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Ultrasensitive detection of *Vibrio cholerae* O1 using microcantilever-based biosensor with dynamic force microscopy

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ABSTRACT

This work presents the first demonstration of a cantilever based *cholerae* sensor. Dynamic force microscopy within atomic force microscope (AFM) is applied to measure the cantilever's resonance frequency shift due to mass of cell bound on microcantilever surface. The *Vibrio cholerae* O1, a food and waterborne pathogen that caused cholera disease in human, is a target bacterium cell of interest. Commercial gold-coated AFM microcantilevers are immobilized with monoclonal antibody (anti-*V. cholerae* O1) by self-assembled monolayer method. *V. cholerae* O1 detection experiment is then conducted in concentrations ranging from 1×10^3 to 1×10^7 CFU/ml. The microcantilever-based sensor has a detection limit of $\sim 1 \times 10^3$ CFU/ml and a mass sensitivity, $\Delta m/\Delta F$, of ~146.5 pg/Hz, which is at least two orders of magnitude lower than other reported techniques and sufficient for *V. cholerae* detection in food products without pre-enrichment steps. In addition, *V. cholerae* O1 antigen–antibody binding on microcanilever is confirmed by scanning electron microscopy. The results demonstrate that the new biosensor is promising for high sensitivity, uncomplicated and rapid detection of *V. cholerae* O1.

1. Introduction

Vibrio cholerae is a causative agent of cholera and belongs to a group of organisms whose natural inhabitant is in an aquatic environment. Even though the endemic areas of cholera are reported only in Africa, Asia and Latin America, there are many imported cases reported in many countries worldwide (WHO). Until now, *V. cholerae* has been classified into 155 serogroups based on somatic antigen (O antigen), but only O1 and O139 are found to associate with cholera outbreaks (Louis et al., 2003; Gubala, 2006). *V. cholerae* O1 is gram-negative curved-rod bacterium and oxidase-positive (Jyoung et al., 2006), which is identified as a food and waterborne pathogen that causes cholera or severe diarrhea disease (Bhowmick et al., 2009). One of common sources of *V. cholerae* O1 is contaminated food, especially, seafood products.

The conventional method for detection and identification of bacterial pathogens relies on microscopic examination and biochemical identification. Although these techniques are inexpensive and give both quality and quantitative information, they are time consuming and cannot detect VNC (viable but non-cultureable) form (Baker et al., 1983; Colwell, 2000). Various monitoring techniques have been developed to detect the toxigenic *V. cholerae* including enzyme-linked immunoassorbent assay (ELISA), polymerase chain reaction (PCR) (Koch et al., 1993; Lyon, 2001; Martinez et al., 2001), real time PCR (Blackstonea et al., 2007), multiplex PCR (Rivera et al., 2003) and DNA probe hybridization technique (Yoh et al., 1993; Wright et al., 1992). These techniques provide high sensitivity for toxigenic *V. cholerae* detection but they require pre-enrichment, molecule labeling, high skill operator and multiple detection steps.

Therefore, an easy-to-use *cholerae* biosensor with sufficiently high sensitivity is needed. Various *bacterial* sensors based on surface plasmon resonance (SPR) (Jyoung et al., 2006; Koubova et al., 2001), quartz crystal microbalance (QCM) (Mao et al., 2006), waveguide-based immunosensors (Horvath et al., 2003) and amperometric immunosensors (Rao et al., 2006) have been demonstrated. However, these techniques have several disadvantages including limited assay life-time, complicated detection steps and insufficient sensitivity for detection of *V. cholerae* O1 in food products.

