# *In-situ* real-time monitoring of biomolecular interactions based on resonating microcantilevers immersed in a viscous fluid

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We report the precise (noise-free) *in-situ* real-time monitoring of a specific protein antigen-antibody interaction by using a resonating microcantilever immersed in a viscous fluid. In this work, we utilized a resonating piezoelectric thick film microcantilever, which exhibits the high quality factor (e.g. $Q$ = 15) in a viscous liquid at a viscosity comparable to that of human blood serum. This implies a great potential of our resonating microcantilever to *in-situ* biosensor applications. It is shown that our microcantilever enables us to monitor the C reactive protein (CRP) antigen-antibody interactions in real-time, providing an insight into the protein binding kinetics.

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Nanomechanical microcantilevers have played a vital role in understanding the various physical phenomena such as temperature,[1] mass sensing,[2] molecular interactions,[3] protein conformations,[4] and protein/polymer conformation transitions.[5] In a recent decade, a resonating microcantilever has allowed the highly sensitive detection of various molecules. The ultrahigh sensitivity of a resonating cantilever is attributed to scaling down that increases the dynamical response ranges as well as the sensitivity. For instance, a recent study by Yang, et al.[6] provided that a resonating micron-scale cantilever enabled the molecular mass sensing in the order of zeptogram. Moreover, a resonating microcantilever has allowed the highly sensitive label-free detection of biomolecules.[7-10]

For a sensitive, reliable real-time monitoring of biomolecular interactions, it is desirable for a resonating microcantilever to perform vibration modes with a high quality factor and a high-frequency dynamical range in a viscous liquid environment. However, most of resonating microcantilevers possess the low quality factor in a liquid environment (e.g. $Q$ = ~ 5 in a liquid environment for Ref. 11), in spite of their high quality factor in normal air.[8,11] Consequently, the dynamical response change (resonant frequency shift) to biomolecular interactions was typically measured in normal air before and after bioassay.[10,12] It is, thus, demanded to develop a resonating microcantilever that are able to overcome the viscous liquid damping effects such that it exhibits the high quality factor in a liquid environment. Recently, we developed the piezoelectric thick film microcantilever that bears a high quality factor in a liquid environment (e.g. $Q$ = ~ 25 in water environment).[13] In this work, we report that our piezoelectric thick film microcantilever exhibits the high quality factor (e.g. $Q$ = 15 ~ 25) in a viscous liquid, even at a viscosity comparable to that of blood serum (i.e. ~ 4.5

cPs). This suggests that our microcantilever may be applicable to an *in-situ* biosensor. Remarkably, in this work, it is shown that our microcantilever enables the precise (noise-free) *in-situ* real-time monitoring of protein-protein interactions.

For an *in-situ* real-time monitoring of biomolecular interactions, we utilized a piezoelectric thick film microcantilever, which are capable of self-actuating/sensing by using piezoelectric and converse piezoelectric effects. The piezoelectric thick film microcantilever, whose dimension is 500 × 35 × 580 $\mu m^3$ or 500 × 35 × 500 $\mu m^3$ (width × thickness × length), fabricated by MEMS process coupled with screen-printing method (See Fig. 1).[13] In order to be operated in a viscous liquid environment, a piezoelectric thick film microcantilever was coated with 1 μm thick parylene-C, which serves as an electrically insulating biocompatible barrier against moisture and bio-fluids. For biomolecular recognitions, the surface of our microcantilever was functionalized by Calixcrown self-assembled monolayer (SAM) that can bind the amine group of protein antibodies, consequently enabling one to immobilize protein antibodies on a cantilever surface.[8] After antibody immobilization process, bovine serum albumin (BSA) was used as a blocking agent to inhibit the non-specific binding.[8,10] The biologically functionalized microcantilever was, then, mounted in a liquid cell that has 300 μm wide micro-channels and 16.5 μl volume reaction chamber. For measuring a resonant frequency of a microcantilever immersed in a viscous liquid environment, an electrically insulating liquid (Fluorinert$^{TM}$, 3M), whose viscosity is in a range of 1.4 cPs to 4.7 cPs, was injected into the inlet of a liquid cell until the channel of a liquid cell was filled with fluid. For an *in-situ* real-time monitoring of biomolecular interactions, C reactive protein (CRP) antigen dissolved phosphate buffered saline (PBS) solution (pH 7.4) was injected into a liquid cell, in which a biologically functionalized

