

Bioassay of Proteins in Stable Solution State Using a Novel Cantilever-based Liposome Biosensor

Z. Zhang, T. Akai, K. Takada,
K. Yamashita, and M. Noda

Graduate School of Science and Technology
Kyoto Institute of Technology
Kyoto, Japan
E-mail: zhang-zy@kit.ac.jp, noda@kit.ac.jp

M. Sohgawa

Graduate School of Science and Technology
Niigata University
Niigata, Japan

Abstract—We have developed a micro-cantilever biosensor with embedded NiCr thin film strain gauge. Meanwhile, DPPC liposomes have been selected as sensing biomolecules to be immobilized on the surface of the micro-cantilever. The liposome-protein interaction was detected by measuring the resistance change rate of the strain gauge. In this work, a PDMS-based sealed reservoir structure was newly fabricated and added on the sensor to keep the target solution stable, where a long-time stable detection was achieved. The resistance of the cantilever-based biosensor increased with time in lysozyme or carbonic anhydrase from bovine (CAB) aqueous solution, and the characteristic of chronological resistance change varied with the concentration and kind of the proteins. It is expected that the micro-cantilever sensor with droplet-sealing structure can be used for bioassay of various proteins in future.

Keywords—micro-cantilever; NiCr strain gauge; droplet-sealing structure; liposome; protein

I. INTRODUCTION

As biological protein detection is widely employed in the fields of medical care, food, agriculture and so on, the development of novel technologies for simple, fast and low-cost detection of trace amount of biological proteins without labeling is significantly required. In the past years, the interactions between liposomes and some important proteins have been deeply investigated [1-3], which suggests that liposomes could be used as sensing biomolecules to realize lab-on-a-chip detection of proteins by recognizing the special phenomena of liposome-protein interactions. For example, we have developed a sensitive microbolometer as a thermochemical detector with immobilized intact liposomes on its surface to identify proteins by measuring the heat variation of the bio-thermochemical reactions [4]; we have also developed a leakage current micro-sensor for protein detection by measuring the leakage of electrolyte from perturbed liposomes caused by liposome-protein interactions [5]. In this study, by further utilizing liposome-protein interactions, a novel scheme of cantilever-based liposome biosensor is proposed.

The principle of cantilever-based biosensor is that sensing biomolecules are immobilized on the surface of a micro-cantilever, then the detection of the target protein is carried out

by measuring the deflection of cantilever caused by the interaction between the sensing biomolecules and target protein [6]. However, there still seems to be some obstacles to realize the full potential of cantilever-based biosensor, although many researches have been done [6-12]. First of all, there are some drawbacks in the existing methods for measuring the deflection of cantilever. The optical method, which is the most conventional one, needs precise optical read-out systems like AFM equipment, therefore becomes huge, complex, and expensive. On the other hand, the signal detection is influenced by the change in the refractive indexes of solutions, and the detections in opaque solutions such as blood are impossible [6-9].

Recently, the cantilever with the silicon (or silicon compound) piezoresistance has been developed, where the deflection can be translated into electrical signal [10-14]. Although the detection has become more convenient, it has been found that the signal of p^+ -Si piezoresistance is not stable due to its high temperature coefficient of resistance (TCR) [14]. Therefore, further improvement in the electrical method for measuring the deflection of cantilever is necessary. Specific biomaterial such as antigen is selected as sensing biomolecule to be immobilized on the surface of cantilever for detecting the corresponding protein such as antibody [6-8]. In another aspect, there are mainly two ways for detection in liquid, one is static measurement by immersing the cantilever in a big open liquid cell (0.8~10 mL) filled with the target protein solution [8,11], and the other is dynamic measurement in the fluid solution which flows through a cell and is controlled by a pump [6,7,13]. However, using any of the two methods, large amounts of reagents are required. In particular, the fluid liquid mentioned in the second method may have more influence on the stability and accuracy of measurement than the static liquid if the liquid flow is not precisely controlled.

In view of the above problems, some design modifications of the cantilever-based biosensor, including 1) insertion of NiCr thin film strain gauge in the cantilever instead of silicon piezoresistance, 2) immobilization of liposomes as sensing biomolecules, and 3) fabrication of a PDMS-based sealed reservoir structure, have been done in this work. To be specific, firstly it has been confirmed that TCR of NiCr is much lower than those of Si and Si compounds [14,16].

