Nanomechanical Microcantilever Operated in Vibration Modes with Use of RNA Aptamer as Receptor Molecules for Label-Free Detection of HCV Helicase

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ABSTRACT

We report the nanomechanical microcantilevers operated in vibration modes (oscillation) with use of RNA aptamers as receptor molecules for label-free detection of hepatitis C virus (HCV) helicase. The nanomechanical detection principle is that the ligand-receptor binding on the microcantilever surface induces the dynamic response change of microcantilevers. We implemented the label-free detection of HCV helicase in the low concentration as much as 100 pg/ml from measuring the dynamic response change of microcantilevers. Moreover, from the recent studies showing that the ligand-receptor binding generates the surface stress on the microcantilever, we estimate the surface stress, on the oscillating microcantilevers, induced by ligand-receptor binding, i.e. binding between HCV helicase and RNA aptamer. In this article, it is suggested that the oscillating microcantilevers with use of RNA aptamers as receptor molecules may enable one to implement the sensitive label-free detection of very small amount of small-scale proteins.

1. Introduction

The nano- and/or micro-mechanical cantilever has played a significant role in biophysics and biochemistry. Specifically, miniaturized cantilever has enabled one to measure the force (Best *et al*. 2003, Hansma *et al*. 2000, Lavery *et al*. 2002), in the order of a pico (10^{-12}) Newton, generated on the biomolecules through the biophysical and/or biochemical process such as DNA unzipping (Allemand *et al*. 2003, Strick *et al*. 2000), protein unfolding (Fisher *et al*. 2000, Marszalek *et al*. 1999, Strick *et al*. 2003), 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 (Allison *et al*. 2002) Recently, this principle has also enabled one to develop the cantilever-based bioassay for the label-free detection (Backmann *et al*. 2005). The biophysical process and/or biochemical process, i.e. ligand-receptor binding, on the microcantilever surface drives the buckling force, and consequently, the deflection change of a microcantilever (Wu *et al*. 2001b). For instance, the cantilever-based bioassay has allowed one to implement the label-free detection of several marker proteins such as myoglobin (Arntz *et al*. 2003), prostate specific antigen (Wu *et al*. 2001a), Taq DNA polymerase (Savran *et al*. 2004), etc.

In spite of capability of a microcantilever operated in static mode (to measure the deflection change) for label-free 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 (Hwang *et al*. 2004b, Lee *et al*. 2005, Lee *et al*. 2004a, Lee *et al*. 2004b). The principle is that the biomolecular interaction drives the dynamic response change of microcantilevers (Ilic *et al*. 2005, Ilic *et al*. 2004). 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 (Dorignac *et al*. 2006, Hwang *et al*. 2006, Lee *et al*. 2004a). The recent studies show that the oscillating microcantilevers can detect the biomolecules in the low concentration in the solution (Hwang *et al*. 2004b, Lee *et al*. 2005, Lee *et al*. 2004b).

It is recently suggested that the surface stress on the microcantilever surface due to ligand-receptor binding is originated from the intermolecular interactions such as steric repulsion and entropic effect corresponding to the configuration change of protein molecule (Wu *et al*. 2001b). It provides that the entropic effect is negligible for antigen-antibody interaction so that the intermolecular interaction is the dominant parameter for the microcantilever motion. This indicates that the configuration change due to antigen-antibody interaction may not be detectable in the existing cantilever-based assay. Consequently, the cantilever-based bioassay may be unable to detect the small-scale proteins in the very low concentration such that the intermolecular interaction is not quite paramount. In order to resolve this challenging issue, it is recently attempted to use the biomolecules, which can undergo large configuration change, as receptor molecules so that one may conduct the highly sensitive detection of biomolecules in the very low concentration in the blood serum. Specifically, the recent studies report that one may use the single chain Fv (scFv) antibody fragment (Backmann *et al*. 2005) and/or DNA aptamers (Savran *et al*. 2004), as receptor molecules, which may allow one

to implement the highly sensitive label free detection.