microcantilever was mounted. In addition, in order to confirm the specific binding on a cantilever surface, the negative control experiment was conducted by injecting BSA dissolved PBS solution into a liquid cell. The resonance behavior of our microcantilever in a liquid cell was measured by using a laser doppler interferometric vibrometer (NEO ARK Co., Japan).

The resonance behavior of a piezoelectric thick film microcantilever is very consistent with classical elasticity theory. In our previous work,[13] it was reported that the resonant frequency of our microcantilever in normal air is well described by simple harmonic oscillator model. As shown in Fig. 2, our resonating microcantilever with a length of 580 μm in normal air possesses the resonant frequency of 47 kHz with a high quality factor $Q$ = ~ 65. Remarkably, our microcantilever exhibits the high quality factor even in a viscous liquid environment. Specifically, for our microcantilever the quality factor $Q$ in an electrically insulating liquid, whose viscosity in a range of 1.4 cPs to 4.7 cPs, ranges from 15 (for 4.7 cPs) to 25 (for 1.4 cPs) (See Fig. 2). This $Q$ value is much higher than any $Q$ values of any other microcantilevers reported in literatures[11]. This may shed light on that our microcantilever enables the precise (noise-free) *in-situ* real-time monitoring of biomolecular interactions. The resonance behavior of our microcantilever in a liquid environment is also well depicted by elasticity theory. The elasticity theory[9] provides the resonant frequency $\omega_i$ of a cantilever immersed in a viscous fluid such as

$$\omega_i = \sqrt{\theta\omega_{0,i}^2 - \eta^2} \tag{1}$$

Here, $\theta$ is a dimensionless parameter defined as $\theta = m_c/(m_c + m_l)$, where $m_c$ is a cantilever's mass and $m_l$ is the hydrodynamic loading arising from surrounding fluid acting on a cantilever.[14] $\omega_{0,i}$ is a resonant frequency of a cantilever in normal air. $\eta$ is a

dimensionless damping coefficient given by $\eta = \gamma L/2(m_c + m_l)$, where $L$ is a cantilever length and $\gamma$ is a viscosity (i.e. $\gamma$ = 1.4 cPs ~ 4.7 cPs). It should be noted that the hydrodynamic loading $m_l$ is given by[14]

$$\frac{m_l}{m_c} = \left(\frac{w}{t_c}\right)\left(1 + \frac{4}{(\lambda_i w/L)\sqrt{w^2\omega_{0,i}/\nu}}\right)\left(\frac{\rho_l}{\rho_c}\right) \quad (2)$$

where $t_c$ is a thickness of a cantilever, $w$ is a width of a cantilever, $v$ is a kinetic viscosity ($v = 10^{-6}$ m$^2$/s), $\lambda_i$ is a constant satisfying the transcendental equation (i.e. $\lambda_i = 1.87$), $\rho_l$ is a density of a liquid ($\rho_l$ = 1000 kg/m$^3$), and $\rho_c$ is a density of a cantilever ($\rho_c$ = 4543 kg/m$^3$). With the cantilever's mass $m_c$ given by $m_c = \rho_c V$ (i.e. $m_c = \sim 4 \times 10^{-8}$ kg), where $V$ is a cantilever's volume, the hydrodynamic loading, $m_l$, is estimated as $m_l = \sim 1.2 \times 10^{-7}$ kg (See Eq. 2). With given parameters for Eq. 1, it can be easily shown that a hydrodynamic loading effect rather than a damping effect plays a role in dynamical response of our microcantilever immersed in a liquid (i.e. $\theta\omega_{0,i}^2/\eta^2 >> 1$).[15] Hence, the resonant frequency of our microcantilever immersed in a viscous liquid is given by $\omega_i = \omega_{0,i}\sqrt{\theta}$. This suggests that our microcantilever operated in an electrically insulating liquid is expected to exhibit the resonant frequency of ~ 23 kHz, consistent with our experimental data (See Fig. 2).