This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI Grant No. 25249048) from the Japan Society for the Promotion of Science (JSPS).

Therefore, it is considered that the application of NiCr thin film strain gauge would improve the stability of the electrical method of deflection measurement. Secondly, the usage amount of target protein solution would be reduced using the PDMS-based reservoir with a volume of 7 μL , and the reservoir is covered with a cover glass to prevent the evaporation of solution during measurement. It is expected a long-time stable detection in the static liquid can be obtained. In this study, the detection of different kinds of target proteins (lysozyme and carbonic anhydrase from bovine (CAB)) as a parameter of concentration is adopted to investigate the protein detection capability of the cantilever-based liposome biosensor.

II. EXPERIMENTAL PROCEDURE

A. Fabrication of micro-cantilever

The micro-cantilever with the NiCr thin film strain gauge was fabricated by surface micromachining process as described previously [16]. In brief, a silicon-on-insulator (SOI) wafer was used as a substrate, on which the films of Si_3N_4 , NiCr, and Au were deposited in sequence by rf sputtering and patterned by photolithography. Then, a Cytop film as a passivation layer was spin-coated and cured at 200°C for 1 hour. Finally, the cantilever structure was formed by the etching of the buried Si oxide sacrificial layer in buffered hydrofluoric acid solution for 5 hours, and then dried in vacuum after rinsing. Figure 1 exhibits that the NiCr thin film strain gauge with the thickness of 40 nm was formed on the micro-cantilever, where the Ni:Cr ratio was adjusted to 8:2 to optimize the sensitivity in this work.

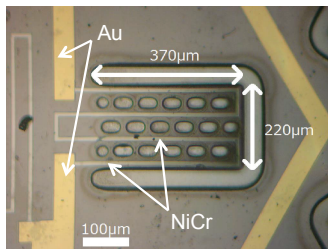


Fig. 1. An optical microscopic image of the micro-cantilever with NiCr thin film strain gauge.

B. Preparation and immobilization of liposome

Dipalmitoyl phosphocholine (DPPC) and phosphatidyl ethanolamine (PE) were purchased from Avanti Polar Lipids (Birmingham, Wales, UK). The liposomes were prepared using DPPC supplemented with 1 wt% of PE as the same manner as reported in elsewhere [4]. In short, the phospholipids were dissolved in a chloroform solution and then evaporated to a dry lipid film. The lipid film was kept under high vacuum for at least 3 hours, and then hydrated with ultrapure water. The suspension was frozen in -80°C and thawed for five cycles, and finally extruded through a polycarbonate filter of 100-nm pore size at room temperature by using a Liposofast extruder (Avestin, Ottawa, Canada) [17].

As for the immobilization of liposomes, first the micro-cantilever was coated with Au ultrathin film by vacuum evaporation, and SAM were formed on the surface of the Au

film via thiol-metal bond by immersing the cantilever in ethanol solution of 16-mercaptoundecanoic acid. Then, the carboxyl radicals on the SAM surface were activated by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide in the mixed solution of 1,4-dioxane (90 vol%) and deionized water (10 vol%) in order to attach the amino radicals of other biomolecules via amide linkage. Finally, the DPPC liposomes containing PE phospholipid molecules which have amino radicals were immobilized on the SAM surface by immersing the cantilever in the liposome suspension for at least 12 hours. The micro-cantilever with immobilized liposomes was kept in ultrapure water before being used. The surface morphology of the intact immobilized liposomes was observed by atomic force microscopy (AFM), as shown in Fig. 2. It is observed that the spherical liposomes are immobilized intact on the SAM and their diameters are 50~100 nm, which agrees well with the mesh size of the filter used in the final liposome preparation process of extruding.

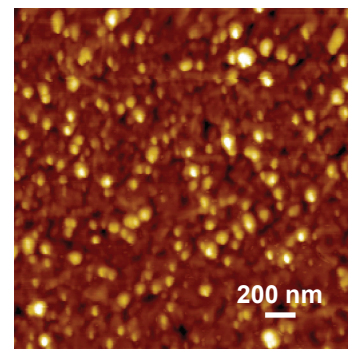


Fig. 2. An AFM image of DPPC liposomes immobilized on SAM/Au layer.