The aptamers have recently been used as receptor molecules due to several features. The aptamers that are usually selected from large randomized oligonucleotides libraries exhibit the specificity to the target protein with high affinity (Famulok *et al*. 2000, Wilson and Szostak 1999). In general, most of antibodies possess the sensitivity to temperature (thermal unfolding) and denaturation upon contact with surfaces, whereas the aptamers have the stability during the long term storage and reversible denaturation (Kirby *et al*. 2004). The small scale of aptamers enables one to construct the denser receptor layers so that one may apply the aptamer layers to the biosensors with increase in the sensitivity (Liss *et al*. 2002). Moreover, the aptamers can be easily immobilized on the functionalized surface of biosensors through chemical modification at terminal sites, because they can be modified with functional groups (Liss *et al*. 2002). With these characteristics, the aptamers has been widely used as receptor molecules for the biosensor applications such as enzyme-linked immunosorbent assay (ELISA) (Drolet *et al*. 1996), fluorescence microscopy (Blank *et al*. 2001), and quartz crystal microbalance (QCM) (Liss *et al*. 2002). Recently, the DNA aptamers has been utilized as receptor molecules for the microcantilevers operated in static mode (Savran *et al*. 2004).

For the highly sensitive label-free detection, it may be desirable to utilize the oscillating microcantilevers exhibiting the small length with use of DNA and/or RNA aptamers as receptor molecules. In this article, we report the label-free detection of Hepatitis C Virus (HCV) helicase in the concentration of 100 pg/ml with use of oscillating microcantilevers utilizing the RNA aptamers as receptor molecules. Specifically, we implement the label-free detection of HCV helicase by measuring the

dynamic response change, i.e. the resonant frequency shift, due to binding between HCV helicase (ligand) and RNA aptamers (receptor). Moreover, we estimate the surface stress induced by ligand-receptor binding on the microcantilever surface for the oscillating microcantilevers. This study may suggest that the oscillating microcantilevers using RNA apatmers as receptor molecules provide the sensitive label-free detection in the very low concentration of proteins.

2. Experimental Section

2.1. Preparation of amino group-modified RNA aptamer

The 3′-tail of RNA aptamer (5′-GGGAGAGCGGAAGCGUGCUGGGCCACAUUGUG AGGGGCUCAGGUGGAUCGCAUGGCCGUGUCCAUAACCCAGAGGUCGAUG GAUCCU-3′) was enzymatically aminated with terminal transferase by using 5-(3-aminoallyl)-deoxy-uridine-5′-triphosphate. 121 mM RNA template was mixed with 1 mM 5-(3-aminoallyl)-deoxy-uridine-5′-triphosphate, 25 mM Tris-HCl, 0.25 mg/ml BSA, 2.5 mM $CoCl_2$, 20 units RNase inhibitor, and 20 units terminal transferase at 37℃ for 1 hr, and then, incubated at 80℃ for 3 min. The reaction was quenched by adding 0.5 M EDTA, and the amino group-modified RNA aptamer was purified by phenol extraction and subsequent ethanol purification.

2.2. Preparation of HCV helicase protein

HCV helicase was cloned as follows (Hwang *et al*. 2004a). HCV helicase cloned vector was inserted in BL21(DE3) and each cell was cultured in a shaking incubator at 37℃. 1mM IPTG was added to a cell when a density reaches 0.5 in OD_{600}. A cell extraction from induction for 5 hr was prepared by sonication for 20 min in a phosphate buffer

containing 1 mM PMSF and 1 mM EDTA. Proteins that possess six consecutive histidine residues on C termini were purified with Ni-NTA agarose resin (Qiagen, Hilden, Germany) from cell extraction. The elution solution of 250 mM imidazole, which was used in purifying processing step, was removed by dialysis process, and quantitation of protein was performed by a Bradford assay.

2.3. Microcantilever biosensor

We fabricated microcantilevers with dimension of 50 μm × 150 μm × 2.15 μm (width × length × thickness) by the micromachining process. We have previously reported the details of microcantilever fabrication procedure in Refs.(Hwang *et al*. 2004b, Lee *et al*. 2005, Lee *et al*. 2004b). Figure 1 shows the scanning electron microscope (SEM) image of the cantilever-based array and a piezoelectric cantilever (in an array) that is able to actuate (oscillate) with use of piezoelectric and converse piezoelectric effects. Our fabricated microcantilevers consist of multiple layers with silicon nitride (SiN_x), silicon dioxide (SiO_2), platinum (Pt), and piezoelectric (PZT) material. The silicon 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 and sensing 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.

