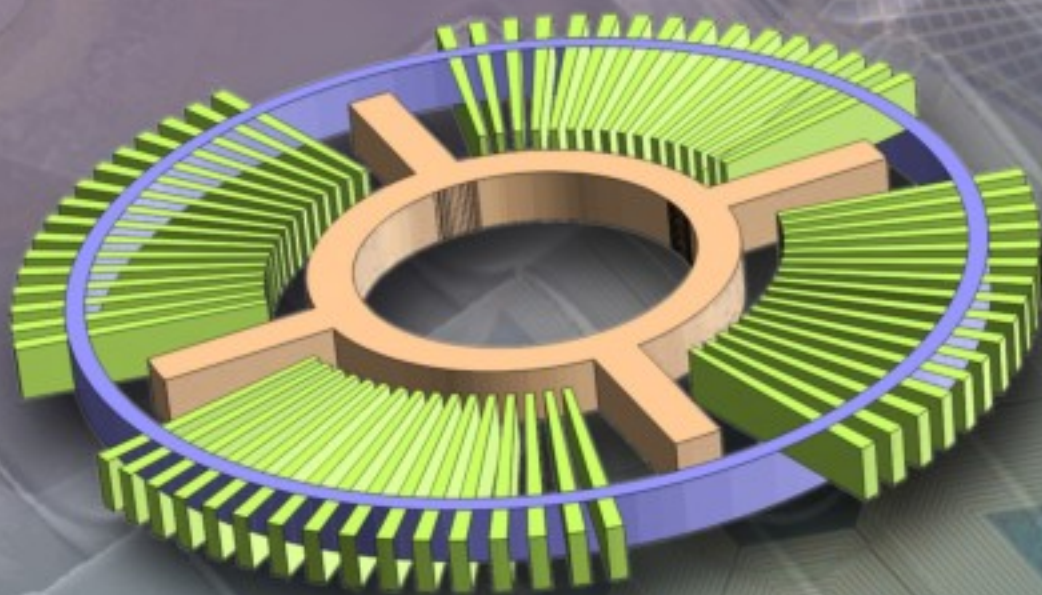


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Optimization of Phage-Based Magnetoelastic Biosensor Performance

¹S. Huang, ¹S.-Q. Li, ¹H. Yang, ¹M. Johnson, ¹J. Wan, ²I. Chen, ³V. A. Petrenko, ²J. M. Barbaree, and ¹B. A. Chin

¹Dept. of Materials Engineering, Auburn University, Auburn, AL 36849, USA

²Dept. of Biological Sciences, Auburn University, Auburn, AL 36849, USA

³Dept. of Pathobiology, Auburn University, Auburn, AL 36849, USA

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Abstract: A magnetoelastic (ME) platform coated with a bio-molecular recognition element (bacteriophage) for selective and specific recognition of *Bacillus anthracis* spores is described. As a wireless sensor, the resonance frequency of the ME sensor decreases in response to the binding of target biological species, which enables the possibility of real time and *in vivo* bio-detection. In this work, fabrication parameters, such as annealing temperature and phage immobilization condition, were investigated to optimize the sensor. In addition, the detection strategy was studied to further improve the performance of ME sensor. It was found that annealing at 200-250 °C in a vacuum oven can effectively increase the ME platform's stability, *Q*-factor. Based on the sensor response and SEM results, a phage concentration of 1×10^{11} vir/ml and a salt concentration of 420 mM in 1xTBS provide the best sensor performance in terms of binding sensitivity. The feasibility of multiple-sensor detection was also demonstrated.

Keywords: Magnetoelastic, Biosensor, Phage, Annealing, Bundling, *Bacillus anthracis* spores

1. Introduction

Fears of deliberate contamination of our food supply and the threat of terrorist attacks using *Bacillus anthracis* have created increasing concerns. Traditional methods of detection such as PCR [1] and antibody-based ELISA techniques [2] can be complicated and exhibit poor specificity in harsh environments. New detection methodologies are needed to address the threat of bioterrorism. Recently, filamentous phage fd has been successfully shown to be an alternative to antibodies as a robust bio-molecular recognition element for biosensors [3]. By immobilizing the phage on magnetoelastic (ME)

sensor platforms, we have fabricated ME biosensors and successfully used them in biological detections [4-6]. The sensor platforms were prepared from ribbons of Metglas® alloy 2826MB. The genetically engineered phage for binding *B. anthracis* spores was produced by Dr. Petrenko [7] of Auburn University's College of Veterinary Medicine. Biosensors are formed by immobilizing phage directly onto the surface of the ME platforms. ME biosensors provide simple, specific, and wireless detections.