Microcantilever is a relatively new sensing platform, which offers excellent sensitivity and very low detection limit (Thundat et al., 1995). By combining the microcantilever and dynamic force microscopy (DFM) in atomic force microscope (AFM), adsorbed molecules can be detected from the information of resonance frequency shift. In DFM, a microcantilever is driven to its resonance

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frequency by piezoelectric actuator. If target molecules adsorbed onto microcantilever, its resonance frequency will decrease due to mass loading (Lang et al., 2002). Thus, the variation of mass on the cantilever causes a resonance frequency shift and the shift is proportional to the amount of adsorbed molecules (Lang et al., 2005; Nugaeva et al., 2007). In addition, this sensing system offers high sensitivity by the aid of optical detection in AFM. Recently, numerous microcantilever-based biosensors have been reported (Hwang et al., 2009). These include microcantilevers for the detection of prostate specific antigen (PSA) (Lee et al., 2003), DNA (Su et al., 2003; Illic et al., 2005), vaccinia virus particle (Gunter et al., 2003; Gupta et al., 2004a,b, 2006) and microorganisms such as *Escherichia coli* O157:H7 (Campbell and Mutharasan, 2005; Ilic et al., 2000). However, there has been no report of microcantilever-based biosensor for *V. cholerae* O1 detection.

This work presents the first demonstration of a cantilever based *cholerae* sensor. In this system, antibody of *V. cholerae* O1 is immobilized on gold-coated microcantilever surface by self-assembled monolayers (SAMs) method and the resonance frequency shift of microcantilever due bacteria binding is measured by DFM as a function of *V. cholerae* concentration. In addition, the sensing structure is examined by scanning electron microscope (SEM). This sensing device will be useful in clinical microbiology laboratories for prevention and control endemic of cholera.

2. Materials and methods

2.1. The principle of dynamic force microscopy

The principle of dynamic force microscopy is illustrated in Fig. 1a. Cantilever is vibrated over a sample surface at its resonance frequency with a fixed resonance amplitude, *A*. This resonance frequency differs from eigen frequency f_0 of free standing cantilever because interaction force between tip at the end of cantilever and sample. The average distance between tip and sample surface is called *d* and the nearest distance between tip at the oscillation peak and sample surface is assigned as *D*. The resonance amplitude *A* is a function of *d*, which can be changed by adjusting the cantilever position relative to sample surface.

Characteristics of frequency shift (Δ_f) of cantilever can be illustrated by considering interaction potentials as shown in Fig. 1b. When the cantilever is far away from sample surface, cantilever is vibrated under harmonic oscillation in parabolic potential (dotted line). In this case, tip motion is sinusoidal and the resonance frequency is eigen frequency of cantilever, f_0. As distance between tip and sample surface is reduced, tip–sample interaction potential (dashed line) would interact with parabolic oscillation potential (dotted line), resulting in effective potential (solid line). Effective potential is asymmetric and oscillation of tip becomes inharmonic. Thus, the oscillation amplitude, *A*, and resonance frequency, *f*, is decreased when *d* is reduced. As a result, the frequency shift (Δ_f) is controlled by two parameters including *d* and *A* (Holscher et al., 1999).

2.2. Bacteria and culture method

V. cholerae O1 and *Vibrio parahemolyticus* were obtained from Faculty of Medical Technology, Mahidol University, Thailand. These bacteria were grown in Luria–Bertani (LB) plate overnight at 37 °C. The pure colony of each bacterium was picked up into PBS buffer solution and the bacterial concentration was adjusted to of 1×10^8 CFU/ml using spectrophotometer. The bacterial suspensions were boiled in 80 °C water bath for 20 min to kill undesired bacteria and then serially diluted with PBS buffer solution (pH 7.4) to concentrations ranging from 10^2 to 10^7 CFU/ml. One milliliter of