2.4. Functionalization of the microcantilever surface

The microcantilever surface, for the interactions between RNA aptamer and HCV helicase, 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_3Cl, at room temperature for 3 hours.

The immobilization of RNA aptamer on the functionalized surface of the microcantilever is ascribed to the feature of Calixcrown SAMs that SAMs possess the interaction with ammonium ions of biomolecules (Lee *et al*. 2003). The RNA aptamer was modified with an amine group at the terminal site to immobilize using Calixcrown SAMs. The immobilization process is summarized as follows: The microcantilever with the functionalized surface was immersed in the RNA aptamer diluted sterilized PBS (phosphate buffered saline), with a concentration of 100nM, at room temperature for 2 hours. The microcantilever was then washed with sterilized PBST (PBS with 0.5% Tween 20, pH 7.8, St. Louis, MO, USA) of 30 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 μg/ml, for 1 hour at room temperature. Then, the microcantilever was rinsed with sterilized PBST (pH 7.4, PBS with 5% Tween 20, St. Louis, MO, USA), and finally the microcantilever was washed with sterilized PBS solution only.

2.5. Detecting the interaction between RNA aptamer and HCV helicase

The microcantilevers allow one to detect the interactions between RNA aptamer and HCV helicase through measurement of the resonant frequency shift. All measurements were carried out in the temperature- and relative humidity-controlled chamber (25℃, 70% [R.H.]). The resonant frequencies, before and after the interaction between RNA aptamer and HCV helicase, were measured with the use of electrical measurement system as shown in Fig. 3(a). By changing the sweeping frequency of the sinusoidal wave on the cantilever's electrode, a sensing signal can be simultaneously measured. This is achieved through the use of an electrode on the cantilever via a special sensing mechanism that can read the sensing signal after inducing the driving signal at each frequency within a few micro seconds. The induced charge through the resonance of the piezoelectric cantilever was measured through the electrode in a DSP (digital signal processing) signal processed voltage variation form and was controlled by a PC (Lee *et al*. 2004a). The AC sine wave form of $1V_{pp}$ (peak to peak) was applied to the actuation electrode in order to vibrate the cantilever while the bottom electrode was grounded. The piezoelectric output from the electrode at the maximum deflection at the 1st resonant frequency was detected. The resonant frequency shift induced by the interaction between RNA aptamer and HCV helicase was evaluated as follows: (*i*) We measured the resonant frequency for the microcantilever before dipping into HCV

helicase dissolved solution. (*ii*) The microcantilever is dipped into HCV helicase dissolved solution with four different concentrations such as 0.1 ng/ml, 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 RNA aptamer-HCV helicase interaction is evaluated from the difference between frequencies obtained from step (*i*) and (*iii*).

3. Results and Discussions

3.1. Detection principle

The resonant frequency ω_i of a microcantilever in normal air is given by elasticity theory such as

$$\omega_i = \left(\frac{\lambda_i}{L}\right)^2 \sqrt{\frac{\xi}{\mu}} \tag{1}$$

where L is a cantilever length, ξ is a cantilever flexural rigidity, μ is an effective mass of a cantilever, and λ_i is a parameter that satisfies the transcendental equation such as $\cos\lambda_i \cosh\lambda_i + 1 = 0$. Upon ligand-binding on a cantilever surface, the resonance frequency, ω_i, of a cantilever (given by Eq. 1) is shifted into a resonant frequency, ν_i (Hwang *et al*. 2006).

$$\nu_i \equiv \omega_i + \Delta\omega_i = \left(\frac{\lambda_i}{L}\right)^2 \sqrt{\left[\frac{\xi}{\mu + \Delta\mu}\right]\left[1 + \frac{2}{\pi^2}\frac{\tau L^3}{\xi}\right]} \tag{2}$$

