

Quantitative Label-free Biodetection of Acute Disease Related Proteins Based on Nanomechanical Dynamic Microcantilevers

Kyo Seon Hwang^{***}, Byung Hak Cha^{*}, Sang Kyung Kim^{*}, Jung Ho Park^{**}, and Tae Song Kim^{*†}

Abstract— We report the label-free biomolecules detection based on nanomechanical microcantilevers operated in dynamic mode for detection of two marker proteins (myoglobin and creatin kinase-MB (CK-MB)) of acute myocardial infarctions. When the specific binding between the antigen and its antibody occurred on the functionalized microcantilever surface, mechanical response (i.e. resonant frequency) of microcantilevers was changed in lower frequency range. We performed the label-free biomolecules detection of myoglobin and CK-MB antigen in the low concentration (clinical threshold concentration range) as much as 1 ng/ml from measuring the dynamic response change of microcantilevers caused by the intermolecular force. Moreover, we estimate the surface stress on the dynamic microcantilevers generated by specific antibody-antigen binding. It is suggested that our dynamic microcantilevers may enable one to use the sensitive label-free biomolecules detection for application to the disease diagnosis system based on mechanical immuno-sensor.

Index Terms—Microcantilever, resonant frequency, Myocardial infarctions, surface stress, nanmechanics

I. INTRODUCTION

Biomolecules detection technology based on numerous biosensors has been used in a variety of applications including recognition of biomaterials, such as, disease-related DNA sequence, specific protein, and enzyme with several functions [1]. One major goal of a biosensor system is to detect biomolecules of very small amounts with good accuracy, reliability, and a simple process step. To achieve this goal, the highly sensitive biosensors development is in the limelight of the biological field. In this point of view, there has been growing interest in ultra-highly sensitive biosensors that use micro-electro-mechanical systems (MEMS) and nano-electro-mechanical systems (NEMS). Especially, microcantilevers have been proposed as nanomechanical transducers to detect the presence of specific compounds with selectivity and quantity [2-4]. Recent studies reports nanomechanical cantilever-based label-free biomolecules detection which have important meaning in clinical diagnosis [5-10]. Moreover, the nanomechanical microcantilever biosensor has played a significant role in biophysics and biochemistry. Specifically, miniaturized cantilever has enabled one to measure the force [11], in the order of a pico (10^{-12}) Newton, generated on the biomolecules through the biophysical and/or biochemical process such as DNA unzipping [12], protein unfolding [13, 14], etc. The principle to measure this force resides in that the biophysical and/or biochemical process generates the force, and consequently, the deflection of a cantilever [15]. Recently, this principle has also enabled one to develop the cantilever biosensor for the label-free detection [16]. The biophysical process and/or biochemical

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process, i.e. specific analyte-receptor binding, on the microcantilever surface drives the buckling force, and consequently, the deflection change of a microcantilever [17]. For instance, the cantilever biosensor has allowed one to implement the label-free detection of several marker proteins such as myoglobin [10], prostate specific antigen [5], Taq DNA polymerase [18], etc.

In spite of these capability of a microcantilever biosensor operated in static mode (to measure the deflection change) for label-free biomolecules detection, the static-mode microcantilevers have the restrictions such that it requires the large length scale for measuring the deflection (see Stoney's equation). That is, a submicron-scale cantilever operated in static mode may not be utilized for label free detection. On the other hand, the cantilevers operated in vibration modes (oscillation) allow one to implement the highly sensitive label-free detection through the decrease of length scale that broadens the dynamic response range. Recently, the microcantilevers operated in the vibration modes provide the highly sensitive label-free detection of biomolecular interactions [6-9]. The principle is that the biomolecular interaction drives the dynamic response change of microcantilevers [19, 20]. It is recently reported that the dynamic response change is mainly driven by the surface stress induced by biomolecular interactions rather than mass change effect. The recent studies show that the oscillating microcantilevers can detect the biomolecules in the low concentration in the solution [6, 7, 9].