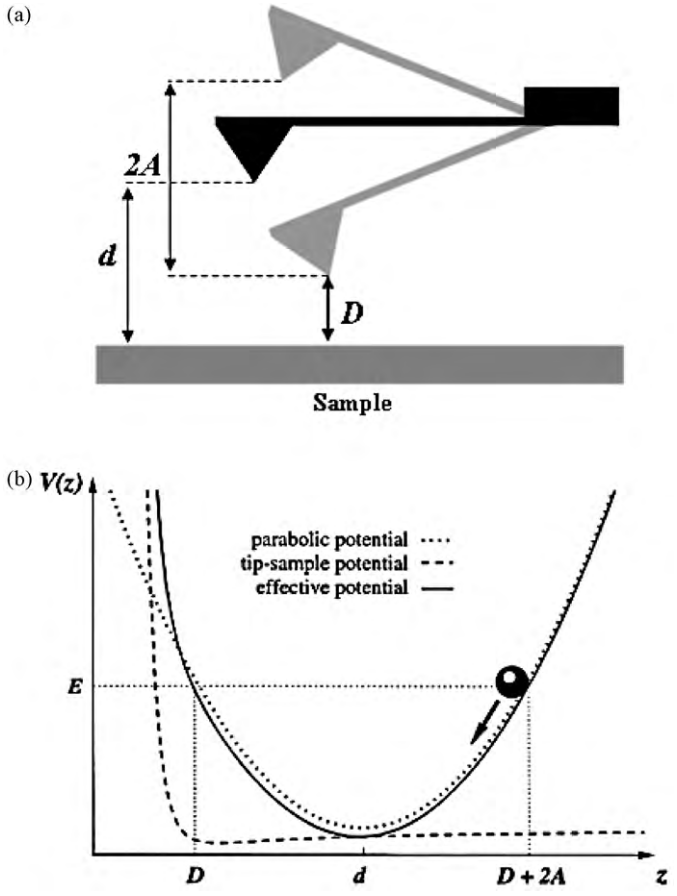

Fig. 1. (a) Scheme of the principle of dynamic force microscopy. (b) Potentials concerning cantilever oscillation in dynamic force microscopy.

bacterial suspension was aliquoted into 1 ml sample container and kept at −20 °C before use.

2.3. Antibody immobilization by self-assembled monolayers

The 250 μm long, 35 μm wide and 1 μm thick gold-coated microcantilever was purchased from NT-MDT Co., Ltd. (NSG10, NT-MDT Co., Ltd., RUSSIA). Before usage, the microcantilever was thoroughly cleaned with piranha solution (conc. H_2SO_4 in 30% H_2O_2; 1:1, v/v) for 5 min to remove organic substance on the surface and subsequently rinsed with deionized water. The freshly cleaned microcantilever surface was modified by simply dipping into the ethanol solution containing 10 mM 3-mercaptoproprionic acid (MPA) for about 3 h at room temperature to form a self-assembled monolayer and then washed with ethanol and deionized water, respectively. In this step, MPA reacted with Au surface leaving free carboxylic groups for further reaction.

The microcantilever was then immersed in the mixture of 200 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma–Aldrich, USA) and 50 mM sulfo-N-hydroxysuccinimide (NHS, Sigma–Aldrich, USA) for 30 min at room temperature to activate the carboxylic groups so that they can form peptide bond with primary amine of antibody. The solution must be prepared immediately before use to avoid loss of activity. After washing with deionized water, 0.5 mg/ml monoclonal antibody (anti-*V. cholerae* O1) in PBS (pH 7.4) was spread over the microcantilever surface. This step was carried out for 1 h at room temperature and excess antibody solution was then washed

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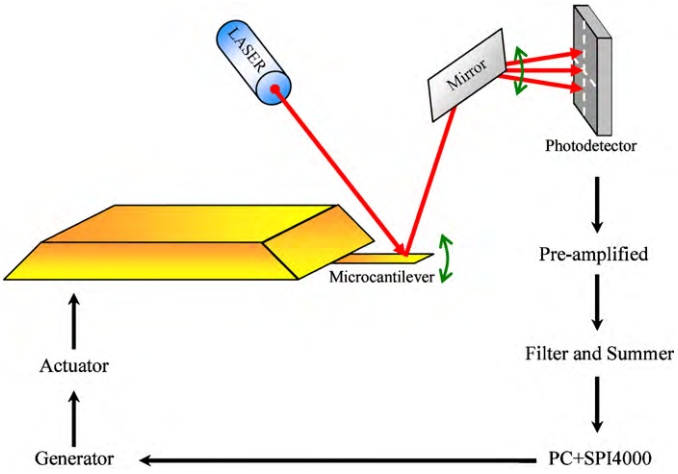

