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PAPER

Application of a new microcantilever biosensor resonating at the air–liquid interface for direct insulin detection and continuous monitoring of enzymatic reactions†

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Here we describe the application of a recently developed high-resolution microcantilever biosensor resonating at the air–liquid interface for the continuous detection of antigen–antibody and enzyme–substrate interactions. The cantilever at the air–liquid interface demonstrated 50% higher quality factor and a 5.7-fold increase in signal-to-noise-ratio (SNR) compared with one immersed in the purified water. First, a label-free detection of a low molecular weight protein (insulin, 5.8 kDa) in physiological concentration was demonstrated. The liquid facing side of the cantilever was functionalized by coating its surface with insulin antibodies, while the opposite side was exposed to air. The meniscus membrane at the micro-slit around the cantilever sustained the liquid in the microchannel. After optimizing the process of surface functionalization, the resonance frequency shift was successfully measured for insulin solutions of 0.4, 2.0, and 6.3 ng ml^{−1}. To demonstrate additional application of the device for monitoring enzymatic protein degradation, the liquid facing microcantilever surface was coated with human recombinant SOD1 (superoxide dismutase 1) and exposed to various concentrations of proteinase K solution, and the kinetics of the SOD1 digestion was continuously monitored. The results showed that it is a suitable tool for sensitive protein detection and analysis.

1 Introduction

Protein detection methods permitting analysis of protein–protein interactions are essential in modern biology and medical sciences. Some of the conventional protein detection methods utilizing antigen–antibody interactions include such standard techniques as Western blot analysis¹ and ELISA.² However, in spite of their sensitivity, these methods are often labour intensive, time consuming, highly variable, demand long optimization curves and do not permit protein detection in real time. Therefore, there has been a great need for approaches allowing continuous and sensitive detection of antigen–antibody or enzyme–substrate interactions in real time.

An array of microfabricated biosensors represents a promising alternative to detect low concentrations of biomolecules in real time.³ Micromechanical resonators including a microcantilever,^{4–11} quartz crystal microbalance (QCM),^{12–16} and surface

acoustic wave (SAW) sensors^{17–19} measure the resonance frequency shift associated with the loaded mass by selective trapping of target molecules. These methods exhibit high mass-resolution in air or vacuum owing to their high quality factor and have superior sensitivity. However, the quality factor of the resonators decreases dramatically in liquids due to viscous damping on the structure. Although the detection of macromolecules (T5 virions, 7 × 10⁴ kDa) has been achieved at subpicomolar concentrations,²⁰ measurements of smaller biomolecules at physiological concentrations remains challenging, with only a few successful attempts at nanomolar concentrations (C-reactive protein, 25 kDa).²¹ The suspended microchannel resonator (SMR) has been invented to avoid the damping effect surrounding a resonator that embeds a microchannel in the cantilever. It could achieve high resolution with a noise level below 27 ag (10^{−18} g) with a 1 Hz bandwidth. In spite of its high sensitivity and functionality, difficulty with *in situ* monitoring due to its closed beam structure remained a concern.

We have recently developed a microcantilever biosensor resonating at the air–liquid interface. The cantilever is placed at the bottom of a microchannel, and the meniscus membrane at the micro-slit around the cantilever suspends the liquid by surface tension. One surface facing the liquid layer is functionalized by incorporating specific antibodies (e.g. anti-insulin), while the other side is exposed to air to improve resonance characteristics of the quality factor. Having no additional layers

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between the cantilever and the laser detector contributed to further noise reduction. This design showed 50% higher quality factor and a 5.7-fold larger signal-to-noise ratio (SNR).²²

There are numerous kinds of low molecular weight chemicals, proteins and hormones playing crucial physiological roles in human metabolism. Insulin (5.8 kDa), a hormone produced by the pancreas, is essential for the regulation of glucose levels.²³ Insulin regulation is a cause of several metabolic syndromes including Diabetes mellitus. Here, in order to demonstrate the utility of the device, insulin detection was performed for a range of physiological concentrations. Self-assembled monolayer of anti-insulin antibodies was formed on the upper (liquid facing) side of the cantilever *via* amino-group formed peptide coupling.^{24,25} Cantilevers functionalized in such a way was exposed to insulin solutions from 68 pM (0.4 ng ml⁻¹) to 1.08 nM (6.3 ng ml⁻¹) in PBS (phosphate buffered saline). A resonance frequency decrease of about 2 kHz was detected in each case with a reaction time corresponding to a specific insulin concentration. Further application of the device for the detection of protein–protein interactions was shown using enzymatic degradation by proteinase K of human recombinant superoxide dismutase (SOD1) immobilized on the cantilever.