In this paper, we report the cantilever biosensor-based marker proteins (myoglobin and creatin kinase-MB antigen) detection of acute myocardial infarctions (AMIs) and dynamic response analysis of microcantilevers through our beam model in order to understand the relationship between dynamic response and surface stress induced by protein-protein interaction. The dynamic response is induced by protein-protein interactions and described by the resonant frequency shift of our fabricated nanomechanical microcantilever. Specifically, we consider the dynamic response of microcantilevers driven by the myoglobin and creatin kinase-MB (CK-MB) antigen-antibody interactions even for low concentration of antigen such as 1 ng/ml to 100 ng/ml. We also estimate the surface stress on the oscillating microcantilevers induced by antigen-antibody interactions. The results indicate that the quantitative study of the surface stress on the microcantilevers can provide insight into biomolecular interactions.

II. MATERIALS AND METHODS

1. Microcantilevers.

We fabricated microcantilevers with dimension of $50 \mu\text{m} \times 150 \mu\text{m} \times 2.15 \mu\text{m}$ (width \times length \times thickness) by the micromachining process as shown in Fig. 1. (for details of fabrication process of microcantilever, see Ref. 7). Our fabricated microcantilevers consist of multiple layers with silicon nitride (SiN_x), silicon dioxide (SiO_2), platinum (Pt), and piezoelectric (PZT) material. The silicon

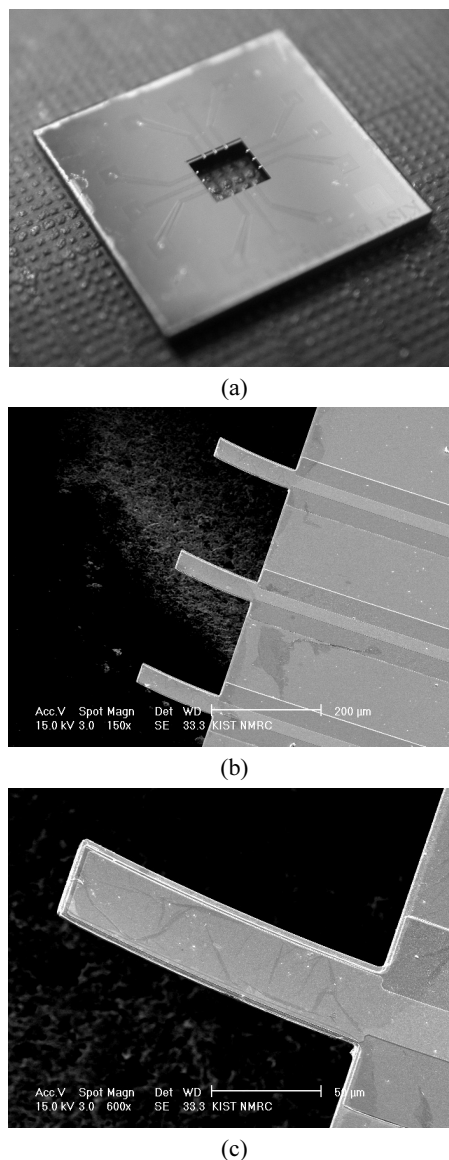


Fig. 1. (a) Photograph showing whole device with twelve cantilevers, and a SEM images of (b) microfabricated cantilever array, (c) unit microcantilever with the dimension of $50 \mu\text{m} \times 150 \mu\text{m}$ (width \times length).

nitride plays a significant role in the actuation such that it allows one to enhance the resonant frequencies due to high elastic performance, and it also provides the role of supporting layer material for a microcantilever. The silicon dioxide performs the function as a biological passivation layer to prevent the physical and/or chemical denaturalization of piezoelectric material during the biological surface treatment process. The sandwich structure that the piezoelectric material is embedded in the platinum layers performs the self-actuation through the piezoelectric and converse piezoelectric effect. Consequently, our microcantilevers do not require any external actuators for vibration so that it may possess the higher sensitivity with miniaturization than mono-layered microcantilevers requiring the external actuator. The microcantilever has a length scale on the order of $10^2 \mu\text{m}$, and thus the resonant frequency is on the order of 10^2kHz . Specifically, the resonant frequencies for our microcantilever arrays (6 cantilevers for each array) are $74 \pm 3 \text{kHz}$ for the first mode. This is consistent with the analytical prediction of $\omega_1^0 = 77.662 \text{kHz}$ for the first mode. The small deviation of experimental data from the analytical predictions for the resonant frequencies may be originated from the etching fabrication process. This process might slightly affect the thickness of the cantilevers, resulting in alternation of the resonant frequencies of the microcantilever.