As stated above, a high quality factor in a viscous liquid environment (e.g. $Q$ = ~ 15 at viscosity of ~ 4.7 cPs) implies a great potential to an *in-situ* real-time monitoring of biomolecular interactions (i.e. CRP antigen-antibody interactions) by measuring the resonance frequency shift induced by biomolecular recognitions. The resonant frequency shift, for a microcantilever with a length of 500 μm, was recorded every 1 min after injecting CRP antigen dissolved solution. It should be noted that the cantilever

with a length of 500 μm exhibits the resonance of 62.18 kHz in normal air and the resonance of 36.11 kHz in a PBS solution, consistent with elasticity theory. The specific interactions between our microcantilever and CRP antigens were proven by negative control experiment, showing no resonant frequency shift, so that non-specific interactions are unlikely to occur in our microcantilever surface (See Fig. 4).

We consider the curvature effect of protein monolayer on the resonance of a cantilever. The resonant frequency, $\chi_i$, of a cantilever after attachment of protein monolayer is given by $\chi_i = \omega_i\sqrt{1+\alpha}$. Here $\omega_i$ is a resonant frequency of a bare cantilever, and a parameter $\alpha$ is given by $\alpha = \xi_p/\xi_c$, where $\xi_p$ and $\xi_c$ are bending rigidities of protein monolayer and a bare cantilever, respectively. Classical elasticity theory provides the bending rigidity of a bare cantilever as $\xi_c = 1.27 \times 10^{-7}$ Nm$^2$, whereas the bending rigidity of protein monolayer is estimated as $\xi_p \approx E_p w t_p (t_c/2)^2 = 8.4 \times 10^{-13}$ Nm$^2$ with given Young's modulus $E_p = \sim 1$ GPa[16] and thickness $t_p = \sim 10$ nm[17]. This indicates that curvature effect of protein monolayer does not play any role on the resonance of a cantilever. Moreover, the surface stress induced by intermolecular interactions between adsorbed proteins may be insignificant for the resonance of a cantilever, because the surface stress effect (intermolecular interactions) may dominate the dynamical behavior of a cantilever when a cantilever's thickness becomes comparable to that of protein layer.[18]

For clarifying the origin of resonant frequency shift due to protein antigen-antibody interactions, we take into account the resonant frequency shift, which was measured in normal air before and after bioassay, due to CRP antigen-antibody interactions (See Fig. 3). Since the curvature effect and/or surface stress effect of protein monolayer are not related to resonance behavior of a cantilever, the resonant frequency

shift in normal air, $\Delta\omega_0$, may be ascribed to the mass of adsorbed proteins.[9]

$$\frac{\Delta\omega_0}{\omega_0} \approx -\frac{1}{2}\frac{\Delta m}{m_c} \tag{3}$$