Fig. 2. Schematic drawing of the resonance frequency measurement by optical leverage method of atomic force microscope (AFM).

with PBS buffer solution (pH 7.4). Finally, 3 mg/ml bovine serum albumin (BSA, Sigma–Aldrich, USA) in PBS buffer solution (pH 7.4) was added onto the antibody-immobilized microcantilever surface to prevent unspecific binding on the empty surface in the sensing system.

2.4. Bacteria binding measurement

Antibody-immobilized microcantilevers were immersed in diluted *V. cholerae* O1 suspensions to assess the sensitivity of the sensor and shaking was required for this antigen–antibody reaction. The PBS buffer solution was used as negative control. The immersion time was 5 min to allow forming of antigen–antibody complex. Cross-reactivity experiment was conducted against *V. parahemolyticus* bacteria. The antibody-immobilized microcantilever was dipped into *V. parahemolyticus* suspension with a concentration of 1×10^8 CFU/ml for 5 min under the same experimental conditions as *V. cholerae* O1 detection.

The measurements were performed using atomic force microscope (AFM, SPA400, Seiko, Japan), operated in DFM (dynamic force microscopy) mode. The resonance frequency was measured before (noted as F_0; baseline resonance frequency signal) and after antigen–antibody binding (noted as F_1; test resonance frequency signal). Before measurement, the microcantilever was mounted on microcantilever holder, having electronic interface to the cantilever chip. The cantilever was then driven by mechanical-acoustic excitation using a piezoelectric actuator in close proximity to the cantilever holder. Fig. 2 shows schematic of the resonance frequency measurement.

The cantilever oscillation was measured by the optical beam deflection method, in which an incident beam from laser diode focused on the back end of cantilever. The reflected light from the cantilever surface was detected by a four quadrant photosensitive detector. The cantilever deflection gave the signal which was proportional to the difference in the photocurrents generated in the upper and lower segments. The photocurrents of the upper and lower segments were pre-amplified by an amplifier. The signal was connected to a data acquisition program (SPI 4000, NT-MDT Co., Ltd., Russia) for processing and analysis. The resonance frequency shift was the difference value between F_0 and F_1.

3. Results and discussion

3.1. V. cholerae O1 detection

3.1.1. Antibody immobilization by physical adsorption

The microcantilever coated with monoclonal antibody by physical adsorption was tested with suspension of *V. cholerae* O1 at different concentrations. From experimental results, the shift of resonance frequency from base line (before antigen–antibody binding) tended to be negative (data not shown). The negative shift occurred due to loss of some protein molecules (protein A, antibody and BSA) that were physically adsorbed on the microcantilever. The loss of the molecules on the microcantilever reduced the mass, leading to the increase of resonance frequency. Thus, physical adsorption of antibody is not a good method for antibody immobilization.

3.1.2. Antibody immobilization by chemical adsorption

The microcantilever that was immobilized with monoclonal antibody by self-assembled monolayers (SAMs) was tested with different known concentrations of *V. cholerae* O1 suspension to determine assay sensitivity of the sensor. From experimental results, the resonance frequency of microcantilever decreased as the concentration of *V. cholerae* O1 suspension increased. Fig. 3a shows the resonance frequency shift of the antibody-immobilized microcantilever with different *V. cholerae* O1 concentrations including 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 CFU/ml. The PBS buffer solution was used as negative control (no cell). The results indicated that immuno-complex of antigen and antibody was successfully formed by antigen–antibody reaction, resulting in mass addition on the microcantilever surface.