2. Functionalization of the Microcantilever Surface

The microcantilever surface, for the interactions between antibody and antigen, is functionalized as follows (see Fig. 2): The gold layer (50 nm thickness) is deposited on the microcantilever surface, and then the microcantilevers were cleaned in a fresh piranha solution (a 4:1 ratio of H_2SO_4 [98.08%] and H_2O_2 [34.01%]) for 1 min for removing the experimental contamination of the gold layer. Further, the microcantilevers were rinsed with deionized water, and then dried under a stream nitrogen gas. Then, the self-assembled mono-layer (SAM) driven by Calixarene was formed on the microcantilever surface through the immersion of the gold-deposited microcantilevers into a solution of Calixcrown in CH_2Cl_2 , at room temperature for 3 hours.

The immobilization of antibody on the functionalized surface of the microcantilever is ascribed to the feature of Calixcrown SAMs that SAMs possess the interaction

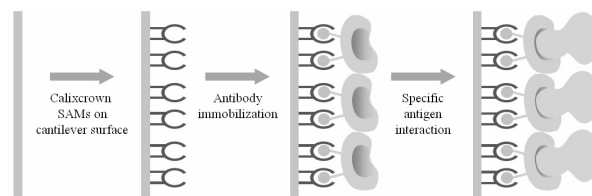


Fig. 2. A schematic diagram explains interaction between antigen and monoclonal antibody using Calixcrown SAMs on Au surface of cantilever. The major binding force could be attributed to the antibody which binds to the crown moiety of the linker molecule through host-guest interaction.

with ammonium ions of biomolecules. The major binding force between the Calixcrown and the antibody could be attributed to the ionized amine groups of the antibody, which bind to the crown moiety of the linker molecule via a host-guest interaction. Hydrophobic interactions between the hydrophobic residues of the antibody and methoxy groups of Calixcrown may also be involved in protein immobilization. This immobilization technique onto Calixcrown SAMs could be performed by minimized nonspecific antibody binding and increased antigen binding to the antibody caused by the correct orientation of the antibody immobilized in high density on the Calixcrown SAMs [21]. The immobilization process is summarized as follows: The microcantilever with the functionalized surface was immersed in the antibody diluted sterilized PBS (phosphate buffered saline), with a concentration of $10 \mu\text{g/ml}$, at room temperature for 1 hour. The microcantilever was then washed with sterilized PBST (PBS with 0.5% Tween 20, pH 7.8, St. Louis, MO, USA) of 1 ml and dried under nitrogen gas. Subsequently, in order to prevent the non-specific binding, the immobilized microcantilever was dipped into dissolved bovine serum albumin (BSA – Sigma, St. Louis, MO, USA) in sterilized PBS with a concentration of $10 \mu\text{g/ml}$, for 1 hour at room temperature. Then, the microcantilever was rinsed with sterilized PBST (pH 7.4, PBS with 1% Tween 20, St. Louis, MO, USA), and finally the microcantilever was washed with sterilized PBS solution only.

The myoglobin and CK-MB antibody-antigen binding experiments were performed using Cy5 (Amersham Biosciences, Piscataway, NJ, USA) fluorescence labeled antigen (Fitzgerald Industries) in order to confirm specific binding after antibody-antigen interaction. To label proteins with fluorescence probes, Fluorolinker Cy5 mono reactive dye (Amersham Biosciences, Piscataway, NJ, USA) was dissolved in 1 M sodium bicarbonate buffer (pH 9.3)

and allowed to react with target proteins for 1 hour. Free dye was then separated from labeled proteins by gel filtration in a Sephadex G-50 column (Amersham Biosciences).