ME-based biosensors have great potential to advance the biological analysis technology. To fully develop the ME biosensor technology, several sensor fabrication variables were investigated in this paper to optimize the performance of ME biosensors for the detection of *B. anthracis* spores. Meanwhile, a new detection methodology was studied to further improve the performance of the ME biosensor. Both the ME sensor platform fabrication and the phage immobilization process greatly affect the quality and performance of the biosensor. Since ME materials are iron-based, a gold surface layer is required for corrosion resistance, as well as biocompatibility (phage immobilization). Also, since these sensor platforms are deposited by sputtering, they must be annealed prior to use in order to reduce the effect of as-sputtered defects. Experiments were performed to determine the annealing condition for the prevention of corrosion in aqueous environments. Another set of variables involves the ideal conditions for the immobilization of the phage onto the gold surfaces of the sensor platforms. Filamentous phage tends to form bundles due to polarization-based attractive interactions. Bundling leads to a reduction in exposed protein binding sites and reduces the overall binding affinity of the biosensor surface. Key parameters that influence bundling are the concentration of phage in the solution, as well as the chemistry of the phage solution, specifically salt concentration.

As wireless free-standing sensors, multiple ME biosensors can be employed in one measurement and easily monitored simultaneously. Utilizing multiple ME biosensors rather than one single sensor can further improve the sensor performance in the detection. One of the challenges facing micro-biosensors is that the chance of the target pathogen cells binding on the sensor platform is very low when the sensors are in very small size or when they are used to detect pathogens in very low concentration cultures. This causes a dramatic increase in the response time of the sensor. By using multiple ME biosensors, the chance of the target cells binding on the sensor is greatly increased. Since ME sensors are magnetic, the binding chance can be further increased by stirring the ME sensors in the sample solution using a magnetic field. More importantly, each ME sensor is an independent sensor and the response of one ME sensor can be measured out of many other MSPs. That is, even if only one sensor bonds with the target cells, it can be detected. This allows a quick detection even for samples with very low concentration of target species.

The feature discussed above also enable the design of a simple approach to simultaneously detect different targets using ME sensors. Several groups of multiple MSPs can be employed for the detection. Each group consists of ME sensors with same dimensions and immobilized with the same biological recognition element (specific phage). For different groups, the dimensions of ME sensors and the immobilized biological recognition elements are different. Therefore, ME sensors in each group could capture a different designed target species. For each individual group, the shift in resonance frequency of one sensor can be identified from other sensors. With this approach, multiple targets of interest can be easily detected simultaneously in a sample with very low concentration. Also, if it is needed, a group of reference ME sensors on which no bio-recognition elements are immobilized can be employed to eliminate the effect of environmental change.

2. Materials and Methods

2.1. Biosensor Platform

ME sensors were made from Metglas alloy 2826 MB ($\text{Fe}_{45}\text{Ni}_{45}\text{Mo}_7\text{B}_3$) magnetoelastic strips (Conway, SC) and hand-polished to 15 μm thickness. A micro-dicing saw was then used to cut the alloy into the size of 2 x 0.4 mm. These platforms were rinsed with fresh acetone, methanol, and finally dried in air to remove the adhesive used in the dicing process.

2.2. Pre-coating and Annealing

The magnetoelastic materials are principally Fe and are easily corroded. In addition, the buffer (i.e. TBS) and bacteria solutions used for the experiments contain a certain amount of salt, which will accelerate corrosion. Thus, after annealing, some pre-coating is necessary to protect the ME platform. Two layers of thin films (Cr & Au) were sputtered onto both sides of the sensor platforms by using a Denton Vacuum Discovery-18 magnetron sputtering system (Moorestown, NJ) with two cathodes (RF and DC). The Cr layer was deposited first in order to improve the adhesion between the ME platform and the Au layer. Additionally, it provides extra electrochemical resistance to corrosion. The Au layer was then deposited not only to protect the ME material from degradation in the saline solutions, but also to provide a bioactive surface upon which the biological agents (bacteriophage here) may be easily adsorbed. The whole deposition process is accomplished entirely within the sputtering system without breaking the vacuum. Table 1 lists the sputtering conditions.