Here, $\Delta\omega_i$ is the resonant frequency shift due to ligand-binding, $\Delta\mu$ is the total mass of ligands, and τ is the surface stress induced by biomolecular interactions due to ligand-binding. It was recently reported that resonant frequency shift, $\Delta\omega_i$, is dominated by the

surface stress induced by biomolecular interactions rather than the total mass of ligands (Dorignac *et al*. 2006, Lee *et al*. 2004a). Hence, one is able to estimate the surface stress due to ligand-binding from the following equation.

$$\tau = \frac{\tau_0}{2}\left[2\left(\frac{\Delta\omega_i}{\omega_i} \right) + \left(\frac{\Delta\omega_i}{\omega_i} \right)^2 \right] \tag{3}$$

where a parameter τ_0 is the buckling load per unit length of a cantilever, i.e. $\tau_0 = \pi^2\xi/L^3$.

3.2. Dynamic response of microcantilevers for ligand-receptor binding.

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 Eq. (1). With the mechanical properties and geometric parameters of a microcantilever, the theory [Eq. (1)] predicts the primary resonant frequency such as ω = 68.724 kHz. This magnitude is comparable to the experimental data, that is, the primary resonant frequency of our microcantilever is about ω = 66 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.

We investigated the dynamic response change of a microcantilever driven by the ligand-receptor binding, i.e. binding between HCV helicase and RNA aptamers. Specifically, we consider the ligand-receptor binding in the several concentrations of ligands dissolved in the solution such as 0.1 ng/ml, 1 ng/ml, 10 ng/ml, and 100 ng/ml. Further, the specific binding property of RNA aptamers was validated by monitoring the resonant frequency of microcantilevers when they are exposed to the prostate specific antigen (PSA). There is no resonant frequency shift of the microcantilever due to PSA,

indicating that RNA aptamers exhibit the specificity of ligand-binding. Fig. 3(b) represents characteristic spectrum revealing the resonant frequency shift corresponding to specific interaction between RNA aptamer and HCV helicase (concentration of 10 ng/ml). The dynamic response change of a microcantilver induced by the binding between HCV helicase and RNA aptamers are presented in Fig. 4. This shows that the resonant frequency of a microcantilever is reduced when the binding between HCV helicase and RNA aptamers occurs. Further, it shows that the magnitude of resonant frequency shift is closely related to the packing density of ligand-bound protein, i.e. RNA aptamers bound with HCV helicase.

3.3. Surface stress induced by ligand-receptor binding.

The ligand-receptor binding induces the surface stress playing a dominant role on the dynamic response change of a microcantilever. The relationship between the dynamic response change and the surface stress induced by the ligand-receptor binding is represented as Eq. (3). As stated earlier, the surface stress is linearly proportional to the resonant frequency shift $\delta\omega_i/\omega_i$, because the term $(\delta\omega_i/\omega_i)^2$ is ignorable when it is compared with $\delta\omega_i/\omega_i$. From Eq. (3), the surface stress due to binding between HCV helicase and RNA aptamers is suggested in Fig. 5. Specifically, the theoretical model provides us that the binding between HCV helicase and RNA aptamers generates the compressive surface stress inducing the buckling of the microcantilever. The compressive surface stress due to binding between HCV helicase and RNA aptamers on our microcantilever ranges from 1.2×10^{-2} J/m^2 to 3.8×10^{-2} J/m^2 for the HCV helicase concentration in the range between 0.1 ng/ml and 100 ng/ml. As stated earlier, the resonant frequency shift due to binding between HCV helicase and RNA aptamers is a