The calibration curve for *V. cholerae* O1 detection is shown in Fig. 3b. The curve shows relationship of the resonance frequency shift versus the concentration of *V. cholerae* O1 suspension in log scale. It can be seen that the resonance frequency shift is linearly proportional to the log of *V. cholerae* O1 suspension in concentration ranging from 1×10^3 to 1×10^6 CFU/ml. From the experiment, the detection limit of the sensor for *V. cholerae* O1 detection was found to be $\sim 1 \times 10^3$ CFU/ml. This is at least two orders of magnitude lower than that of standard ELISA method (1×10^5 CFU/ml) (Rao et al., 2000) and another reported amperometric immunosensor (1×10^5 CFU/ml) (Rao et al., 2000).

The antibody-immobilized microcantilever was also tested with PBS buffer solution (pH 7.4) in the negative control experiment, in which bacterial cells were not present. The result showed no significant resonance frequency shift (~14 Hz). The additional mass, Δm due to antigen–antibody binding can be straightforwardly related to the shift of resonance frequency of microcantilever according to

$$\frac{1}{f_1^2} - \frac{1}{f_0^2} = \frac{\Delta m}{(4n\pi^2 k)} \quad (1)$$

where k is the spring constant of the cantilever, f_0 is the initial resonance frequency prior to the mass loading, f_1 is the resonance frequency after mass addition (mass of bacteria bound on the microcantilever surface) and n is a geometry-dependent correction factor ($n = 0.24$) if the additional mass is uniformly distributed over a rectangular shaped microcantilever (Gupta and Akin, 2004). Thus, the additional mass of the bacteria cells bound on the microcantilever surface is estimated from Eq. (1) and the results are listed in Table 1. The detection mass sensitivity of the sensor defined by $\Delta m/\Delta F$ is determined to be ~146.5 pg/Hz. High sensitivity and very low detection limit of the sensor have thus been achieved. These can be attributed to very high mass sensitivity of microcantilever sensor and effective antibody immobilization on the cantilever structure by SAMs method.

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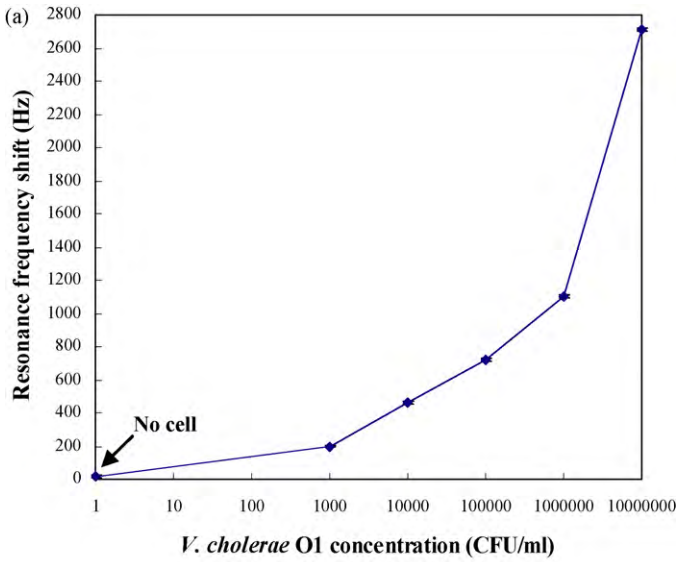