2 Cantilever resonating at the air–liquid interface

The size of the cantilever was 80 μm × 20 μm × 5 μm. It was cut out from the bottom of a microchannel by the micro-slit of 6 μm gap. A meniscus membrane over the slit separated the air and liquid phases. The cantilever device was fabricated and integrated in the microchannel. On the device, an SiO₂ layer was sputtered on the surface of the cantilever facing the microchannel for surface modification. Then, a PDMS cover was bonded to the device to form a microchannel after punching two holes for the inlet and outlet. The surface of the cantilever was cleaned and activated by a piranha solution (H₂O₂: H₂SO₄ = 1 : 3) for 20 min, and rinsed carefully in purified water. Finally, the PDMS cover with an inlet and an outlet was placed on the device and then bonded.

A modulated laser excited the cantilever at the fixed-end by photothermal effect, and a laser Doppler velocimeter (LDV) measured its amplitude at the free-end (Fig. 1A, B). The observed signal by the LDV was fed back to a lock-in-amplifier to compare the two signals between the LDV and the

photothermal laser (Fig. 1D). The signal was recorded with a sampling rate of 10 Hz, and a frequency drift of the system was below 100 Hz.^{26,27} The liquid was introduced by a push/pull syringe pump (KD scientific, KD270) connected to both sides of the microchannel in order to stabilize the liquid pressure on the cantilever. In addition, the new bio-solution was loaded in an injector placed between the pump and the inlet of the microchannel (Fig. 1C).

The cantilever had a quality factor of 15 that is 50% higher than for one submerged in liquid, and it showed a 5.7-fold better signal-to-noise-ratio. The method had 25-fold higher frequency resolution than the cantilever emerged in liquid, corresponding to a mass resolution of 10⁻¹⁶ g.²²

In general, a change in mass loaded on the surface of cantilever translates into the resonance frequency shift (Δf_R). Furthermore, the meniscus membrane around the cantilever works as an additional spring effect ($k_{\Delta\gamma}$) on a resonator

$$f_R + \Delta f_R = \frac{1}{2\pi} \sqrt{\frac{k_{Si} + k_{\Delta\gamma}}{M_{Si} + \Delta M_b}} \quad (1)$$

k_{Si} and $k_{\Delta\gamma}$ are the spring constant of the silicon cantilever and the surface tension at a meniscus membrane, respectively. M_{Si} and ΔM_b are the mass of the resonator and the additional mass bonded with cantilever, respectively. Park *et al.* showed numerical modeling and empirical experiments for the cantilever with a meniscus membrane.²² The resonance frequency shift due to changes of the surface tension is negligible compared to the additional mass of the cantilever.

3 Surface functionalization and optimization

The process of functionalizing the surface is optimized by using fluorescent observation and resonance frequency measurements. The self-assembled monolayer of APTES ((3-aminopropyl) triethoxysilane) was deposited on the cantilever, generating evenly distributed amino groups. Antibodies were covalently immobilized onto the amino groups with EDC (*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide), which catalyzed the formation of covalent bonds between APTES amino groups and the carboxyl group of antibodies.

Briefly, the cantilever was exposed to the APTES solution (APTES : diH₂O: Ethanol = 5% : 5% : 90%) for 30 min, followed

Fig. 1 Device structure and experimental setup: (A) cross-sectional view of cantilever at the air–liquid interface, (B) bottom view, (C) balance microfluidic system by push/pull syringe pump, and (D) continuous measurement system.

by a rinse in ethanol then purified water carefully.²⁵ Remaining fluid was removed with nitrogen gas and the device was dried at 60 °C for 20 min.

