ANALYTICAL SCIENCES MARCH 2015, VOL. 31

Label-free Detection of Antigen Protein Using a Metal Mesh Device Surface-modified by an Antibody

Hirokazu SETO,* Seiji KAMBA, Takashi KONDO,** Yuichi OGAWA,*** Yu HOSHINO,* and Yoshiko MIURA*†**

**Graduate School of Engineering, Kyushu University, 744 Motooka, Nishi, Fukuoka 819–0395, Japan*
***Murata Manufacturing Co., 1-10-1 Higashikotari, Nagaokakyo 617–8555, Japan*
****Graduate School of Agriculture, Kyoto University, Kitashirakawaoiwake-cho, Sakyo, Kyoto 606–8502, Japan*

A biosensor for protein detection was developed using antibody-immobilized metal mesh devices. Antihemoglobin antibodies were covalently immobilized on a metal mesh device. Extraordinary transmission with a dipped structure was observed for a metal mesh device immobilized with antihemoglobin antibodies as well as for the original metal mesh device. Hemoglobin in the mixture solution containing albumin at a hundred-fold concentration was detectable using antihemoglobin-immobilized MMDs. The detectability using the antihemoglobin-immobilized metal mesh device was similar to that of a commercially-available kit for the qualitative determination of hemoglobin.

Keywords Metal mesh device, antigen-antibody, hemoglobin, label-free biosensor

(Received December 5, 2014; Accepted January 29, 2015; Published March 10, 2015)

Introduction

Recently, there have been significant developments in biosensors. Specific biomolecular recognitions, such as antigen-antibody, enzyme-substrate, saccharide-lectin, and DNA hybridization, are used in many biosensors.[1] In biosensing, biochemical reactions are indicated by various signals, *e.g.*, microcalorimetric sensors detect heat changes, electrochemical and field-effect transistor sensors detect voltage-current changes, surface plasmon resonance (SPR) and reflectometric interference spectroscopy sensors detect refractive index changes, and quartz crystal microbalance (QCM) and surface acoustic wave sensors detect frequency changes.

In the 1960s, metal mesh devices (MMDs) were found to exhibit frequency selectivity, and could be used as band-pass filters.[2] MMD surfaces have periodic micrometer-sized square holes, and electromagnetic waves in the terahertz region are transmitted through the MMDs. The extraordinary transmittance frequency is inversely proportional to the periodic size of the MMD.[3] When the complex refractive index of the MMD surface is changed by attaching a substance, the extraordinary transmission peak with a dipped structure is shifted toward lower frequency. Based on this phenomenon, MMD sensors, which detect the quantity of substance from the shift in the spectrum, have been developed.[4–12] Metal species for manufacturing are not limited, because MMDs made from various metals have extraordinary transmittance. MMDs offer high-throughput, compared with QCM and SPR, and can be used in screening and initial diagnosis. MMD sensing covers targets with a wide range of sizes, including nanometer-order proteins and micrometer-order cells, by selecting appropriate operating frequencies, which is the biggest advantage. Recently, we determined particulate matter of various sizes in air using MMDs with different periodic sizes.[11]

Recently, it was found that the area of the electric field of the MMDs surface and the target size have a good correlation.[9] Therefore, when we detect a small-size target, such as proteins, we had better design MMDs, which can work at a much higher frequency region than the far-infrared (IR) region. To realize this detection, we have developed MMDs, that have an extraordinary transmission with a dipped structure at around 40 THz (40 THz-MMD).[8] The sensitivity, which is affected by changes in the complex refractive index, decreases exponentially with leaving from the MMD surface.[8,9,12] Detection by the sensing system, in which biomolecular targets are directly adsorbed on the MMD surface, is therefore high. MMD surfaces have been modified by biotin and saccharide to achieve the direct adsorption of biomolecular targets, in which 100 THz-MMDs with small periodic sizes were used.[10,12] The detection limits of 100 THz-MMD sensors are low, and are similar to those achieved using QCM and SPR. The transmittance selectivities of 40 THz- and 100 THz-MMDs are in the middle-IR range (1000 – 4000 cm^{-1} of wavenumber), and therefore ordinary IR spectrometers can be used.