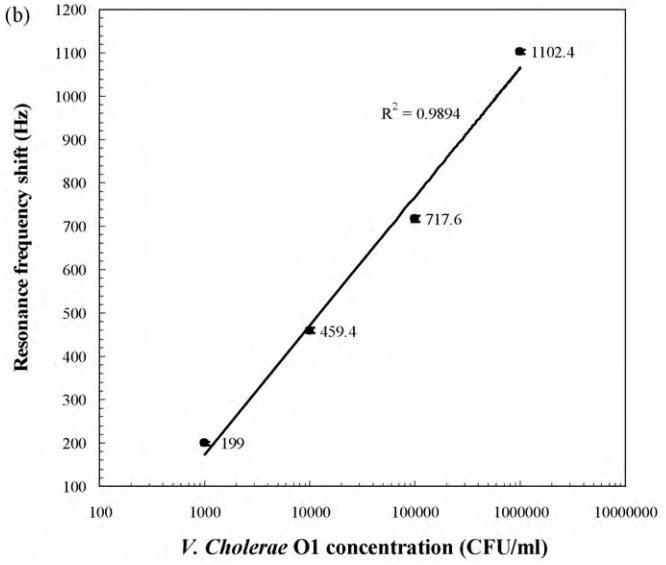

Fig. 3. (a) Relationship of resonance frequency shift versus *V. cholerae* O1 concentration. The control experiment (no cell) was an antibody-immobilized microcantilever tested in PBS buffer solution. Note that 0 is substituted by 1 since it cannot be plotted in log scale. (b) Calibration curve of the resonance frequency shift versus *V. cholerae* O1 concentration. The result shows linearity of *V. cholerae* O1 detection in the range of 10^3–10^6 CFU/ml.

The mass sensitivity and detection limit of the sensor is comparable to those of reported microcantilever sensors for detection of *E. coli* O157:H7 (Campbell and Mutharasan, 2005; Detzel et al., 2006), *Samonella typhimurium* (Zhu et al., 2007) and *Aspergillus niger* (Nugaeva et al., 2007). These reports demonstrate the mass sensitivity of ~50 pg/Hz and the detection limit of ~1×10^3 CFU/ml. Nevertheless, the mass sensitivity is still several orders lower than those of microcantilevers for detection of *Bacillus anthracis* spores (9.2 fg/Hz) (Davila et al., 2007) and vaccinia virus (6.3 attogram (ag)/Hz) (Gupta et al., 2004a,b). These ultrasensitive microcantilever sensors are specially designed with sophisticated sensing layer and nanometer scale cantilever thickness. It is thus possible to further reduce detection limit of microcantilever sensor for *V. cholerae* O1 detection by employing a specially designed microcantilever. However, detection limit of ~1×10^3 CFU/ml by the commercial microcantilever presented in this work is already satisfactory for use in general microbiological laboratories.

Table 1
Estimation of mass change on microcantilever surface.

	Resonance frequency (kHz)			
Concentration (CFU/ml)	1×10^3	1×10^4	1×10^5	1×10^6
ΔF (Hz)	199	459.4	717.6	1100.9
Δm (ng)	30.4	82.6	104.4	121.5
$\Delta m/\Delta F$ (pg/Hz)	152	179	145	110
Average			146.5 pg/Hz	

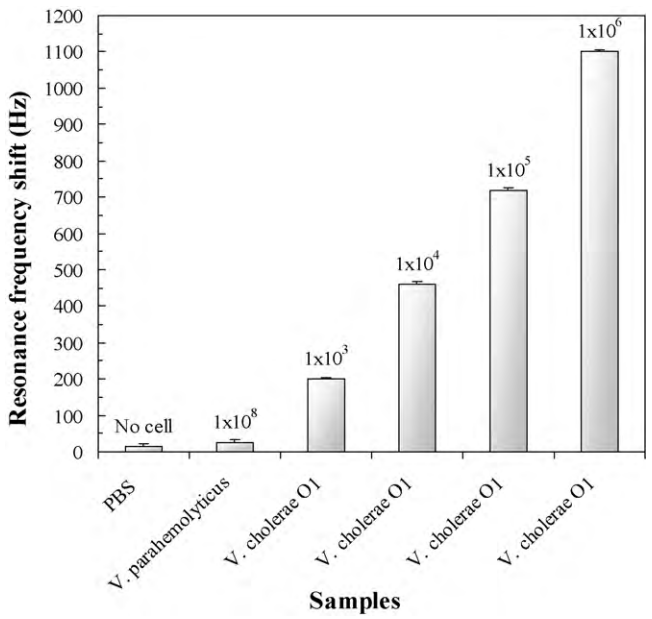

Fig. 4. Cross-reactivity of the sensor: comparison of resonance frequency shift response when the antibody-immobilized microcantilevers were tested with suspension of *V. parahemolyticus*, *V. cholerae* O1, and PBS buffer solution.