where $\omega_0$ is the resonant frequency of a cantilever operated in normal air before bioassay, and $\Delta m$ is the mass of adsorbed molecules. With $\Delta\omega_0$ = 2.79 kHz and $\omega_0$ = 62.18 kHz, the mass of adsorbed proteins is estimated as $\Delta m$ = 3.5 ng. Fig. 4 shows the resonant frequency shift that was measured in the liquid environment during protein antigen-antibody interactions. It is remarkable that, for protein antigen-antibody interactions, the resonant frequency shift measured in liquid environment is larger than that estimated in normal air. It is consistent with previous works[19] which reported that, for protein antigen-antibody interactions, the resonant frequency shift for a mass sensor (e.g. quartz crystal microbalance) was estimated in liquid environment larger than that measured in normal air by factor of ~4. This phenomenon is attributed to protein antigen-antibody interactions increasing the hydrophilicity that changes hydrodynamic loading coupled to resonance behavior of a mass sensor.[19] Accordingly, the resonant frequency shift induced by protein antigen-antibody interactions for a cantilever immersed in a liquid is originated from the change of hydrodynamic loading due to increase of hydrophilicity during antigen-antibody interactions.

$$\frac{\Delta\omega}{\omega} = \frac{1}{2}\frac{\Delta m_l}{m_l}(1-\theta) + \frac{1}{2}\frac{\Delta m}{m_c}\theta \tag{4}$$

Here, $\Delta\omega$ and $\omega$ are the resonant frequency shift and the reference resonant frequency (before bioassay) which are measured in liquid, respectively, $\Delta m_l$ is the change of hydrodynamic loading induced by antigen-antibody interactions, and $\Delta m$ is the mass of adsorbed proteins. It provides that the change of hydrodynamic loading $\Delta m_l$ due to CRP antigen-antibody interactions is estimated as $\Delta m_l = 9.3 \times 10^{-8}$ g. Moreover, as shown in

Fig. 4, the resonant frequency shift follows the Langmuir kinetic model, indicating that our microcantilever may allow for gaining insight into kinetics of protein-protein interactions. Further, high quality factor of our microcantilever operated in liquid enables the noise-free real-time monitoring of protein-protein interactions, since $1/Q$ represents the intrinsic noise of a system.[20]

In summary, we report the *in-situ* real-time monitoring of CRP antigen-antibody interactions by using a piezoelectric thick film microcantilever that possesses the high quality factor even in a viscous liquid environment. It was shown that resonance of an *in-situ* cantilever is well depicted by hydrodynamic loading, and that the protein antigen-antibody interactions increase the hydrophilicity resulting in a change of hydrodynamic loading coupled to resonance behavior of a cantilever. Moreover, the precise *in-situ* real-time monitoring of protein-protein interactions is ascribed to high quality factor of our microcantilever. Consequently, our microcantilever enables us to precisely gain insight into kinetics of protein-protein interactions. In the long run, our piezoelectric thick film microcantilevers may allow the precise real-time monitoring of various biomolecular interactions such as DNA-DNA interactions, DNA-protein interactions, and protein-small-molecule interactions.