Table 1. Sputtering conditions for ME sensor pre-coating.

	DC	RF
	Cr	Au
Pre-sputter Power (W)	100	100
Pre-sputter Time (s)	300	300
Sputter Power (W)	100	100
Sputter Time (s)	300	600
Sputtering rate (nm/s)	0.308	0.231
Pressure (mTorr)	5-6	
Sputter Gas	Ar	
Vacuum (Torr)	5×10^{-6}	

In order to remove the residual stress as well as the as-sputtered defects, an annealing process was employed. This was carried out at 70 °C, 150 °C, 200 °C, 250 °C or 300 °C for 2 hours in a vacuum oven, under a vacuum of at least 10^{-3} Torr. Afterwards, the samples were cooled to room temperature in the oven while still under vacuum.

2.3. Bacteriophage Immobilization and Spore Binding Measurements

The filamentous phage clones JRB7 with a concentration of 1.06×10^{12} vir/ml were kindly provided by Dr. Barbaree of Department of Biological Sciences at Auburn University. In order to prepare a phage solution containing different amounts of NaCl, dry NaCl was added to 1xTBS (25 mM Tris, 3 mM KCl, and 140 mM NaCl at a pH of 7.4) solution separately to obtain five different salt concentrations: 140 mM, 280 mM, 420 mM, 560 mM, and 840 mM. Then, JRB7 phage solution was diluted by the series of the 1xTBS solutions above to obtain a phage concentration of 1.06×10^{11} vir/ml in five

different concentrations of NaCl. 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. Each NaCl condition was repeated for 5 individual sensors.

Phage was immobilized on the sensor surface by incubating the ME particles in the phage solutions (300 μ l). 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, and then transferred to clean PCR tubes. Spore solutions were then added into these tubes and allowed to incubate for another 1 hour, followed by another distilled water rinse.

2.4. Operation Principle and Analyte Binding Measurement

ME materials, when exposed to a time-varying external magnetic field, can be resonated due to the change in their dimensions. For a thin (i.e. length is much larger than the thickness), ribbon-shaped sensor of length L , vibration will be mainly along the length direction at its fundamental resonant frequency. This frequency is given as equation (1) [8-9]:

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

where E , ρ and ν are the Young's modulus of elasticity, density, and the Poisson's ratio of the sensor material, respectively. The resonance frequency is dependent on geometry as well as mass. With an additional mass load on the sensor surface, a shift in the resonance frequency (Δf) will result as described by equation (2) [10]:

$$\Delta f = -\frac{f}{2} \frac{\Delta m}{M} \quad (2)$$

where f is the fundamental resonance frequency, Δm is the mass change on the ME material, and M is the initial mass of the ME material. Fig. 1 illustrates the wireless nature of the individual sensor and the basic principle for detecting bound mass (spores) in air. In our research, after phage immobilization, the characteristic resonance frequency f_0 for each sensor was measured. After exposure to the spore solution, the frequency was measured as f_{mass} . As spores are captured by the specific phage on the sensor's surface, the mass added onto the sensor increases resulting in a frequency shift to a lower value.

2.5. Microscopic Analysis

A JEOL-7000F (JEOL USA, Peabody, MA) scanning electron microscope (SEM) was used to confirm and quantify the binding of target antigens to the phage-coated ME biosensors. In order to count the number of spores bound to the sensor's surface, the entire surface of one side of the sensor was photographed and the spores were counted individually. This number was then multiplied by 2 to account for the total number of spores on both sides of the sensor. In preparation for SEM observations, the 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 Philips 301 (Philips Electronic Instruments, Mahwah, NJ) transmission electron microscope (TEM) was used to verify the interaction of bacteria and spores with the corresponding phage. The TEM samples were prepared on 400 mesh formvar/carbon coated nickel grids. The grid was floated on a drop of sample solution for one hour. 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 (PTA) and floated on another drop of stain solution for 3 min to obtain a negative stain of the sample. The grid was allowed to dry before examination.