function of the concentration of HCV helicase, and consequently, the surface stress is also a function of the packing density of RNA aptamers bound with HCV helicase. This may indicate that the surface stress might be generated by the intermolecular interactions between ligand-bound proteins. Moreover, even for the small amount of HCV helicase such as the concentration of 1 ng/ml, the order of the surface stress is quite comparable to that for the higher HCV helicase concentration such as 1 ng/ml ~ 10 ng/ml. It may suggest that the configuration change of RNA aptamers induced by ligand-binding (i.e. binding with HCV helicase) may play a role on the surface stress as well as intermolecular interactions between ligand-bound proteins. Specifically, it is known that RNA aptamer is the long nucleotide chain with loop region where protein can be bound to, and that the loop is very flexible when the ligand-binding occurs on the loop. This might provide that the ligand-binding to the RNA aptamers may drive the entropic change that may induce the surface stress on the microcantilever. This may shed light on that the RNA aptamers as receptor molecules may allow one to implement the label-free detection of very small amount of small-scale proteins. That is, the long nucleotide chain with flexible loop exhibiting the specific binding may perform the better function as a receptor molecule than the protein antibody that exhibits too small configuration change to generate the surface stress enough to be measured experimentally for the small amount of small-scale proteins.

4. Conclusion

We demonstrate the label-free detection of HCV helicase even in the low concentration with piezoelectric microcantilevers, operated in vibration mode, which utilize the RNA aptamers as receptor molecules. The specific binding property of RNA aptamers that

have the flexible loop regions where ligand-binding occurs may allow one to use the RNA aptamers as receptor molecules for enhancement of the sensitivity for the label-free detection. Specifically, the RNA aptamers as receptor molecules may enable one to construct the sensitive microcantilever for the ligand-binding even in the very small amount of the small-scale proteins. This may be ascribed to that the configuration change that induces the entropy change may play a role on the surface stress for the microcantilever. It indicates that, if one is able to construct the engineered RNA aptamers that exhibit the specific binding on the loop region, then it may be possible to improve the sensitivity of label-free detection of small-scale proteins in the small amount. That is, the engineered RNA aptamers as receptor molecules may provide the sufficient surface stress for the ligand-binding to RNA aptamers on the microcantilever surface. As a result, this may suggest that one may develop the sensitive cantilever-based bioassay that is capable of label-free detection of small-scale proteins in the very small amount in the blood serum.

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Figure Captions

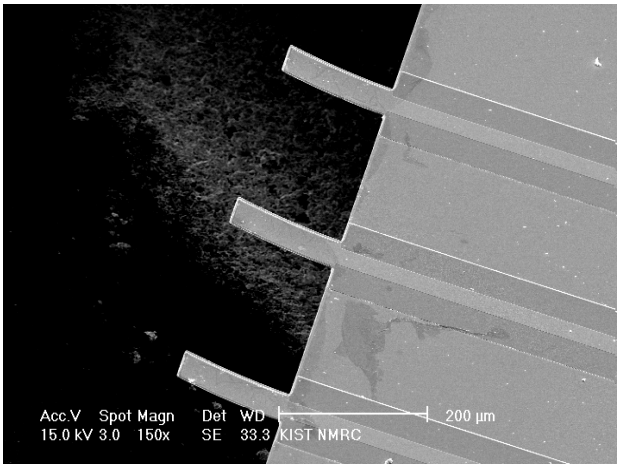
Figure 1. (a) A SEM image of microfabricated cantilever with the dimension of 50 μm × 150 μm (width × length). (b) Photograph showing whole device with three cantilevers.

Figure 2. Schematic showing the bottom-side view of functionalized microcantilever. (a) The amine-modified RNA aptamers were immobilized on the Calixcrown self-assembled monolayers. (b) The HCV helicase was specific interacted with the RNA aptamer on the microcantilever surface. When this happen, the resonant frequency of microcantilever was shifted in lower frequency range.

Figure 3. (a) Schematic of electrical measurement system for resonant frequency measurement of nanomechanical microcantilever (b) Measured dynamic response of cantilever before (blue) and after (red) a binding event between immobilized RNA aptamer and HCV helicase of 10 ng/ml.