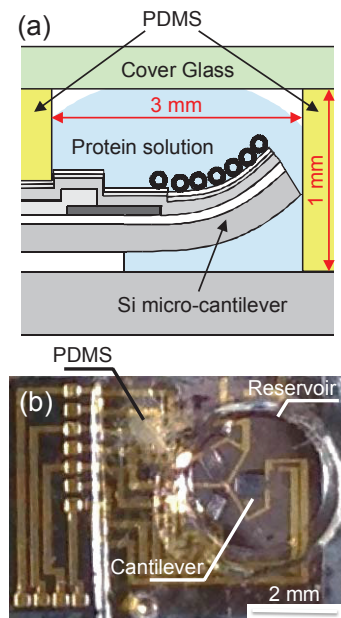


Fig. 3. A schematic cross-sectional view (a) and a bird's eye view (b) of the droplet-sealed structure.

C. Detection of target proteins in droplet-sealing structure

Lysozyme and CAB were obtained from Sigma Aldrich (St. Louis, MO, USA). Their aqueous solutions with various concentrations (100~1200 μM) were selected as detection targets. In order to keep the target solution stable during measurement, a newly developed PDMS-based sealed structure was added on the cantilever sensor [18]. Figure 3 illustrates the droplet-sealing structure with a 7- μL reservoir. After the target solution (7 μL) was filled in the reservoir, the reservoir was covered with a cover glass. Then, the resistance of the micro-cantilever sensor was measured every 0.5 s using a digital multimeter (R6581, Advantest, Japan). The deflection of the cantilever was evaluated by the resistance change rate of the strain gauge, $\Delta R/R_0$, where R_0 represents the initial resistance (approximately 4.5 k Ω) of the NiCr thin film strain gauge, and ΔR represents the change of resistance with time.

III. RESULTS AND DISCUSSION

In Fig. 4 is plotted the resistance change rate of the micro-cantilever sensor in lysozyme aqueous solutions as a function of time. As expected, the resistance is almost stable in water, owing to no interaction occurring on the cantilever. Moreover, it is clear that a long-time stable micro-detection more than 30 minutes has been achieved by using the droplet-sealing structure. By contrast, the previous results (not shown here) showed that, after about 10-minute stable measurement in 8-microliter water droplet without any droplet-sealing structure, the resistance dramatically increased and sharply decreased within only 1 minute caused by the complete evaporation of the water around the cantilever. It is suggested that the newly designed droplet-sealing structure prevents the evaporation of the solution around the cantilever and sufficiently improves the stability of the solution. In this way, some protein solutions with low chemical activity would be accurately detected benefiting from the extended time of stable measurement. Next, it is found that the resistance gradually increases with time in the lysozyme solution. G. Wu [6] and J. Fritz [8] have explained that the interaction of biomolecules on the surface of cantilever leads to the change in surface stress, followed by the deflection of the cantilever. Therefore, it is indicated that the DPPC liposome recognizes the lysozyme molecule, and the deflection of the micro-cantilever caused by the liposome-protein interaction on its surface can be translated to stable electrical signal for detection. In addition, the increase rate of the resistance becomes larger in the lysozyme solution with higher concentration. The reason for this phenomenon is considered to be that more lysozyme molecules interact with the liposomes per unit area of the cantilever surface with increasing the concentration of the lysozyme solution. This suggests that the recognition of the lysozyme concentration can be realized by the cantilever-based liposome biosensor.

The similar phenomena have also been observed for the measurement in the CAB solutions (data not shown here). The resistance increases with time in the CAB solutions, and the increase rate of the resistance is dependent on the concentration of the CAB solution. However, it is found that the increase rates of the resistance in the CAB solutions are much higher than those in the lysozyme solutions. For a

detailed comparison, in Fig. 5 is summarized the resistance change rate of the micro-cantilever sensor vs. protein concentration after filling the reservoir with CAB or lysozyme solution for 30 min. The increasing tendency of resistance change rate with increasing the concentration of either protein is clearly shown in Fig. 5. In particular, by comparing the resistance changes in CAB and lysozyme solutions with the same concentration (e.g., 400 or 800 μM), it is obvious that the increase rate in CAB solution is much larger than that in lysozyme solution. It is considered that this phenomenon is closely related to their different molecular bonding structures [19,20], which play an important role in the chemical activity of proteins. The above results indicate that this cantilever-based liposome biosensor with droplet-sealing structure is capable of detecting the concentration and kind of proteins.