3. Measuring the Resonant Frequency Shift

In order to measure the resonant frequency of the microcantilevers, we used an impedance analyzer (4294A, Agilent technologies, USA), which enabled the monitoring of the phase angle as a function of the sweeping frequency (see Fig. 3). Specifically, while the phase angle of the impedance for a microcantilever that functions as a capacitor is approximately -90° in the off-resonance, the phase angle in the resonant frequency exhibits the peak value is responsible for the microcantilever motion. The resonant frequency shift induced by the interaction between antibody and antigen was evaluated as follows: (i) We measured the resonant frequency for the microcantilever before dipping into antigen dissolved solution. (ii) The microcantilever is dipped into antigen dissolved solution with three different concentrations such as 1 ng/ml, 10 ng/ml, and 100 ng/ml, and then the microcantilever is rinsed with sterilized PBS. (iii) The resonant frequency is measured for the microcantilever, which was processed as step (ii), in the temperature- and humidity-controlled chamber. (iv) The resonant frequency shift due to antibody-antigen interaction is evaluated from the difference between frequencies obtained from step (i) and (iii). The measurement was carried out at 37°C and R.H. 70 % using temperature & climatic test systems (VC4018, Votsch Industrietechnik, Germany).

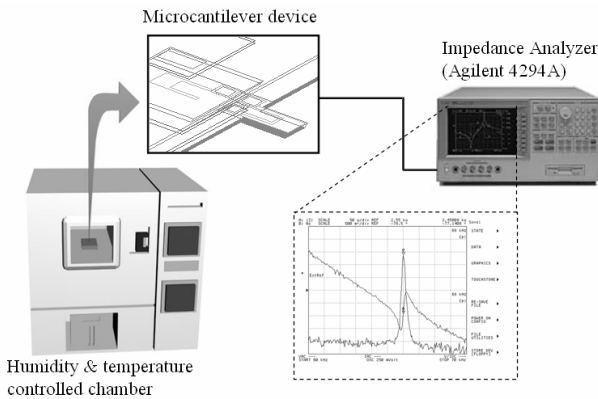


Fig. 3. Schematic of the detection system for resonant frequency measurement of nanomechanical microcantilevers by using an impedance analyzer. The measurement was carried out at 37°C and R.H. 70 % using temperature & climatic test systems for measuring in same environment.

4. Surface Stress Generated by Biomolecular Interaction

The recent study provides that, with the ligand-receptor binding on the cantilever surface, the resonant frequency of a microcantilever represented is shifted into the resonant frequency in the form of

$$\tilde{\omega}_i \equiv \omega_i + \delta\omega_i = \left(\frac{\lambda_i}{L}\right)^2 \sqrt{\left(\frac{\xi}{\mu + \delta\mu}\right) \left(1 + \frac{2}{\pi^2} \frac{\tau L^3}{\xi}\right)} \quad (1)$$

where $\delta\omega_i$ is the resonant frequency shift due to ligand-receptor binding, $\delta\mu$ is the mass change per unit length of a cantilever due to the adsorption of the ligand, and τ is the surface stress originated from the intermolecular interaction and entropic effect. In general, it is known that the contribution of mass of the ligand to the resonant frequency shift is negligible so that the surface stress is the dominant parameter for the resonant frequency shift [8, 22]. Further, the ratio of $\delta\omega_i/\omega_i$ is in the order of 10^{-2} for our case, and consequently, the surface stress can be represented by the linear equation with respect to the parameter $\delta\omega_i/\omega_i$.

$$\tau = \pi^2 \left(\frac{\xi}{L^3}\right) \left(\frac{\delta\omega_i}{\omega_i}\right) \quad (2)$$

One can easily notice that the term $\pi^2\xi/L^3$ in Eq. (2) is the surface stress corresponding to the static Euler buckling load $P_{cr} = \pi^2\xi/L^2$. It indicates that the dynamic response change of a microcantilever is induced by the buckling of the microcantilever due to ligand-receptor binding. The microcantilevers are very sensitive to not only the ligand-receptor binding but also the disturbance such as non-specific binding. Hence, the non-specific binding can be prevented through biological passivation (blocking) the surface with molecules similar to receptors. This was validated by monitoring the resonant frequency of microcantilevers when they are exposed to the non-specific proteins such that there was no resonant frequency shift, indicating that the functionalized microcantilevers exhibit the specific interactions with the specific analytes.

III. RESULTS AND DISCUSSION

1. Dynamic Response of the Fabricated Microcantilever

The impedance analyzer enables the measurement of the resonant frequency of microcantilevers. Fig. 4 shows

the phase angle change as a function of the sweeping frequency of the proposed fabricated microcantilever with a dimension of $50 \mu\text{m} \times 150 \mu\text{m}$ (width \times length). At off-resonance, the phase angle is not quite -90° due to parasitic impedance generated by the cable and the probe connecting the electrode of the cantilever to the impedance analyzer. However, the phase angle shift in the resonance was much larger than phase angle shift originated from the parasitic impedance, thus the parasitic impedance is negligible in the measurement of the resonance frequency.