Figure 4. The resonant frequency shift due to RNA aptamer-HCV helicase interaction with the HCV helicase concentrations from 0.1 ng/ml to 100 ng/ml

Figure 5. The surface stress exerted on our microcantilevers due to RNA aptamer-HCV helicase interaction with the HCV helicase concentration in the range of 0.1 ng/ml ~ 100 ng/ml



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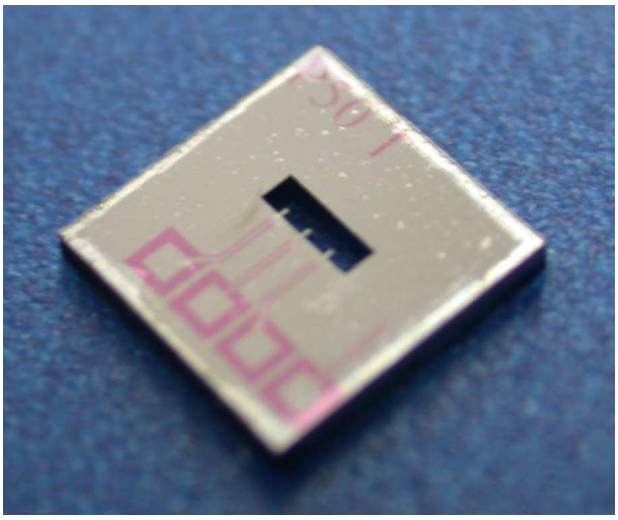
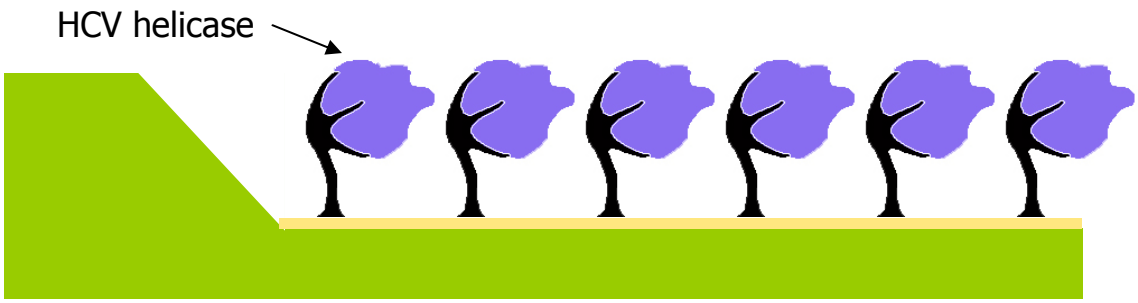


(b)

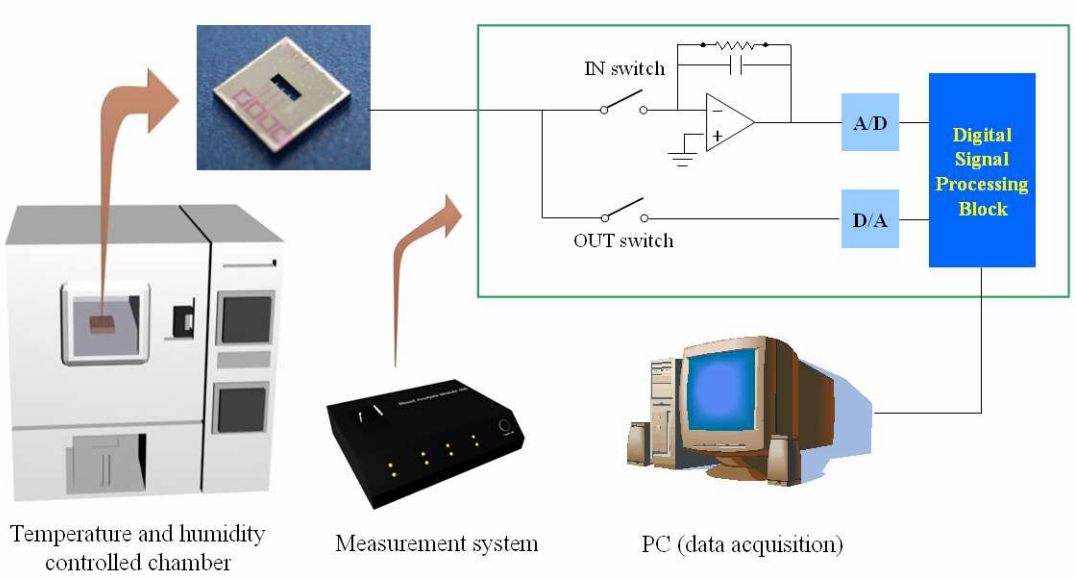
Figure 1. *Hwang et al.*

(a)