In the present study, an antigen protein was detected using a 100 THz-MMDs, on which antibodies were immobilized. Specific antigen-antibody affinities are widely used in biosensing.[13–15] Various biomolecular targets have been sensed by the corresponding antibodies. In the current study, antihemoglobin and hemoglobin were used as the model ligand and target protein, respectively. The detection ability of the antihemoglobin-immobilized MMD was compared with that of

† To whom correspondence should be addressed.
E-mail: miuray@chem-eng.kyushu-u.ac.jp

a hemoccult kit for urine, which has been used in medical centers and at home.

Experimental

Reagents and materials

3-(Trimethoxysilyl)propyl methacrylate (TMSMA; Sigma Co., St. Louis, MO), *N*-acryloxysuccinimide (NAS; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and 2,2′-azobis isobutyronitrile (AIBN; Wako Pure Chemical Industries Ltd., Osaka, Japan) were used to synthesize poly(TMSMA-*r*-NAS) (Fig. S1, Supporting Information). The TMSMA and NAS moieties act as surface-reactive and amine-reactive groups, respectively, and poly(TMSMA-*r*-NAS) has frequently been used for binding a sensor matrix and protein ligands *via* the amine coupling reaction.[16–18] Anti-human hemoglobin monoclonal antibodies and purified human hemoglobin were purchased from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan) and MP Biomedicals, LLC (Santa Ana, CA), respectively. Albumin from bovine serum (Sigma Co., St. Louis, MO) was used as a negative-control protein. We used 100 THz-MMDs. MMDs were manufactured by the electroforming method using nickel, and had a hole size, grid interval, and thickness of 1.8, 2.6, and *ca.* 1 μm, respectively.

Immobilization of anti-human hemoglobin antibodies on MMDs

The MMDs were washed with a mixture of aqueous ammonia and hydrogen peroxide at 60°C for 20 min before use. The MMDs were incubated in a poly(TMSMA-*r*-NAS) solution (DMF, 5 g L^{-1}, 2 mL) at 37°C for 20 h, and were then heated at 110°C for 30 min for covalent bond formation. The amounts of poly(TMSMA-*r*-NAS) immobilized on the MMDs were estimated from the changes in the weight of the MMDs. The poly(TMSMA-*r*-NAS)-immobilized MMDs were incubated in a phosphate-buffered solution (10 mM, pH 7.4) of antihemoglobin (167 mg L^{-1}, 0.5 mL) at 37°C for 20 h. The residual succinimide groups were inactivated by immersion in ethanolamine aqueous solution (10 g L^{-1}, 1 mL). The resulting MMDs were washed with water, and then dried. The MMD transmittance properties were evaluated using an IR spectrometer (Spectrum 100 FTIR spectrometer, PerkinElmer Inc., Waltham, MA) under dry conditions. The wavenumber resolution, accumulation number, and spot diameter were 2 cm^{-1}, four measurements, and 6 mm ϕ, respectively. The transmittance IR spectra were recorded at a frequency range of 20 – 120 THz. The presence of poly(TMSMA-*r*-NAS) and antihemoglobin on the MMDs was confirmed using X-ray photoelectron spectroscopy (XPS) (Supporting Information). The amount of antihemoglobin on the poly(TMSMA-*r*-NAS)-immobilized surface was determined using a QCM (Supporting Information).

