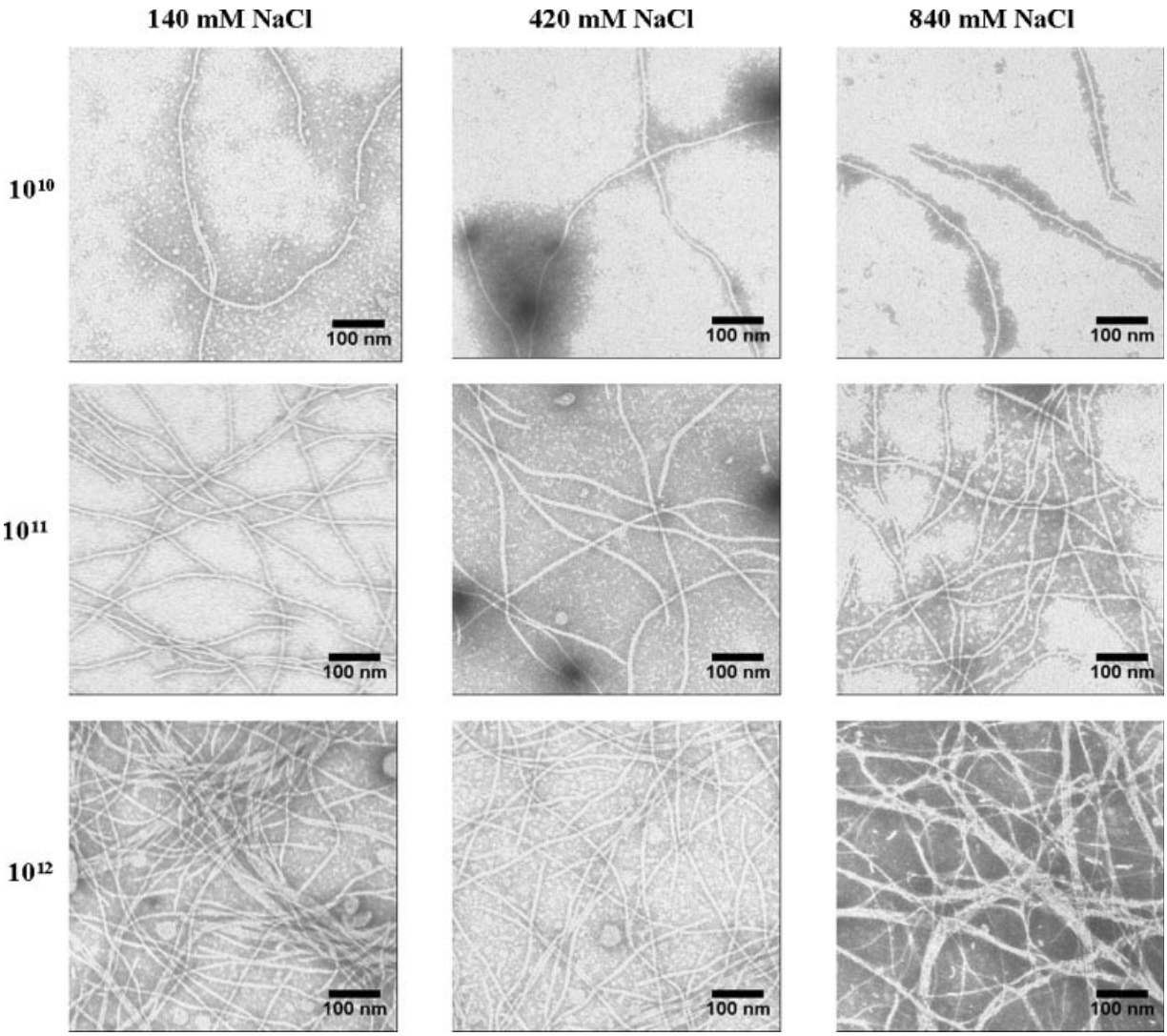

Figure 3. TEM images of different concentration of phage in selected NaCl concentrations.