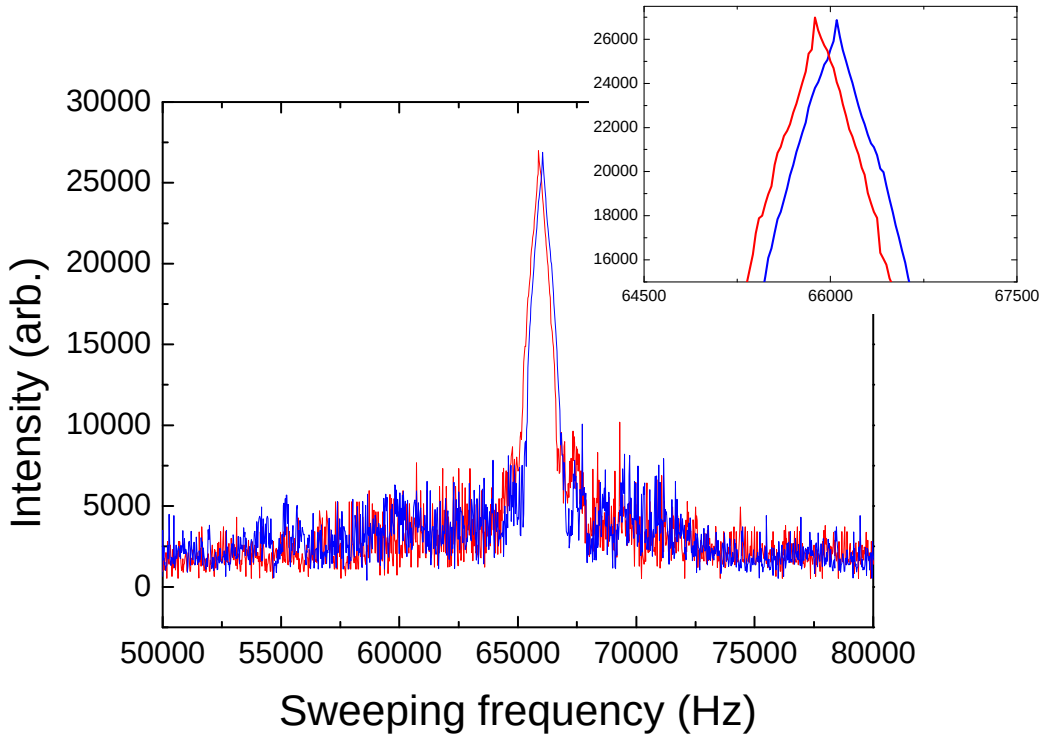


(b)

Figure 2. *Hwang et al.*



(a)



(b)