The dynamic response of a microcantilever in normal air, due to piezoelectric effect of PZT material that could enable the energy conversion between mechanical and electrical energy, is described by equation of cantilever's resonant frequency (see Ref. 23). With the mechanical properties and geometric parameters of a microcantilever, the theory predicts the first mode resonant frequency such as $f = 67.878 \text{ kHz}$ ($\omega = 2\pi f$). This magnitude is comparable to the experimental data, that is, the primary resonant frequency of our microcantilever is about $f = 67 \pm 3 \text{ kHz}$. The small amount of discrepancy may be ascribed to the fabrication process such as etching process that may affect the thickness of the SiN_x layer, and consequently, the flexural rigidity and the cross-sectional area.

As a structure approaches resonance, the amplitude of its vibration would increase, its resonant frequency being defined as the point of maximum amplitude. When we find the resonance frequency of microcantilever with phase angle shift, increasing the sharpness of the resonance curve enables the resonant frequency to be more clearly defined and would improved the performance and resolution of the microcantilever. The sharpness of resonance curve can be expressed as a quality factor (Q, Q-factor), Q

$$Q = \frac{f_r}{\Delta f} \quad (3)$$

where f_r is the resonant frequency and Δf is the full-width-half-maximum (FWHM) of resonance curve. Our fabricated microcantilever shows the Q-factor of about 135 that can clearly define the frequency of maximum amplitude point (resonant frequency) for detecting the antibody-antigen interaction.

2. Experimental Quantitative Myoglobin Antigen Analysis using Fluorescence Image

The examination of myoglobin antibody-antigen interaction, as a function of myoglobin antigen concentration, was carried out by using a laser confocal scanner (GSI Lumonics Co.). Fig. 5 shows fluorescence scanner images as a function of interacted myoglobin antigen concentrations with (a) 1 ng/ml, (b) 10 ng/ml, (c) 100 ng/ml, and negative controlled with (d) 10 $\mu\text{g/ml}$ of prostate specific antigen. Fluorescence images from fig. 4 indicated the presence of Cy5 labeled myoglobin antigen (with three different concentration) binding to the immobilized antibody on Calixcrown SAMs formed cantilever. The brightness of cantilever image increased with the increase of myoglobin concentration, indicating that the trend of resonance frequency shift (see Fig. 6)

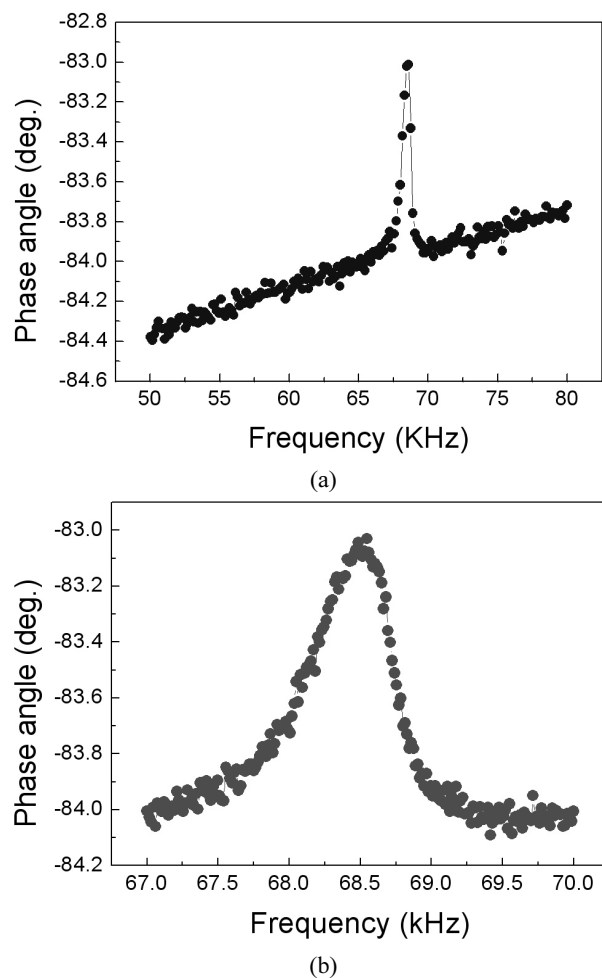


Fig. 4. The phase angle as a function of sweeping frequency of microfabricated nanomechanical cantilever range of (a) 50 kHz to 80 kHz, and (b) 67kHz to 70 kHz.