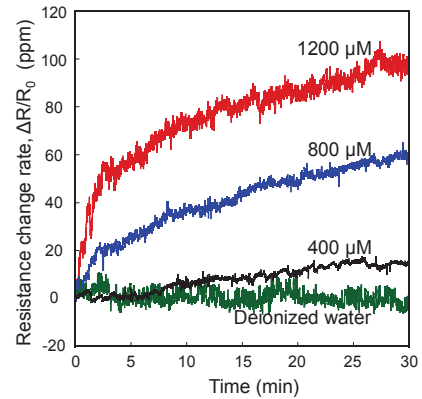


Fig. 4. Resistance change rate ($\Delta R/R_0$) of the micro-cantilever sensor in deionized water and lysozyme aqueous solutions as a function of time.

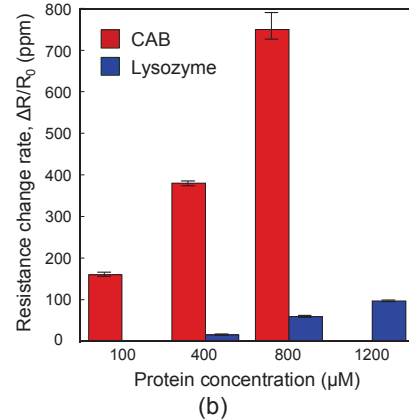


Fig. 5. Resistance change rate of the micro-cantilever sensor vs. protein concentration after filling the reservoir with CAB or lysozyme aqueous solution for 30 min.

IV. CONCLUSION

A droplet-sealing structure with a capacity of 7 μL sustains the stable liquid condition of the target protein, and brings about a long-time stable micro-detection. The resistance of the cantilever sensor increases with time in both lysozyme and CAB aqueous solutions. It is indicated that DPPC liposome is an appropriate sensing biomolecule, and the liposome-protein

interaction can be translated to stable electrical signal for detection by using the cantilever-based biosensor. Furthermore, it is found that the increasing rate of the resistance becomes larger with increasing the concentration of the protein solution. Meanwhile, by comparing the resistance change rate in different kinds of target protein solutions with the same concentration, it is also found that CAB shows much larger interaction with DPPC liposome than lysozyme. These results indicate that the cantilever-based liposome biosensor is excellent at detecting the concentration and kind of proteins. Therefore, it is expected that the novel biosensor becomes a promising candidate device for bioassay of proteins.