Figure 3. *Hwang et al.*

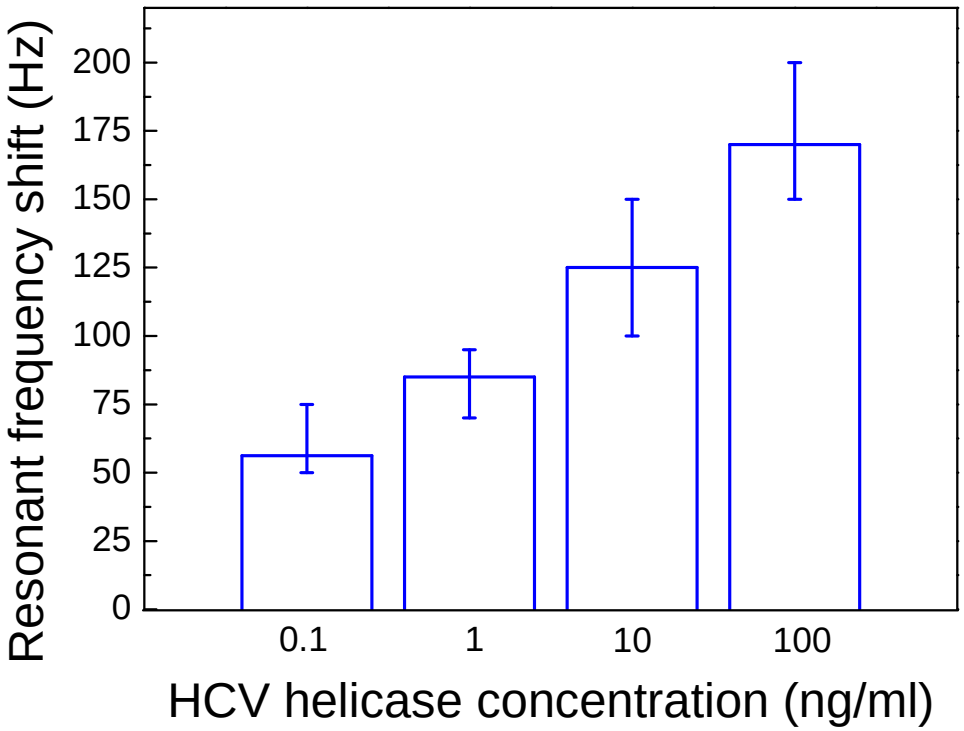


Figure 4. *Hwang et al.*

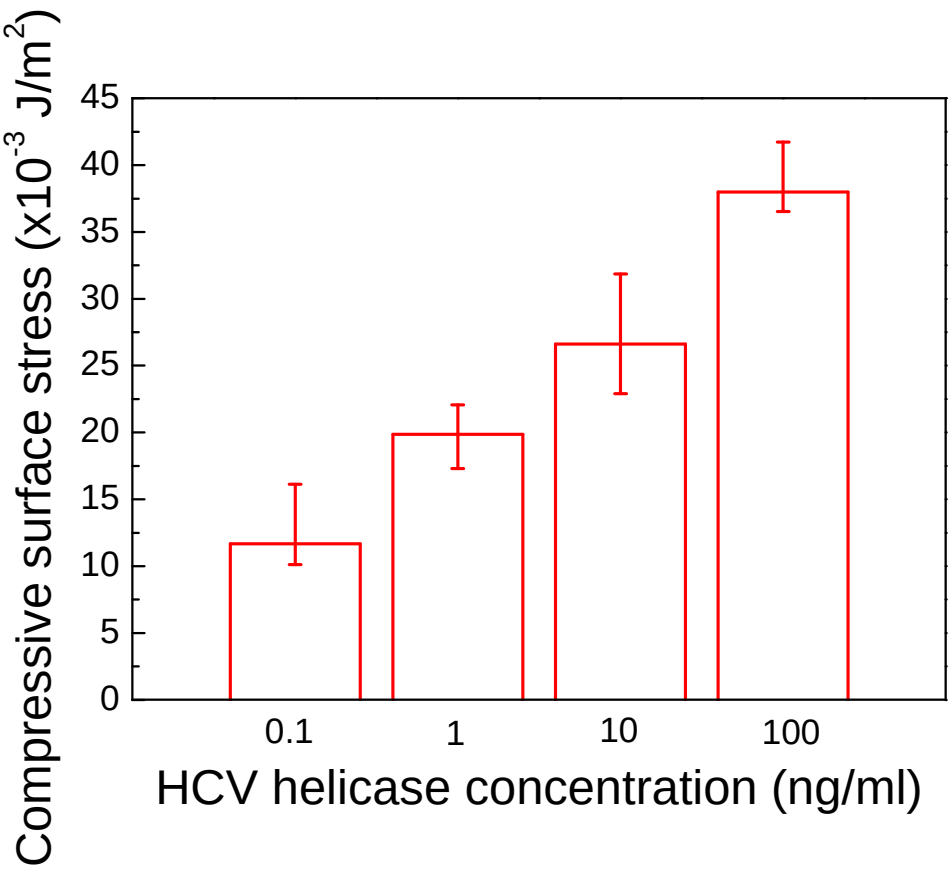


Figure 5. *Hwang et al.*