3.2. Cross-reactivity test

V. parahemolyticus bacteria were used to verify the cross-reactivity performance of the sensor. The test was done under the same condition used for *V. cholerae* O1 detection. The antibody-immobilized microcantilever was immersed into 1×10^8 CFU/ml *V. parahemolyticus* with 5-min shaking. It was then washed with PBS buffer solution (pH 7.4) and DI water and dried by nitrogen gas. The resonance frequency of microcantilever was measured before and after immersion in suspension of *V. parahemolyticus*.

Fig. 4 shows the comparison of the resonance frequency shift of antibody-immobilized microcantilever tested with PBS buffer (pH 7.4), 1×10^8 CFU/ml *V. parahemolyticus* suspension and *V. cholerae* O1 with the concentrations between 1×10^3 and 1×10^6 CFU/ml, respectively. The resonance frequency shifts of PBS buffer and 1×10^8 CFU/ml *V. parahemolyticus* were 14.4 and 26.6, respectively while those of *V. cholerae* O1 at concentration of 1×10^3, 1×10^4, 1×10^5 and 1×10^6 CFU/ml were 199.0, 459.4, 717.6 and 1102.4 Hz, respectively. The resonance frequency shift of the antibody-immobilized microcantilever tested with *V. parahemolyticus* suspension is not significant (~24 Hz) compared to those of *V. cholerae* O1. Thus, it can be assumed that the antibody has no cross-reactivity with other strains of bacteria.

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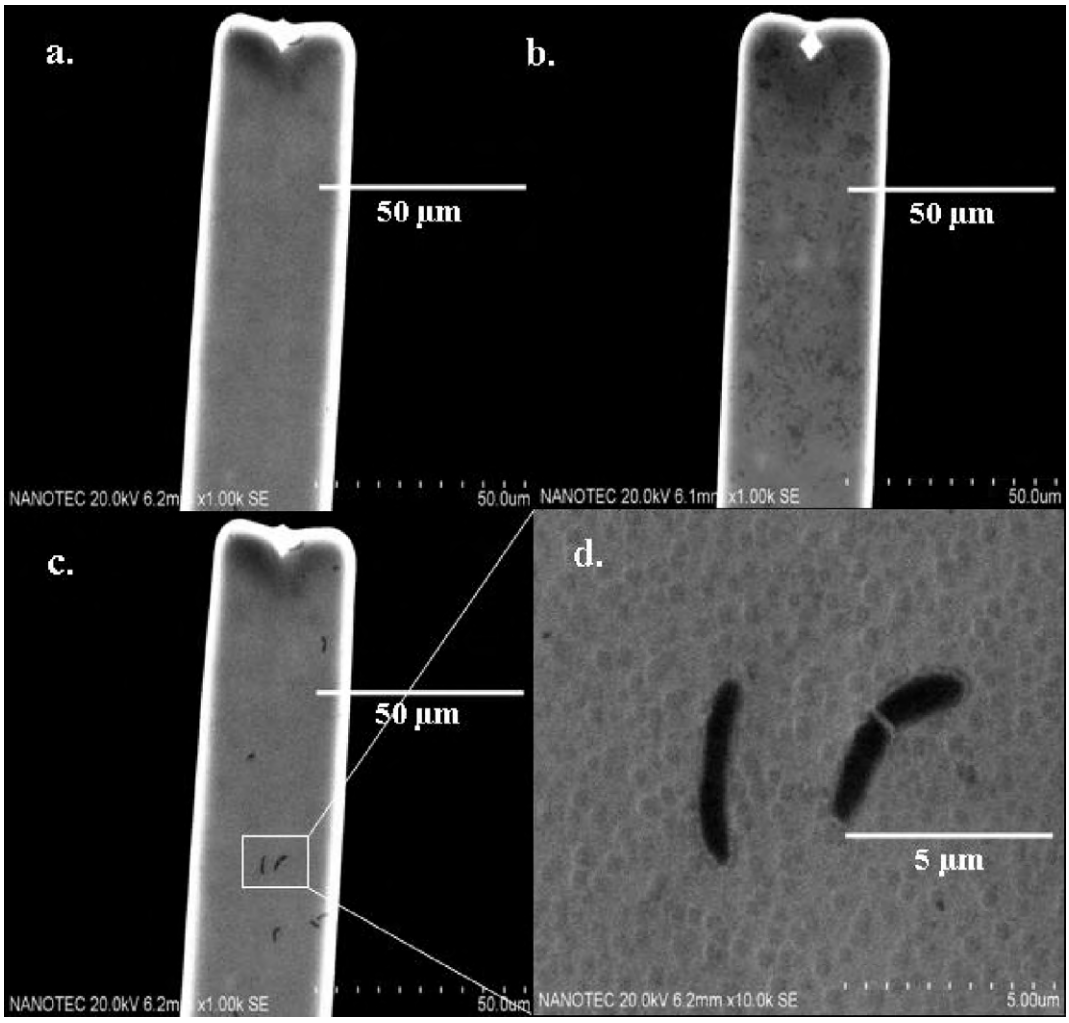