REFERENCES

- [1] M. Dathe, M. Schüman, T. Wiprecht, A. Winkler, M. Beyermann, E. Krause, K. Matsuzaki, O. Murase, and M. Bienert, "Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes," *Biochemistry*, vol. 35, pp. 12612-12622, Sep. 1996.
- [2] N. Yoshimoto, M. Yoshimoto, K. Yasuhara, T. Shimanouchi, H. Umakoshi, and R. Kuboi, "Evaluation of temperature and guanidine hydrochloride-induced protein-liposome interactions by using immobilized liposome chromatography," *Biochem. Eng. J.*, vol. 29, pp. 174-181, Apr. 2006.
- [3] S. Morita, M. Nukui, and R. Kuboi, "Immobilization of liposomes onto quartz crystal microbalance to detect interaction between liposomes and proteins," *J. Colloid Interf. Sci.*, vol. 298, pp. 672-578, Jun. 2006.
- [4] M. Noda, T. Shimanouchi, M. Okuyama, and R. Kuboi, "A bio-thermochemical microbolometer with immobilized intact liposome on sensor solid surface," *Sens. Actuators B*, vol. 135, pp. 40-45, Dec. 2008.
- [5] M. Noda, P. Lorchirachoonkul, T. Shimanouchi, K. Yamashita, H. Umakoshi, and R. Kuboi, "Sensitivity enhancement of leakage current microsensor for detection of target protein by using protein denaturant," *IEEE Sens. J.*, vol. 11, pp. 2749-2755, Nov. 2011.
- [6] G. Wu, R. H. Datar, K. M. Hansen, T. Thundat, R. J. Cote, and A. Majumdar, "Bioassay of prostate-specific antigen (PSA) using microcantilevers," *Nat. Biotechnol.*, vol. 19, pp. 856-860, Sep. 2001.
- [7] R. Raiteri, G. Nelles, H. Butt, and W. Knoll, "Sensing of biological substances based on the bending of microfabricated cantilevers," *Sens. Actuators B*, vol. 61, pp. 213-217, Dec. 1999.
- [8] J. Fritz, M. K. Baller, H. P. Lang, H. Rothuizen, P. Vettiger, E. Meyer, H. J. Güntherodt, C. Gerber, and J. K. Gimzewski, "Translating biomolecular recognition into nanomechanics," *Science*, vol. 288, pp. 316-318, Apr. 2000.
- [9] C. Ziegler, "Cantilever-based biosensors," *Anal. Bioanal. Chem.*, vol. 379, pp. 946-959, Aug. 2004.
- [10] N. S. Kale, M. Joshi, P. N. Rao, S. Mukherji, and V. R. Rao, "Bio-functionalization of silicon nitride-based piezo-resistive microcantilevers," *Sadhana-Acad. P. Eng. S.*, vol. 34, pp. 591-597, Aug. 2009.
- [11] Y. Chen, P. Xu, M. Liu, and X. Li, "Bio/chemical detection in liquid with self-sensing Pr-Oxi-Lever (piezo-resistive SiO₂ cantilever) sensors," *Microelectron. Eng.*, vol. 87, pp. 2468-2474, Dec. 2010.
- [12] S. Hyun, H. Kim, Y. Kim, and H. Jung, "Mechanical detection of liposomes using piezoresistive cantilever," *Sens. Actuators B*, vol. 117, pp. 415-419, Oct. 2006.
- [13] K. Yoo, K. Na, S. Jong, B. Nahm, C. J. Kang, and Y. Kim, "Microcantilever-based biosensor for detection of various biomolecules," *Jpn. J. Appl. Phys.*, vol. 45, pp. 515-518, Jan. 2006.
- [14] H. Ohnishi, M. Sohagawa, H. Tachibana, Y. Huang, T. Kanashima, M. Okuyama, K. Yamashita, M. Noda, and H. Noma, "Stability improvement of tactile sensor of normal and shear stresses using Ni-Cr thin film gauge," *IEEJ Trans. SM.*, vol. 129, pp. 411-416, Mar. 2009.
- [15] N. K. Chaki, and K. Vijayamohan, "Self-assembled monolayers as a tunable platform for biosensor applications," *Biosens. Bioelectron.*, vol. 17, pp. 1-12, Jan. 2002.
- [16] M. Sohagawa, D. Hirashima, Y. Moriguchi, T. Uematsu, W. Mito, T. Kanashima, M. Okuyama, and H. Noma, "Tactile sensor array using microcantilever with nickel-chromium alloy thin film of low temperature coefficient of resistance and its application to slippage detection," *Sens. Actuators A*, vol. 186, pp. 32-37, Oct. 2012.
- [17] R. C. MacDonald, R. I. MacDonald, B. Ph. M. Menco, K. Takeshita, N. K. Subbarao, and L. Hu, "Small-volume extrusion apparatus for preparation of large, unilamellar vesicles," *Biochim. Biophys. Acta*, vol. 1061, pp. 297-303, Jan. 1991.
- [18] M. Sohagawa, T. Fujimoto, K. Takada, K. Yamashita, and M. Noda, "Detection of interaction between biological proteins and immobilized liposomes by a micro-cantilever with NiCr thin film strain gauge," in *Proc. IEEE Sens. 2013*, Nov. 2013, pp. 846-849.
- [19] C. D. Boone, A. Habibzadegan, C. Tu, D. N. Silverman, and R. McKenna, "Structural and catalytic characterization of a thermally stable and acid-stable variant of human carbonic anhydrase II containing an engineered disulfide bond," *Acta Cryst. D Biol. Crystallogr.*, vol. 69, pp. 1414-1422, Aug. 2013.
- [20] V. Touch, S. Hayakawa, K. Fukada, Y. Aratani, and Y. Sun, "Preparation of antimicrobial reduced lysozyme compatible in food applications," *J. Agric. Food Chem.*, vol. 51, pp. 5154-5161, Aug. 2003.