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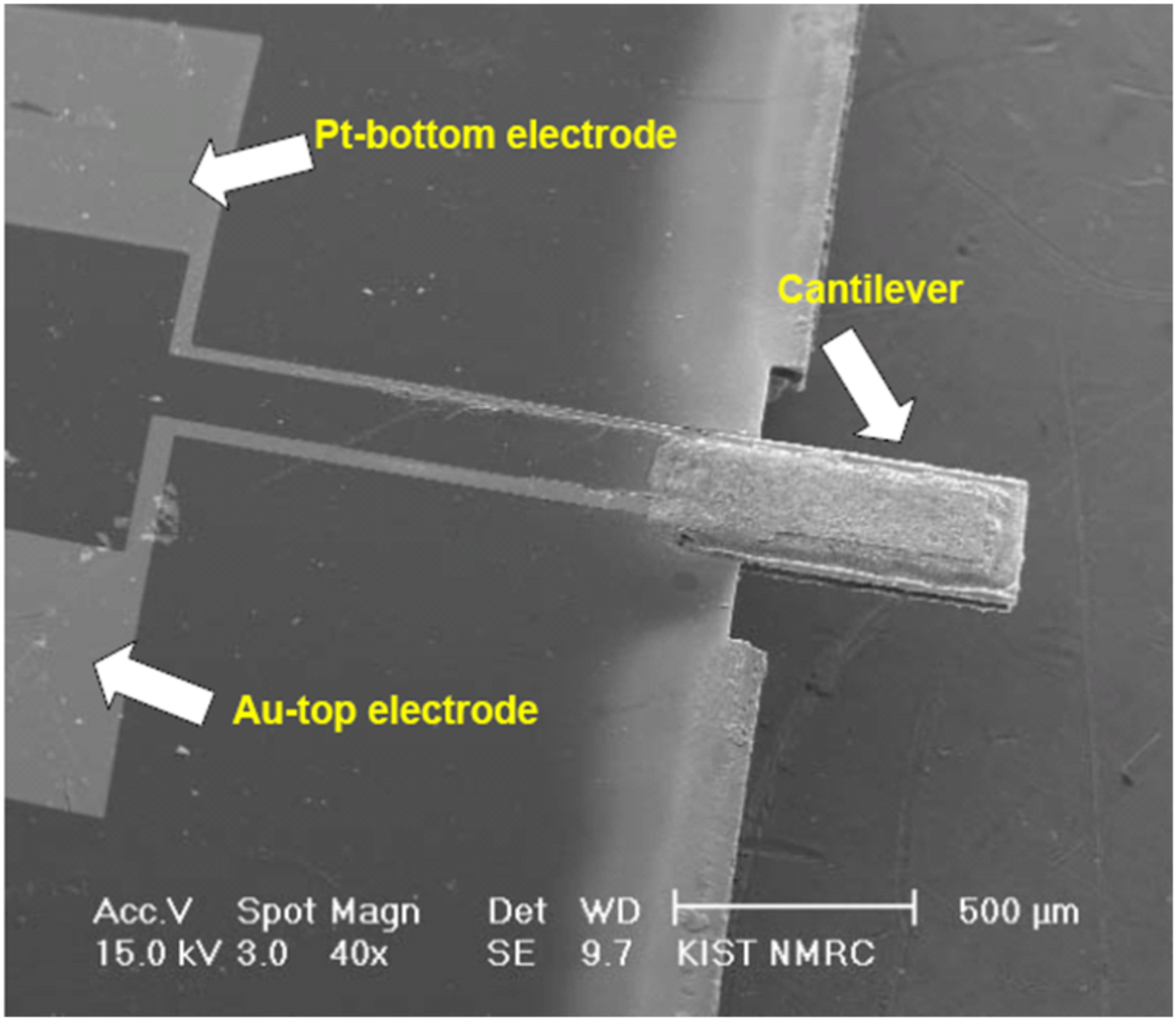
## Figure Captions

Fig. 1. Scanning electron microscopy (SEM) image of a piezoelectric thick film microcantilever, whose dimension is 500 × 35 × 580 $\mu m^3$ (width × thickness × length).

Fig. 2. Resonant frequency shift due to virtual mass and quality factor for our microcantilever in a viscous liquid.

Fig. 3. Resonance behavior of PZT thick film microcantilever, operated in normal air, before and after bioassay