Fig. 5. Scanning electron microscope (SEM) images of antibody-immobilized microcantilever surface (a) before antibody immobilization (b) after antibody immobilization (c) after *V. cholerae* O1 binding at a concentration of 10^3 CFU/ml and (d) high magnification view of *V. cholerae* O1 cells on gold surface.

3.3. Surface characterization

The tested microcantilever was examined by scanning electron microscope (SEM) to characterize *V. cholerae* O1 cells binding on microcantilever surface and to confirm that the resonance frequency shift was generated from the mass of bacteria cells bound on the microcantilever. Fig. 5a and b shows the SEM image of the cantilever beam before and after antibody immobilization, respectively. It can be seen that the cantilever surface before antibody immobilization is very smooth and becomes shaded after the immobilization. However, anti-*V. cholerae* O1 protein molecules immobilized on the gold surface cannot be clearly observed because of its very small size. Fig. 5c demonstrates the SEM micrograph of the microcantilever beam after *V. cholerae* O1 binding at a concentration of 10^3 CFU/ml. It can be observed that thin and long dark cells are now scattered on the cantilever surface and higher magnification view (Fig. 5d) reveals that the cells have curve-rod shaped, which is a typical characteristic of *V. cholerae* bacteria.

It should be noted that the number of *V. cholerae* O1 bacteria may not be precisely counted because of its large size variation and agglomeration. In addition, the cell mass of *V. cholerae* O1 is presently unknown. Thus, it is difficult to correlate the results from surface analysis to the estimated mass change by QCM. Moreover, non-uniform distribution of bacteria would considerably affect the mass change estimation. As a result, the mass change calculated from Eq. (1) may not be accurate. However, it is still useful to estimate the mass change to obtain the order of magnitude of the mass sensitivity.

4. Conclusion

In conclusion, we have developed microcantilever-based biosensor for detection of *V. cholerae* O1, an important food and waterborne pathogen. Commercial gold-coated AFM microcantilevers are immobilized with monoclonal antibody (anti-*V. cholerae* O1) by self-assembled monolayer method. *V. cholerae* O1 detection experiment is performed in concentrations ranging from 1×10^3 to 1×10^7 CFU/ml. The microcantilever sensor has a high sensitivity of ~146.5 pg/Hz and a low detection limit of 1×10^3 CFU/ml, which is at least two orders of magnitude lower than other reported techniques and sufficient for *V. cholerae* detection in food products without pre-enrichment steps. In addition, linear relationship between the resonance frequency and the log of *V. cholerae* O1 concentration is obtained in concentration ranging from 1×10^3 to 1×10^6 CFU/ml. Therefore, the microcantilever-based biosensor is promising for direct detection of *V. cholerae* O1 cells in microbiological laboratories.

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