The solutions were loaded in the injector and continuously introduced to the microchannel using a push/pull syringe pump for one hour with a flow rate of 45 $\mu\text{L h}^{-1}$. Through the PDMS cover, the fluorescent intensity on the cantilever was recorded with a fluorescent camera (Cascadell-512, Photometrics) attached to a microscope (BX51, Olympus).

3.1 Self-assembled monolayer: homogeneous attachment

We evaluated the effect of the APTES self-assembled monolayer. We prepared two devices; one with APTES treatment and the other without treatment. Then, fluorescent insulin antibodies solution (20 $\mu\text{g ml}^{-1}$) was applied on two different cantilever devices using an EDC solution of 2 mg ml^{-1} as a cross-linker in order to examine the difference in surface status.

As illustrated in Fig. 2(A), with APTES treatment, fluorescent antibodies were immobilized over the cantilever surface homogeneously. On the other hand, without APTES treatment, antibodies were tangled over the cantilever. The standard deviation of fluorescent intensity distribution over the surface was 375 a.u. with APTES treatment, while it was 623 a.u. without APTES (Fig. 2A). We confirmed that the antibodies were immobilized by the amino group of the self-assembled monolayer on the cantilever. Without APTES treatment, they were attached and absorbed irregularly on the surface of the cantilever.

3.2 Cross-linker (EDC): peptide bonding

We studied and optimized the EDC concentration to immobilize antibodies to the APTES covered surface. We analyzed the results in terms of fluorescent brightness and durability of binding depending on the EDC concentration of 0.2, 2, and 20 mg ml^{-1} . The fluorescent intensity was measured 3 h after immobilization of antibodies. Before measurement, we rinsed the microchannel carefully with PBS at a flow rate of 3 $\mu\text{L min}^{-1}$ for 2 min.

The fluorescent intensity of the surface was the strongest in EDC solution of 2 mg ml^{-1} , followed by the 20 mg ml^{-1} solution and then 0.2 mg ml^{-1} (Fig. 2A). While low EDC concentration

was not sufficient for efficient amino-carboxyl bonding, a higher concentration of EDC resulted in an excessive and non-specific antibody-antibody bonding prior to their attachment to the surface of the cantilever.

3.3 Optimization of antibodies condition

We optimized the binding conditions of insulin antibodies using resonance frequency measurement. Two antibody solutions of 20 and 2 $\mu\text{g ml}^{-1}$ were prepared and applied to the microchannel after diluting it in EDC coupling solution (2 mg ml^{-1} in EDC, 50 mM MES, pH. 5.2, 0.05% proclin300). The resonance frequency of the cantilever was recorded for one hour at a sampling rate of 10 Hz.

As illustrated in Fig. 2(B), the decrease in the resonance frequency was detected 12 min after the anti-insulin solution was applied to the microchannel and immediately after the dead-volume between the injector and the microchannel ($V = 9 \mu\text{L}$) was gone. The resonance frequency of the cantilever gradually decreased about 5 kHz and 14 kHz when 20 $\mu\text{g ml}^{-1}$ and 2 $\mu\text{g ml}^{-1}$ antibodies solution were applied, respectively (Fig. 2B). The frequency increased about 2 kHz with a higher concentration of antibodies after 30 min. On the other hand, the frequency decreased and saturated in 2 $\mu\text{g ml}^{-1}$ antibody solution. We confirmed the binding reproducibility by observing a consistent resonance frequency shift of about 13 kHz with repeated application of 2 $\mu\text{g ml}^{-1}$ antibody solution. Higher concentration of antibodies promoted self-aggregation prior to their attachment to the cantilever surface generating a similar effect when 20 mg ml^{-1} EDC solution was used (see above). Tangled antibodies have less chance to be immobilized with the amino-group and detached easily by continuous flow in the micro-channel.

We compared the observed resonance frequency shift due to the binding of insulin antibodies with its expected theoretical value. The bonding density of the antibodies could be calculated based on the size of the protein and APTES surface density.^{28,29} According to our calculations, binding of 150 kDa insulin antibody peptides to the amino-groups of APTES at 3.8×10^{-9} mole m^{-2} density should produce a resonance frequency shift of 19 kHz.