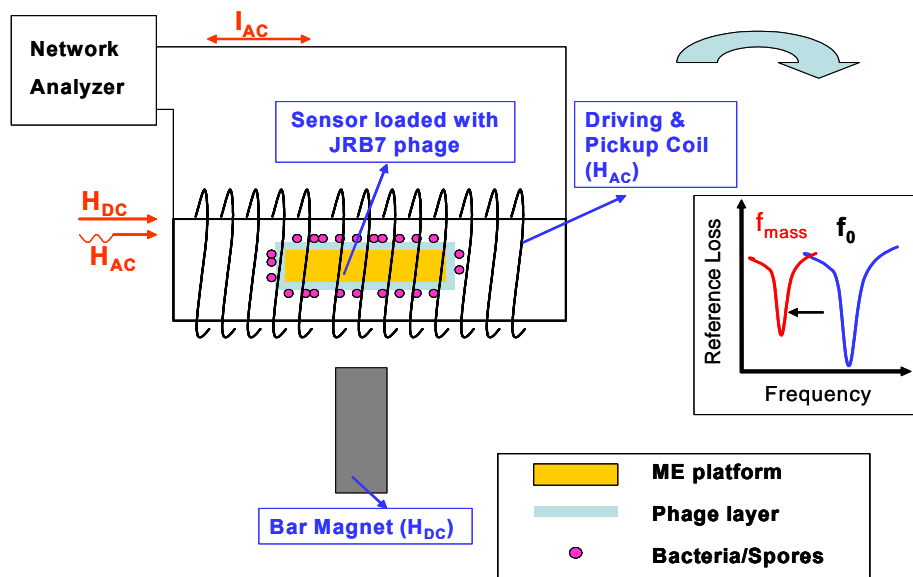


Fig. 1. Setup for ME biosensors measurement.

2.6. Demonstration of Multiple Sensor Detection

To demonstrate the feasibility of multiple sensor detection using ME sensors, the ME sensors were fabricated by dicing the magnetoelastic ribbon into strips with designed dimensions. ME sensors with two lengths – 25 mm and 23 mm – were fabricated. The width and the thickness of all sensors are 5 mm and 30 μm , respectively. After dicing, the sensors were ultrasonically cleaned with acetone for 10 minutes, and then dried by N₂ gas.

Two groups of sensors with different lengths were used in the demonstration. In order to demonstrate the resonance frequency shift due to the mass load, Au was sputtered on the ME sensor surface as the mass load using a sputter coater (PELCO SC-6, Ted Pella, Inc., Redding, CA).

3. Results and Discussion

3.1. Effect of Annealing

Fig. 3 shows the SEM image of a platform before and after annealing. As can be seen, the micro-cracks are the main characteristic of the surface morphology. We hypothesized this was due to internal residual stress generated during the original material manufacture and/or from the polishing/dicing operations. In order to show the effect of annealing, six groups of sensors were annealed under six different temperatures, with the resonance frequency of each sensor being measured and compared before and after annealing. The spectrum quality (Q) factor and the amplitude of frequency signal are the main elements to represent the performance of the sensors. The Q -factor is given by:

$$Q = \frac{f_0}{\Delta w}, \quad (3)$$

where f_0 is the resonance frequency and Δw is defined as the peak width where the amplitude falls to half of its maximum value. Fig. 2 shows the trends of Q -factor and amplitude change after annealing under different temperatures as well as the surface microstructure change under those conditions. As temperature increases from 150 °C to 300 °C, the Q -factor of the sensors can be observed to increase until a maximum is achieved between 200 °C and 250 °C. As temperature is increased further to 300 °C, the Q -factor of the sensors decreases by about 60 %. Since a higher Q -value means a more accurate determination of the resonance frequency, the optimum annealing temperature is in the range of 200 - 250 °C. A similar trend for amplitude was found as well. It is clear to see that the amplitude reached a maximum after annealing at 200 °C.