Effect of Salt Concentration on Spore Binding

To evaluate the effect of NaCl concentration on the phage immobilization and, thus, the sensors' performance, the numbers of spores bound to the sensor's surface were counted using the SEM. The entire surface of one side of the sensor was photographed and spores were counted individually. This number was then multiplied by 2 to account for both sides of the sensor. Table I and Figure 4 shows a comparison of number of spores bound on each ME sensor immobilized with different phage concentrations (10^{10}–10^{12} vir/mL) under different salt concentrations (140–840 mM).

Table I. Binding numbers of spores under different conditions.

	Salt [C] (mM)				
Phage [C] (vir/mL)	140	280	420	560	840
10^{10}	28,800	19,200	10,700	8,200	7,100
10^{11}	112,100	136,200	370,800	80,000	57,800
10^{12}	20,500	19,000	47,600	12,100	8,600

From Figure 4, a noticeable difference in the attachment of spores to the sensors can be observed under each condition. The SEM results were observed to be consistent with the results obtained from the TEM observations. The sensor immobilized with a phage at a concentration of 10^{11} vir/mL showed the most binding. With an increase in the salt concentration from 140 to 420 mM, an increase in number of spores bound to the sensor was observed, with a maximum at the salt concentration of 420 mM. However, a further increase in salt concentration resulted in a decrease in the number of spores bound. A similar trend was found with a phage concentration of 10^{12} vir/mL, but, the number of spores bound was significantly lower than that for phage

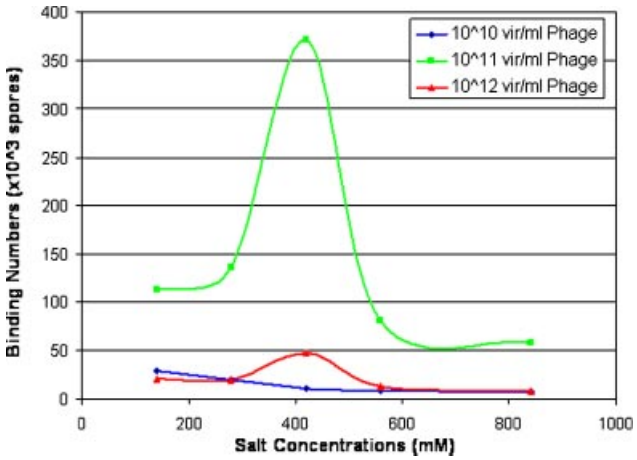

Figure 4. The binding numbers of spores as a function of different NaCl concentrations. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

at a concentration of 10^{11} vir/mL. For phage at a concentration of 10^{10} vir/mL, an increase in salt concentration resulted in a decrease in attached spores. The binding of spores to the sensor with concentrations 10^{10} and 10^{12} vir/mL was significantly lower than that for 10^{11} vir/mL. The lower number of bound spores observed at 10^{12} vir/mL was mainly due to the large number of phage filaments forming bundles. In the case of 10^{10} vir/mL, the low binding is the result of the small number of phage filaments present in the solution. As a result, phage at a concentration of 10^{11} vir/mL with 420 mM NaCl in 1× TBS is the optimum condition resulting in the most favorable condition for binding of spores.

Dynamic Response in Different Concentrations of *B. Anthracis* Spores

Figure 5 shows the dynamic response of the sensor immobilized with phage using the optimum condition (10^{11} vir/mL and 420 mM NaCl) to increasing concentrations of *B. anthracis* spores (5×10^1–5×10^8 cfu/mL). As the concentration of the spore solution was increased, the resonance frequency experienced the expected decrease. The initial frequency remained stable until the solution with a spore concentration of 5×10^3 cfu/mL was introduced, where the first detectable drop of frequency can be seen. At the end of the test, after introduction of the highest spore concentration, the total resonance frequency shift for this sensor was 1,420 Hz. Figure 6 shows the calibration curve of the ME sensor dynamic response and represents the sigmoidal fit of the signals obtained. The biosensors showed a sensitivity of 202 Hz/decade in the linear range (5×10^2 to 5×10^8 cfu/mL).