Fig. 2 Optimization of cantilever surface functionalization. A. Effect of APTES and EDC on surface density of fluorescent antibody (2 $\mu\text{g ml}^{-1}$): no APTES (a) and with APTES (b), 2 mg ml^{-1} EDC (a, b), 20 mg ml^{-1} EDC (c), and 0.2 mg ml^{-1} EDC (d); B. Immobilization of insulin antibodies: antibodies at high concentration (20 $\mu\text{g ml}^{-1}$) could not be immobilized on the surface due to the unspecific binding among them (1), while 2 $\mu\text{g ml}^{-1}$ antibody solution demonstrated good binding rates to the cantilever surface (2, 3).

3.4 Surface tension by various liquids in a microchannel

The elasticity of meniscus membrane at the micro-slit was changed by the surface tension, and affects the resonance frequency shift. We evaluated the surface tension by theoretical and empirical study. Here, using goniometry, we examined the surface tension changes by antibody solutions of 2, 20, and 40 $\mu\text{g ml}^{-1}$ in 2 mg ml^{-1} EDC solution.

The surface tension of antibody solutions of 2, 20, and 40 $\mu\text{g ml}^{-1}$ were 72, 71, and 68 mN m^{-1} , respectively. The resonance frequency shift could be estimated within less than a few Hz based on the precedent study, which means that we could ignore this effect by the surface tension change on resonance frequency shift.²²

4 Results and discussion

4.1 Insulin detection

Using the functionalized cantilever by the optimized process in chapter 3, we detected insulin in the physiological range of concentrations. The insulin solutions of 0.4, 2, and 6.3 ng ml^{-1} (69 pM, 344 pM, and 1.08 nM) were introduced individually in the microchannel continuously at 45 $\mu\text{L min}^{-1}$ for one hour. In this paper, one functionalized cantilever was tested for the consistency of its performance.

The resonance frequency decreased, and then saturated to a constant level. In all cases, the resonance frequency gradually shifted down approximately 2 kHz due to the captured insulin on the functionalized cantilever. However, the reaction times were different depending on the concentrations (Fig. 3). Insulin solutions with higher concentrations had the shortest reaction time. In the highest concentration of 6.3 ng ml^{-1} , the frequency was saturated within 5 min, while it took 7 and 16 min in the insulin solution of 2 and 0.4 ng ml^{-1} , respectively. The result that the final frequency shift was the same for all concentrations suggests that all immobilized antibodies had captured insulin

Fig. 3 Higher concentrations of insulin demonstrate faster reaction time: the insulin solution of 6.3 ng ml^{-1} reacted quickly while lower concentrations show slower reaction times. (A) Conceptual diagram of insulin – anti-insulin binding.

and the surface has reached saturation. The time of frequency shift is directly associated with the insulin concentration.

The mass of insulin immobilized on the cantilever can be estimated as 7.1×10^{-14} g assuming that two insulin molecules bind each antibody. This should result in the 1.53 kHz resonance frequency decrease. Furthermore, the frequency shift due to a surface tension change can be calculated as -120 and -170 Hz for 0.4 and 2.0 ng ml^{-1} insulin solutions respectively.

The anti-insulin monoclonal antibodies used in this study are directed against Insulin/Proinsulin (D6C4 clone) and recognize insulin monomers and oligomers³⁰ but do not react with free C peptide (AbD Serotec, UK). Reported experiments detected total insulin and the set up was not designed for the separate detection of monomers and oligomers which would require oligomer specific antibodies.

At high mM concentrations, insulin tends to form oligomers including di- and hexamers *in vitro*, the process being facilitated by the high temperatures and low pH (< 2.0).³¹ Current experiments performed insulin detection at concentrations within physiological levels at neutral pH (PBS) and room temperature that most likely precluded from severe oligomer formation. In addition, the test was designed for the detection of total insulin and if the need for identification of di- or hexamer insulin species arises, the set up clearly has to be modified by changing functionalization of the cantilever surface that would have to immobilize oligomer specific antibodies.³²

4.2 SOD1 protein degradation by proteinase K

Detection of insulin is based on the capturing of the insulin molecules by the anti-insulin antibodies, and results in a resonance frequency decrease due to the protein mass increase on the surface of the cantilever. To test if the reduction of the immobilized protein load on the surface of the cantilever may be used as an efficient means of detection, we applied SOD1 functionalized cantilever for the monitoring of enzymatic degradation by proteinase K. Proteinase K or endopeptidase K (EC 3.4.21.64) is a broad-spectrum serine protease capable of digesting native proteins.