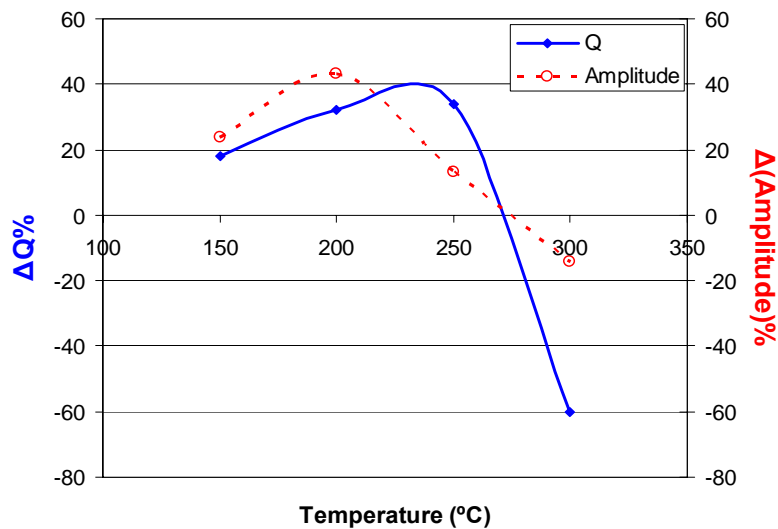


Fig. 2. The effect of annealing temperature on Q factor and amplitude of the resonance frequency.

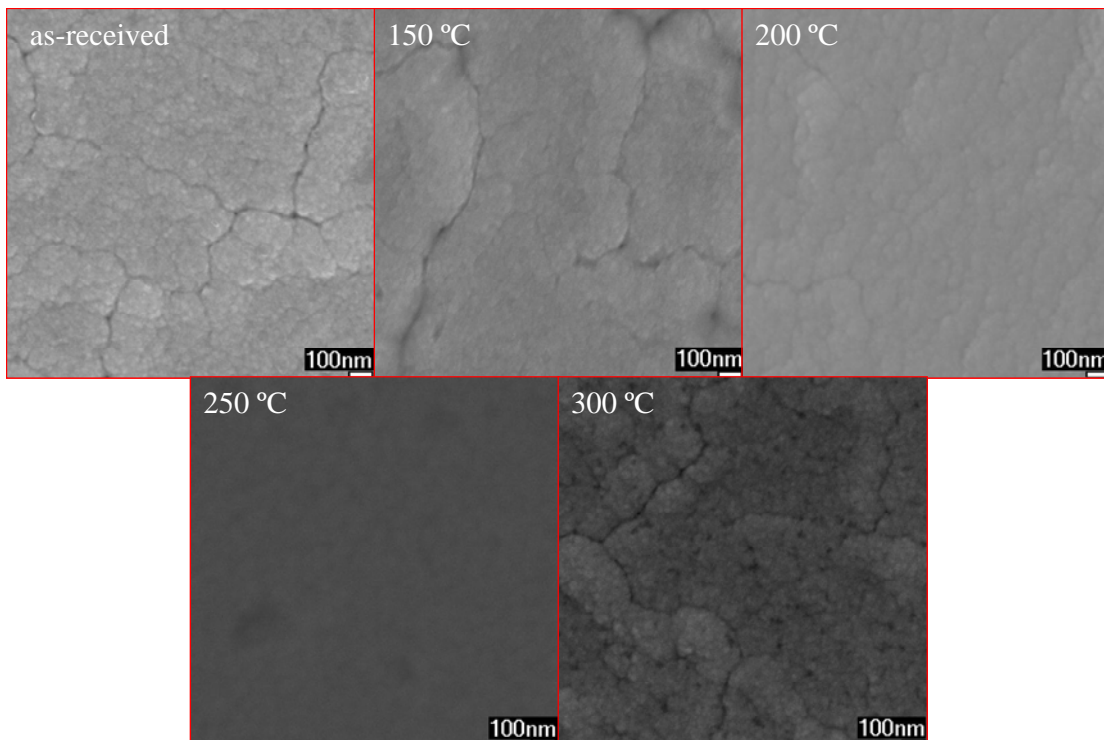


Fig. 3. SEM images of sensor surface before and after annealing under different temperatures (150 °C, 200 °C, 250 °C, 300 °C).

This phenomenon can be explained by SEM images (Fig. 3). Compared with the picture (Fig. 3) of no annealing, there are still some of the cracks on the surface after annealing at 150 °C. When annealed at 200 °C, the surface became much smoother, and the cracks disappeared when the temperature was about 250 °C. However, the cracks started to show up again when the annealing temperature was increased to 300 °C. Within the optimum annealing temperature range of 200 – 250 °C, the residual stresses are removed and the structural defects are also corrected.