Fig. 4. *In-situ* real-time monitoring of resonant frequency shift induced by CRP antigen-antibody interactions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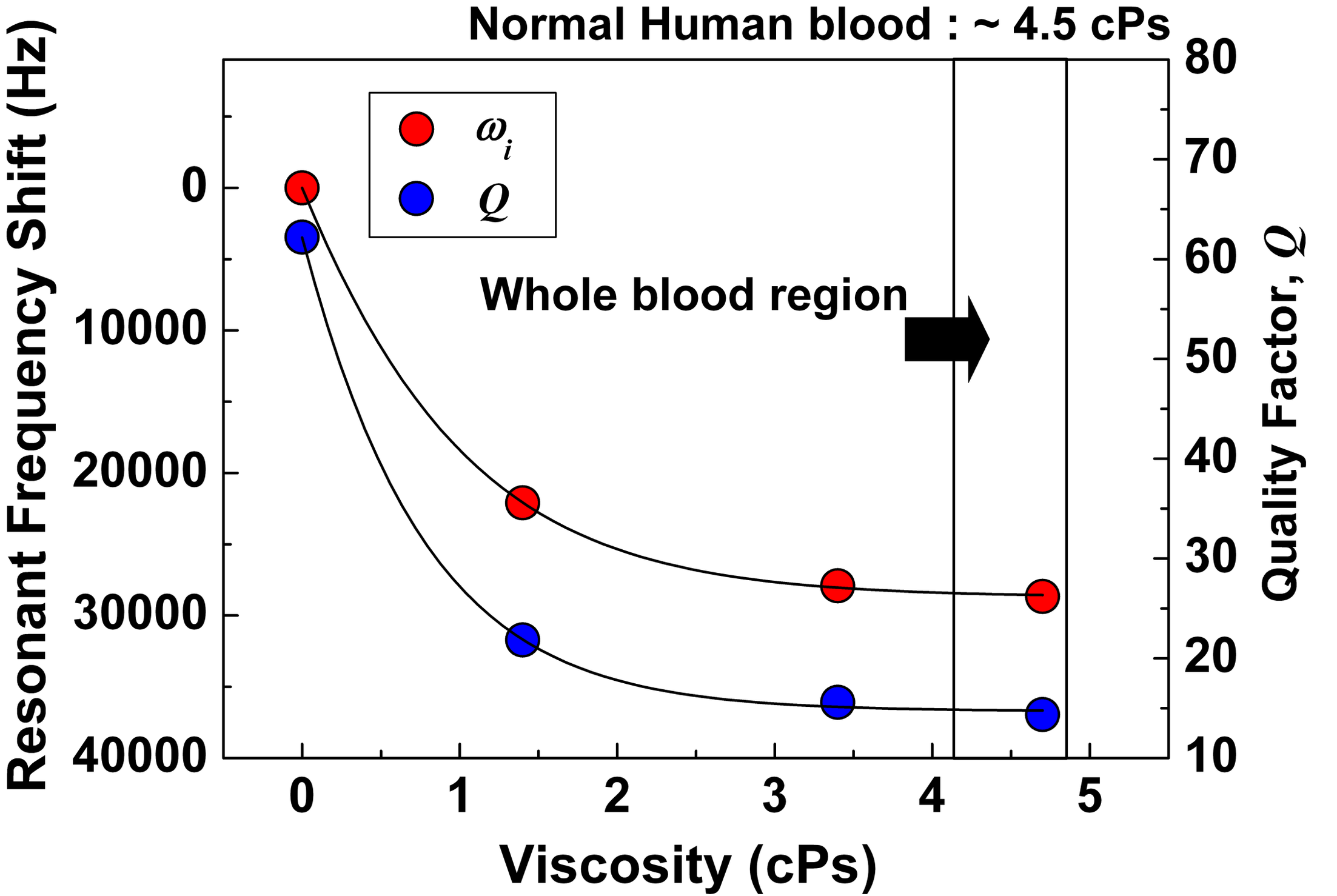
15.0 kV 3.0 40x SE 9.7 KIST NMRC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$Q$