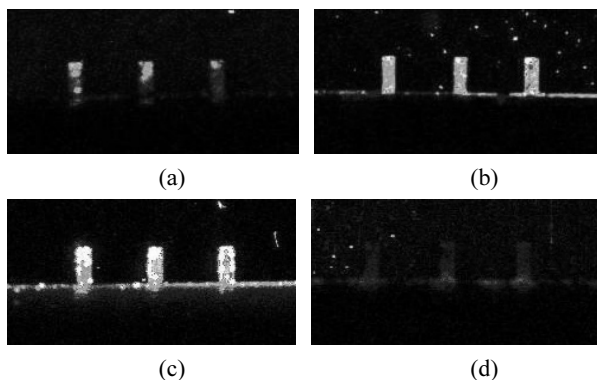


Fig. 5. The fluorescent scanner images as a function of the interacted myoglobin antigen with (a) 1 ng/ml, (b) 10 ng/ml, (c) 100 ng/ml, and, as a negative control, with (d) 1 µg/ml of prostate specific antigen. Fluorescence images indicate the presence of Cy5 labeled myoglobin antigen binding to an active immobilized myoglobin antibody on cantilever's Au surface, whereas nonspecific binding can not be seen on the other side.

was much similar to the fluorescence results. Negative control means that the response when myoglobin antibody immobilized cantilever is exposed to a different kind of protein. For negative control, the PZT nanomechanical cantilever, which was immobilized with myoglobin antibody, were exposed to a solution with a 1 µg/ml of prostate specific antigen. As can be seen in Fig. 5(d), Fluorescence image reveals that the nonspecific binding between capture myoglobin antibody and prostate specific antigen can be negligible. It means high specificity of myoglobin protein against other protein. Fluorescence images also indicate the presence of Cy5 labeled myoglobin antigen binding to an active immobilized myoglobin antibody on cantilever's Au surface, whereas nonspecific binding can not be seen on the other side. This suggests that resonant frequency change occur only due to myoglobin antigen-antibody interaction. In case of the CK-MB antigen-antibody interaction, we can achieve similar result fluorescent images compare with myoglobin antigen-antibody interaction.

3. Quantative Protein (Myoglobin and CK-MB Antigen) Detection using Microcantilever

We consider the resonant frequency shift caused by myoglobin and CK-MB antigen-antibody interactions with antigen concentrations of 1 ng/ml, 10 ng/ml, and 100 ng/ml. The resonant frequency shift for the first mode induced by the myoglobin antigen-antibody interaction