3.2. Effect of Salt Concentrations

Fig. 4 shows the TEM pictures of phage distribution at different salt concentrations. The phage filaments tend to cluster together when the solution contains 140 mM NaCl. This phenomenon is called “phage bundles”. As the amount of NaCl is increased to 420 mM, the resolubilization of bundles is observed. Upon increasing to 840 mM NaCl, the bundles formed again. There are four negative charges for each protein coat of a single phage [11]. When the phage solution contains a small amount of counterions (Na^+), the phage filaments will start to neutralize, which leads to bundling. As the amount of Na^+ ions is increased, disaggregation of the filaments occurs. When the Na^+ concentration becomes too high, there will be not enough water molecules to hydrate all the counterions, which results in formation of bundles again [12].

The frequency shift results as well as the binding numbers of the spores counted based on the SEM pictures is summarized in Fig. 4. Both of them show very similar trends. When the salt concentration is increased from 140 mM to 840 mM, the frequency shift and spore binding numbers increases until a maximum is achieved at a salt concentration of 420 mM. As NaCl concentration is increased further, the shift in resonance frequency and the number of bound spores decreases. As seen in Fig. 4 (b), the binding is uniformly distributed. This is consistent with the results obtained from the TEM observations. When phage bundles, it leads to a reduction in exposed protein binding sites and reduces the overall binding affinity of the biosensor surface. The result of this is that a lower number of spores will be able to bind onto the sensor. Since a higher amount of binding on the sensor corresponds to a higher frequency shift of the biosensor (as per Eq. 2). It is evident that the 420 mM salt concentration represents the optimum binding condition for 10^{11} vir/ml phage solution.

3.3. Multiple ME Sensors Detection

Fig. 5 demonstrates the multiple sensor detection using ME sensors. Two groups of the sensors were used in the test. One group consists of two sensors with the length of 25 mm (sensor L1 and L2), while another group has two sensors with 23 mm length (sensor S1 and S2). Fig. 5 (a) shows the spectrum of the ME sensors as they were put in the test chamber and their resonance frequencies were measured by a single scan. It clearly shows that the resonance frequencies of all the ME sensors separated into two groups, which represented the ME sensors belonging to different groups. In each group, the resonance frequencies of the two sensors can be observed. Ideally, in the same group, the sensors would have the same dimension and have the same resonance frequency. However, in practice, the variation in the dimensions occurs during the dicing, which results in the slight difference in the resonance frequency of sensors in the same group. The precise lengths of the sensors were measured. For the group of ME sensors with the longer length, L1 and L2 are in the length of 25.05 mm 25.23 mm, respectively. For another group, S1 is in the length of 23.05 mm, while the length of S2 is 23.34 mm.