SOD1 (2 $\mu\text{g ml}^{-1}$ in EDC buffer) was applied for one hour on an amino-group coated surface, followed by rinsing by PBS buffer at a flow rate of 3 $\mu\text{L min}^{-1}$ for 2 min. Then, proteinase K solutions of 0.01, 1, and 2 mg ml^{-1} were applied to the microchannel.

Twelve minutes after the application of SOD1 solution, about 4 kHz of the resonance frequency shift was registered suggesting that SOD1 was immobilized on the surface of the cantilever. Two minutes after the application of 2 mg ml^{-1} proteinase K solution, the resonance frequency shifted back to its initial value (Fig. 4), indicating the complete digestion of SOD1 by the enzyme. As expected, lower concentrations of proteinase K (0.01 mg ml^{-1}) required markedly longer time for the resonance frequency to come back to the initial value. The digestion slopes were 2, 0.4, and 0.09 kHz min^{-1} for the 2.0, 1.0, and 0.01 mg ml^{-1} proteinase K solutions, respectively (Fig. 4).

5 Conclusions

In this work, we have applied a cantilever resonating at the air–liquid interface to detect insulin at 0.4, 4, and 6.3 ng ml^{-1}

Fig. 4 Enzymatic reaction: degradation of immobilized SOD1 by proteinase K: Proteinase K digested SOD1 on the cantilever. x -axis represents cantilever recovery rate corresponding to SOD1 degradation and expressed as Hz min^{-1} (A) Conceptual diagram of protein interaction.

concentrations. The microchannel facing side of the cantilever was functionalized with anti-insulin antibodies. The process of surface functionalization was optimized using fluorescent observation and resonance frequency measurement. Application of three different concentrations of insulin solution all caused the resonance frequency to shift down approximately 2 kHz, although the shifting speed was greater for higher concentrations. We confirmed that the experimental values of the resonance frequency shift were in good agreement with theoretical estimates.

Utility of the biosensor was further tested for monitoring enzymatic reaction progression by demonstrating the digestion of immobilized SOD1 by proteinase K solutions at various concentrations.

We believe our sensor opens the way to a continuous and highly sensitive detection and analysis of low molecular weight biomolecules and their interactions with various protein counterparts in biomedical applications.