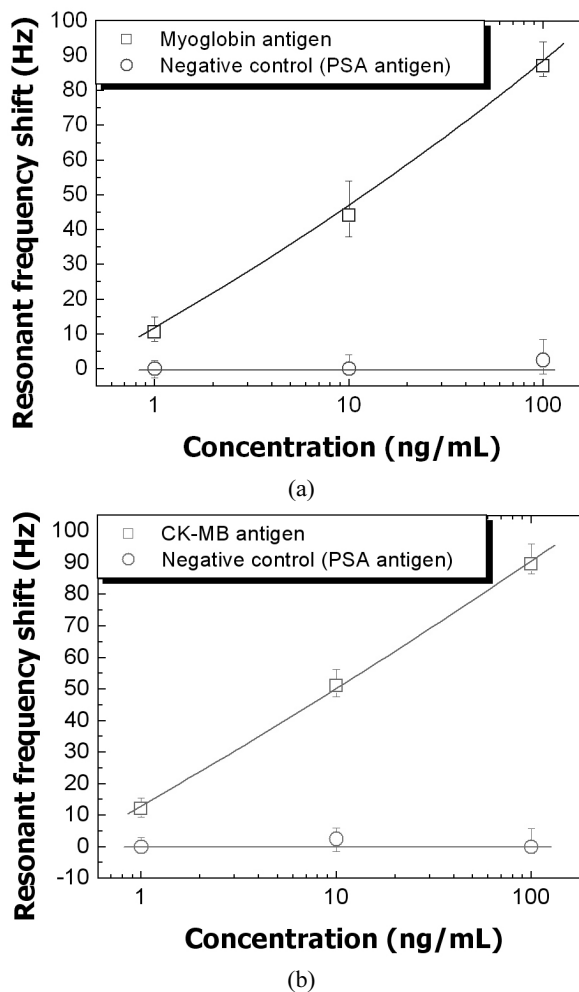


Fig. 6. Experimental resonant frequency shift as a function of (a) myoglobin and (b) CK-MB antigen concentration. The higher antigen concentration, the larger mechanical response change of microcantilever was occurred. Further, when the non-specific antigen was reacted with myoglobin and CK-MB monoclonal antibody immobilized microcantilever, there is no resonant frequency.

interaction is on the order of 1 Hz for given antigen concentrations. Fig. 6 shows the experimental results of the mechanical response as a function of the myoglobin (Fig. 6 (a)) and CK-MB (Fig. 6 (b)) antigen concentration ranging from 1ng/ml to 100 ng/ml and we can achieve the detection limit in the clinical threshold concentration range. Increasing antigen concentrations of 1, 10, and 100 ng/ml let to respective increasing values of the experimental resonant frequency change of 10.5, 44, and 87 Hz in case of myoglobin antigen and 12, 51, 89.5 Hz in case of CK-MB antigen due to spring constant change of cantilever caused by surface stress generation [24].

From these experimental results it is evident that

antibody on the microcantilever surface interact to specific antigen with no labeling being necessary. Moreover, the specific binding property of antigen and its specific antibody was validated by monitoring the resonant frequency of microcantilevers when they are dipped into the nonspecific antigen (prostate specific antigen). When this happened, there was no resonant frequency shift of microcantilevers, indicating that the myoglobin and CK-MB antibody exhibit the specificity in binding of myoglobin and CK-MB antigen. Furthermore, we can obtain that the majority origin of resonant frequency shift of nanomechanical microcantilever is surface stress change due to intermolecular force [24]. In spite of the molecular weight of two protein (myoglobin and CK-MB antigen) is different values, the resonant frequency shift value is very similar. That is, surface stress change of dynamic microcantilever dominates in resonant frequency shift of microcantilever for the biomolecules detection. The quantitative myoglobin and CK-MB antigen detection results indicates that the surface stress with respect to the increase in the population of antibody-antigen pairs, indicating that the intermolecular interactions between antibody-antigen pairs generate the compressive stress.

4. Surface Stress

For the analysis of surface stress generation caused by biomolecular interaction on the microcantilever surface, we performed a comparative study with research Gerber's group [10]. The dynamic motion of the dynamic-mode microcantilevers or the mechanical behavior of the static-mode microcantilevers, for the label-free detection, is originated from the surface stress caused by the biomolecular interactions such as antigen-antibody interactions. We evaluated the surface stress caused by myoglobin and CK-MB antigen-antibody interactions in our microcantilevers with the data of resonant frequency shifts from the Eq. 2, shown as in fig. 7. The surface stress exerted in our microcantilevers ranges from 2×10^{-3} N/m to 2×10^{-2} N/m for the myoglobin and CK-MB antigen concentration from 1 ng/ml to 100 ng/ml. On the other hand, the microcantilevers of Gerber and coworkers exhibit the surface stress in the range between 2×10^{-3} N/m and 8×10^{-3} N/m for myoglobin antigen concentration from 20 μ g/ml to 100 μ g/ml and 4×10^{-3} N/m for CK-MB antigen concentration of 50 μ g/ml. It indicates that our microcantilevers are able to