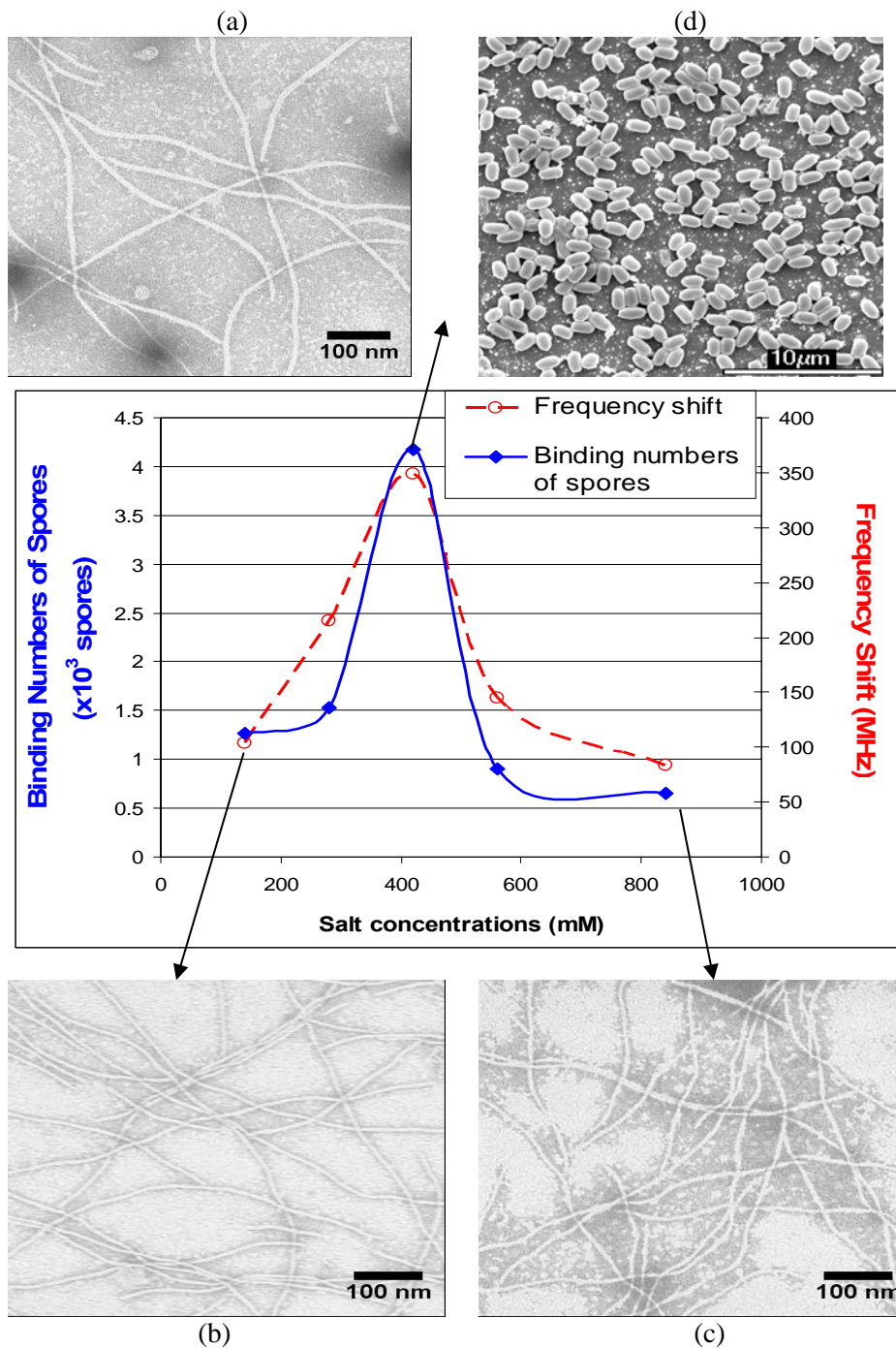


Fig. 4. Frequency shift and spore binding numbers of sensors at different salt concentrations in 10^{11} vir/ml phage solution. TEM photographs of phage in selected NaCl concentrations: (a) 140 mM, (b) 420 mM, (c) 840 mM. SEM photographs (d) of spore binding of sensor immobilized with 10^{11} vir/ml phage in 420 mM NaCl solution.

Fig. 5 shows that the slight dimension difference due to the fabrication does not affect the identification of the signals of the sensors in different groups. Fig. 5 (b) shows the spectrum of the ME sensors after Au was sputtered on L2 and S1 surface as mass load. For each group, the shift in the resonance frequency of the MSP with the mass load can be easily identified. This demonstrated that the mass load change on a sensor can be detected in individual group, as well as in different groups. In real detection, even if only one sensor captures the target cells, it can be identified out of other sensors. By employing multiple ME sensors, two or more interested species can be rapidly detected simultaneously.