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References

- 1 J. Renart, J. Reiser and G. R. Stark, *Proc. Natl. Acad. Sci. U. S. A.*, 1979, **76**, 3116–3120.
- 2 R. S. Yalow and S. A. Berson, *J. Clin. Invest.*, 1960, **39**, 1157–1175.
- 3 G. Wu, R. H. Datar, K. M. Hansen, T. Thundat, R. J. Cote and A. Majumdar, *Nat. Biotechnol.*, 2001, **19**, 856–860.
- 4 J. Fritz, M. K. Baller, H. P. Lang, H. Rothuizen, P. Vettiger, E. Meyer, H.-J. Guntherodt, C. Gerber and J. K. Gimzewski, *Science*, 2000, **288**, 316–318.
- 5 K. Hwang, J. Lee, J. Park, D. Yoon, J. Park and T. Kim, *Lab Chip*, 2004, **4**, 547–552.
- 6 K. Y. Gfeller, N. Nugaeva and M. Hegner, *Biosens. Bioelectron.*, 2005, **21**, 528–533.
- 7 K. Y. Gfeller, N. Nugaeva and M. Hegner, *Appl. Environ. Microbiol.*, 2005, **71**, 2626–2631.
- 8 T. Kwon, K. Eom, J. Park, D. Yoon, H. Lee and T. Kim, *Appl. Phys. Lett.*, 2008, **93**, 173901.
- 9 K. Hwang, S. Lee, S. Kim, J. Lee and T. Kim, *Annu. Rev. Anal. Chem.*, 2009, **2**, 77–98.
- 10 S. Lee, K. Hwang, H. Yoon, D. Yoon, S. Kim, Y. Lee and T. Kim, *Lab Chip*, 2009, **9**, 2683–2690.
- 11 B. Cha, S. Lee, J. Park, K. Hwang, S. Kim, Y. Lee, B. Ju and T. Kim, *Biosens. Bioelectron.*, 2009, **25**, 130–135.
- 12 J. Rickert, A. Brecht and W. Gopel, *Anal. Chem.*, 1997, **69**, 1441–1448.
- 13 M. Z. Atashbara, B. Bejcek, A. Vijha and S. Singamaneni, *Sens. Actuators, B*, 2005, **107**, 945–951.
- 14 S.-R. Hong, S.-J. Choia, H. D. Jeong and S. Hong, *Biosens. Bioelectron.*, 2009, **24**, 1635–1640.
- 15 J. Fattisona, F. Azarib and N. Tufenkji, *Biosens. Bioelectron.*, 2011, **26**, 3207–3212.
- 16 Z.-Q. Shen, J.-F. Wang, Z.-G. Qiu, M. Jin, X.-W. Wang, Z.-L. Chen, J.-W. Li and F.-H. Cao, *Biosens. Bioelectron.*, 2011, **26**, 3376–3381.
- 17 M. D. Schlensog, T. M. Gronewold, M. Tewes, M. Famulok and E. Quandt, *Sens. Actuators, B*, 2004, **101**, 308.
- 18 M. Alvarez, J. Friend and L. Y. Yeo, *Nanotechnology*, 2008, **19**, 455103.
- 19 E. Gizeli, F. Bender, A. Rasmusson, K. Saha, F. Josse and R. Cernosek, *Biosens. Bioelectron.*, 2003, **18**, 1399–1406.
- 20 T. Braun, M. K. Ghatkesar, N. Backman, W. Grange, P. Boulanger, L. Letellier, H.-P. Lang, A. Bietsch, C. Gerber and M. Hegner, *Nat. Nanotechnol.*, 2009, **4**, 179–185.
- 21 N. Kim, D.-K. Kim and Y.-J. Cho, *Sens. Actuators, B*, 2009, **143**, 444–448.
- 22 J. Park, S. Nishida, P. Lambert, H. Kawakatsu and H. Fujita, *Lab Chip*, 2011, **11**, 4187–4193.
- 23 J. M. Berg, J. L. Tymoczko and L. Stryer, 7th edition, 2010, New York: WH Freeman, ISBN-10: 1429229365.
- 24 N. Barie and M. Rapp, *Biosens. Bioelectron.*, 2001, **16**, 979–987.
- 25 H. Yuan, W. M. Mullett and J. Pawliszyn, *Analyst*, 2001, **126**, 1456–1461.
- 26 S. Nishida, D. Kobayashi, T. Sakurada, T. Nakazawa, Y. Hoshi and H. Kawakatsu, *Rev. Sci. Instrum.*, 2008, **79**, 123703.
- 27 S. Nishida, D. Kobayashi, Y. Nishimori and H. Kawakatsu, *J. Vac. Sci. Technol., B: Microelectron. Nanometer Struct.–Process., Meas., Phenom.*, 2009, **27**, 964–968.
- 28 J. E. Sader, *J. Appl. Phys.*, 1998, **84**, 64–76.
- 29 R. R. Bhat and J. Genzer, *Nanotechnology*, 2007, **18**, 025301.
- 30 M. T. Coughlan, F. Y. Yap, D. C. Tong, S. Andrikopoulos, A. Gasser, V. Thallas-Bonke, D. E. Webster, J. ichi Miyazaki, T. W. Kay, R. M. Slattery, D. M. Kaye, B. G. Drew, B. A. Kingwell, S. Fourlanos, P.-H. Groop, L. C. Harrison, M. Knip and J. M. Forbes, *Diabetes*, 2011, **12**, 2523–2532.
- 31 J. Brange, L. Andersen, E. D. Laursen, G. Meyn and E. Rasmussen, *J. Pharm. Sci.*, 1997, **86**, 517–525.
- 32 R. Kaye, E. Head, F. Sarsoza, T. Saing, C. W. Cotman, M. Necula, L. Margol, J. Wu, L. Breydo, J. L. Thompson, S. Rasool, T. Gurlo, P. Butler and C. G. Glabe, *Molecular Neurodegeneration*, 2007, **2**, 18.