Conclusions

The work detailed in this paper focused on improving the mass sensitivity of a phage-based, magnetoelastic biosensor by changing the phage suspension chemistry. The results of this study indicate that the optimum binding condition for gold-coated, magnetoelastic biosensors occurs when phage is immobilized at a concentration of 10^{11} vir/mL.

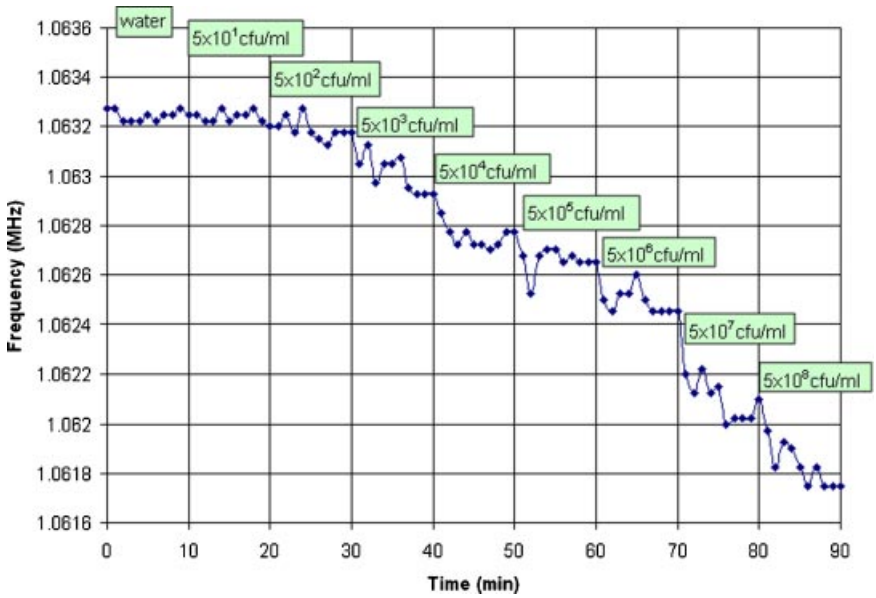

Figure 5. Response of a ME biosensor exposed to different concentrations of *B. anthracis* spores (5×10^1–5×10^8 cfu/mL). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

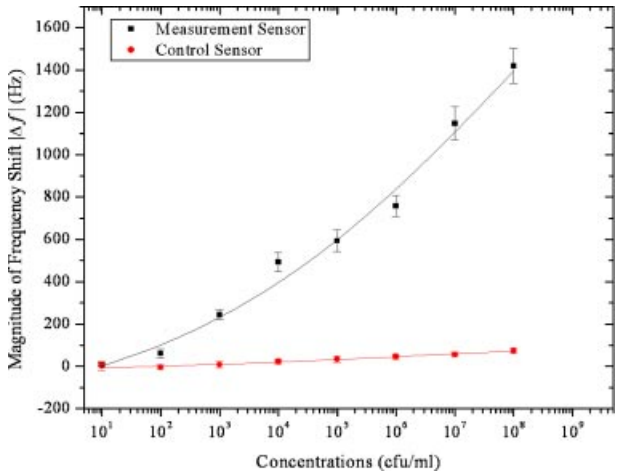

Figure 6. Calibration curve of ME biosensor dynamic response, when exposed to increasing concentrations (5×10^1–5×10^8 cfu/mL) of *B. anthracis* spores suspensions ($R^2 = 0.98$). And the curve represents the sigmoidal fit of signals obtained. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Furthermore, the concentration of salt in the phage suspension will affect the binding affinity of the biosensor. The higher the number of spores bound to the sensor's surface, the better the mass sensitivity of the sensor. For a solution of phage at a concentration of 10^{11} vir/mL in 1× TBS, a salt concentration of 420 mM will promote an optimum number of binding sites for spores due to even distribution of the phage filaments. In contrast, a low-salt concentration will lead to the formation of phage bundles, resulting in a smaller amount of spore binding. Conversely, a salt concentration that is too high will also cause clustering of the phage filaments. Both SEM images of spore binding on the sensor surfaces and TEM images of phage distribution in solution are consistent with our findings.

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