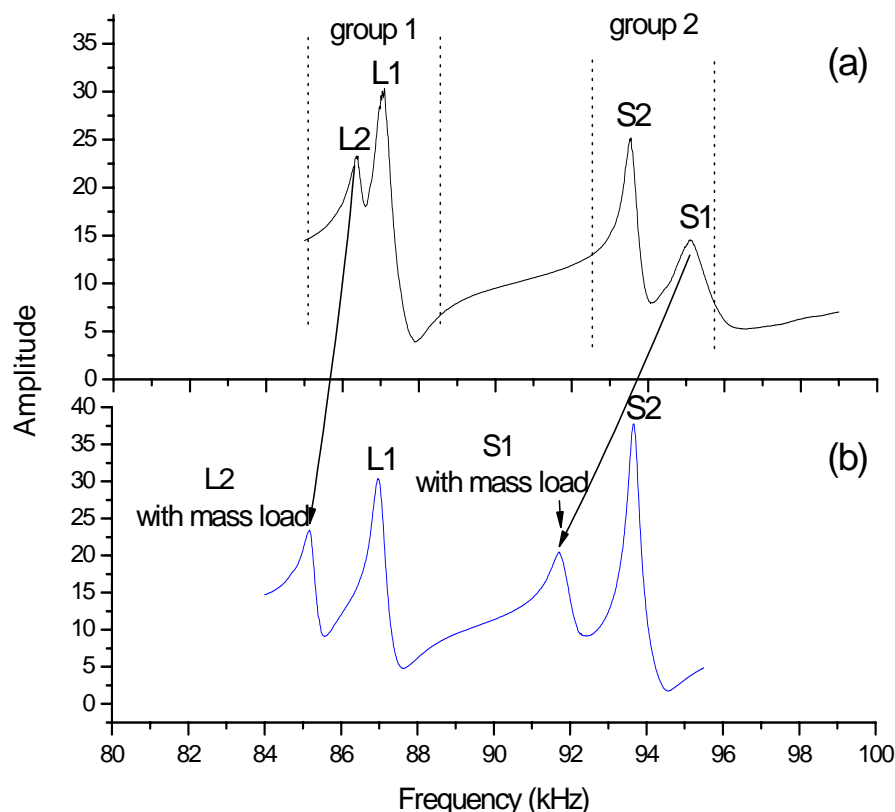


Fig. 5. Demonstration of the multiple-target approach.

4. Conclusions

The optimization of phage based ME biosensors performance by using annealing and modifying the chemistry of filamentous phage solution has been established. By annealing between 200 – 250 °C in vacuum after sputtering, the residual stress created during dicing is largely relieved and many of the structural defects will be removed. Afterwards, the Q -factor of the resonance signal increased by about 30% and the amplitude increased by about 25%. By increasing the amount of NaCl in the 5×10^{11} vir/ml phage solution to 420 mM, the tendency of the phage filaments to bundle will be minimized, thereby increasing the available binding sites on the ME sensor's surface. The result is that more spores will be able to bind to the sensor surface, increasing the binding affinity of the ME biosensor. Furthermore, by using new detection strategy – multiple sensor detection, the performance of the ME sensors can be further improved.

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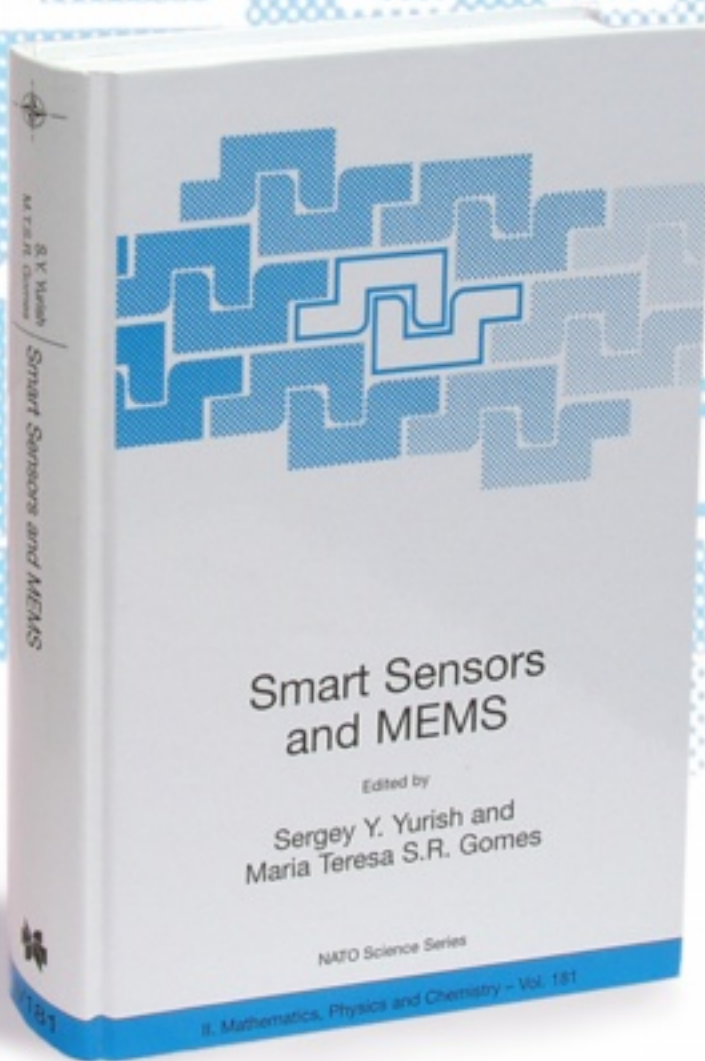
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