Whole blood region
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20000
30000
40000
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1
2
3
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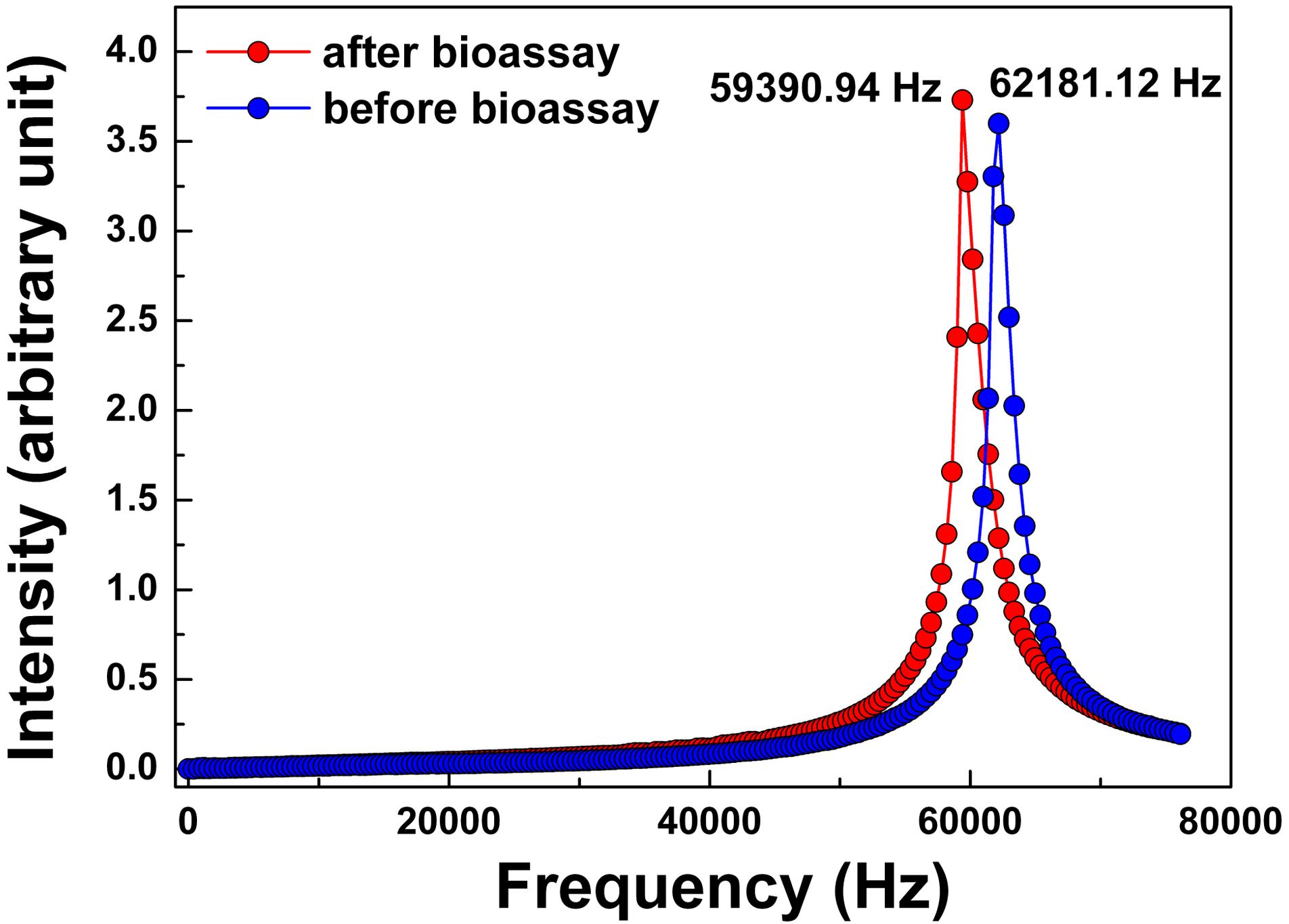
5
Viscosity (cPs)