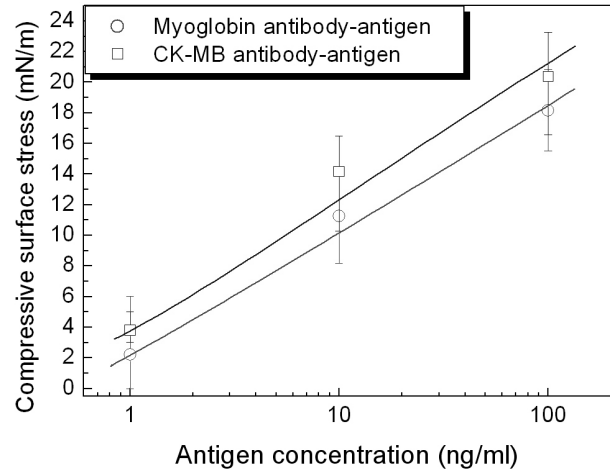


Fig. 7. The surface stress generation as a function of myoglobin and CK-MB antigen concentration in first mode. It indicates that our theoretical model is very consistent with experimental analysis results.

exert the larger surface stress even much lower biomolecular interactions, so that our microcantilever exhibits the much higher analytical sensitivity than the static-mode microcantilevers. The higher sensitivity (larger surface stress) of our microcantilevers may be ascribed to the experimental environment and the mechanical characteristics of microcantilevers. Specifically, our experiment was implemented in the relative humidity-controlled chamber so that the viscous effect (damping effect) might be negligible. The viscous effect may be of the significance for the dynamic motion of microcantilevers in the liquid chamber such that the viscous effect reduces the resonant frequency, and consequently, the surface stress by dissipating the vibration energy into the heat bath (liquid). Furthermore, from Eq. 2, the surface stress may be dependent on the mechanical characteristics of microcantilevers such as the bending modulus and the length scale. The larger bending modulus and smaller length scale might guarantee the larger surface stress, so that our microcantilevers have the larger surface stress than that of Gerber and coworkers because their microcantilevers are more compliant and longer than ours.

For quantitative comparison between our result and the result of Gerber and coworkers, we introduced the dimensionless surface stress defined as $\bar{\tau} = \tau / \tau_0$, where $\tau_0 = \xi / l^3$. This dimensionless quantity for the surface stress allows us to measure the quantity, indicating for the biomolecular interactions, which is independent of the mechanical characteristics of microcantilevers such

as bending modulus and length scale. Our microcantilevers exert the dimensionless surface stress from 0.001 to 0.01 for the myoglobin antigen concentration from 1 ng/ml to 100 ng/ml, while the microcantilevers of Gerber and coworkers have the dimensionless surface stress in the range between 0.82 and 3.79 for the myoglobin antigen concentration from 20 μ g/ml to 100 μ g/ml. This seems rationale in that the dimensionless surface stress of ours is much lower, by about 2-3 orders in magnitude, than that of Gerber and coworkers', because the myoglobin antigen concentration, responsible for biomolecular interactions, is lower than that of Gerber and coworkers' by about 3 orders in magnitude. This indicates that our dynamic-mode microcantilevers enable us to detect the much smaller biomolecular interactions than the static-mode microcantilevers.

IV. CONCLUSIONS

We demonstrate label-free biomolecules detection based on resonant frequency change of nanomechanical dynamic microcantilever. Moreover, we provide the quantitative analysis regarding the role of the surface stress induced by biomolecular interactions on the dynamic response of microcantilevers. It is shown that antigen-antibody interaction induces the compressive surface stress, and consequently, reduces the resonant frequency. Our study shows that our nanomechanical microcantilever is able to detect the biomolecular interactions (i.e. myoglobin and CK-MB antigen-antibody interaction) for the much lower concentration of myoglobin antigen than the nanomechanical microcantilever of Gerber's group. This suggests that the miniaturized self-actuating nanomechanical microcantilever is very appropriate for sensitive detection of biomolecular interactions. This indicates that our nanomechanical microcantilever, composed of multiple layers including PZT material for self-actuating vibration, is very suitable for not only the MEMS application but also the experimental tool for sensitive detection of biomolecular interactions. Our results indicate that the self-actuated vibrating microcantilever with smaller length scale allows us to detect the biomolecular interactions, which is even much smaller than that generated in the conventional microcantilever in the static-mode type, with much higher sensitivity. Hence, our study shed light on the great potential of the

nanomechanical microcantilever for developing the high sensitive biosensor for detecting.

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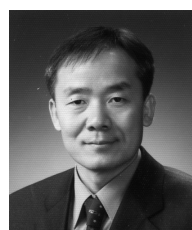


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