after bioassay
before bioassay
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62181.12 Hz
Intensity (arbitrary unit)
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3.0
2.5
2.0
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1.0
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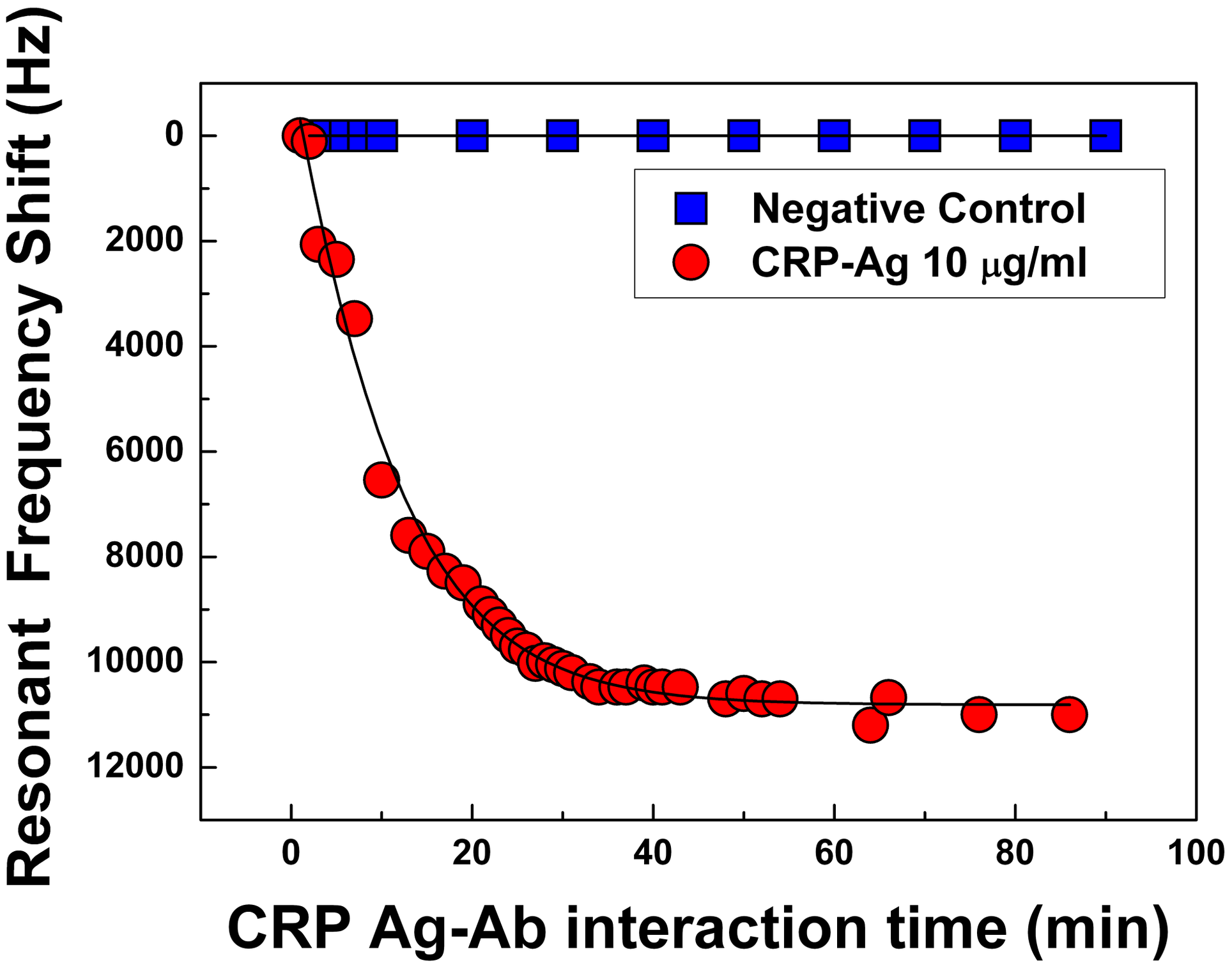
0.0
0
20000
40000
60000
80000
Frequency (Hz)


Resonant Frequency Shift (Hz)
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2000
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6000
8000
10000
12000
0
20
40
60
80
100
CRP Ag-Ab interaction time (min)
Negative Control
CRP-Ag 10 μg/ml