Antigen-antibody detection using antibody-immobilized MMDs

Proteins (hemoglobin and albumin) were detected using the antihemoglobin-immobilized MMDs. A hemoglobin solution was passed through a microfilter before use; the concentration of protein in the filtrate was determined by ultraviolet-visible spectroscopy. The antihemoglobin-immobilized MMDs were immersed in a phosphate-buffered solution (10 mM, pH 7.4) of hemoglobin (5×10^{-5} to 5×10^{-1} g L^{-1}, 1 mL) or albumin (5×10^{-3} to 5×10^{1} g L^{-1}, 1 mL) at 25°C for 2 h. The MMDs were then washed with a sufficient quantity of water using a shaker for over 30 min, and then dried. The transmittance IR spectra of the MMDs were recorded before and after protein adsorption. The shifts in the spectra ($-\Delta f$) were estimated from the difference between the dipped frequencies before and after protein adsorption, and were corrected according to the shifts observed for MMD incubated in buffer. Hemoglobin adsorption on the MMDs was confirmed using XPS. The binding ability of the immobilized antihemoglobin to hemoglobin was determined using a QCM (Supporting Information).

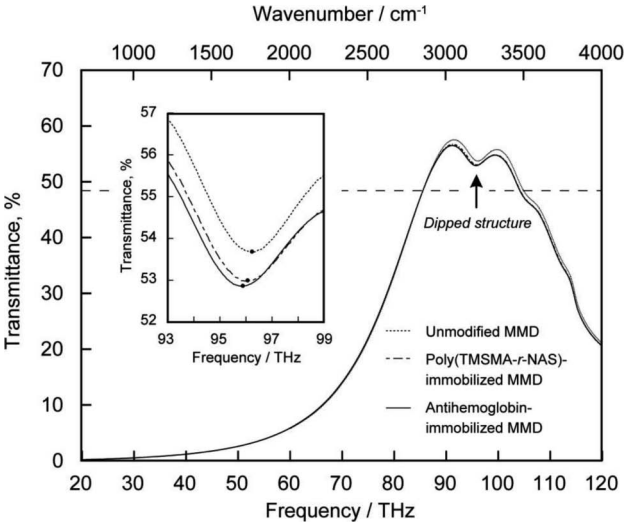

Fig. 1 Transmittance IR spectra of unmodified, poly(TMSMA-*r*-NAS)-immobilized, and antihemoglobin-immobilized MMDs.

Hemoglobin was detected using the antihemoglobin-immobilized MMDs in coexistence with albumin. The antihemoglobin-immobilized MMDs were immersed in a mixture solution of hemoglobin (5×10^{-3} g L^{-1}) and albumin (5×10^{-3} to 5×10^{1} g L^{-1}) at 25°C for 2 h. The MMDs were then washed with a sufficient quantity of water using a shaker for over 30 min, and then dried. The relative frequency changes for hemoglobin were calculated from the $-\Delta f$ for hemoglobin in the absence and presence of albumin.

Results and Discussion

Preparation of antibody-immobilized MMDs

Antihemoglobin-immobilized MMDs were prepared *via* an amine coupling reaction. The transmittance IR spectra of the unmodified, poly(TMSMA-*r*-NAS)-immobilized, and antihemoglobin-immobilized MMDs are shown in Fig. 1. A higher transmittance was observed at between 85 and 105 THz, although the opening ratio of the MMDs was 48%. Surface plasmon polariton-like waves are excited by anomalous diffraction through an MMD with periodic holes.[3,19] As a result, the electromagnetic field is intensified and localized at the MMD surface. A dipped structure was observed at around 95 THz. The MMD transmittance spectra were affected by the incursion of a substance into the localized electromagnetic field. When poly(TMSMA-*r*-NAS) was immobilized on the MMD, the dipped structure was shifted toward lower frequency. The amount of poly(TMSMA-*r*-NAS) immobilized on the MMD was 19 μg per sheet. The dipped structure was then shifted toward lower frequency by the immobilization of antihemoglobin. The extraordinary transmission with a dipped structure in these IR spectra indicates that the antihemoglobin-immobilized MMDs retained the periodic lattice geometry. The antihemoglobin-immobilized MMDs were used in the middle-

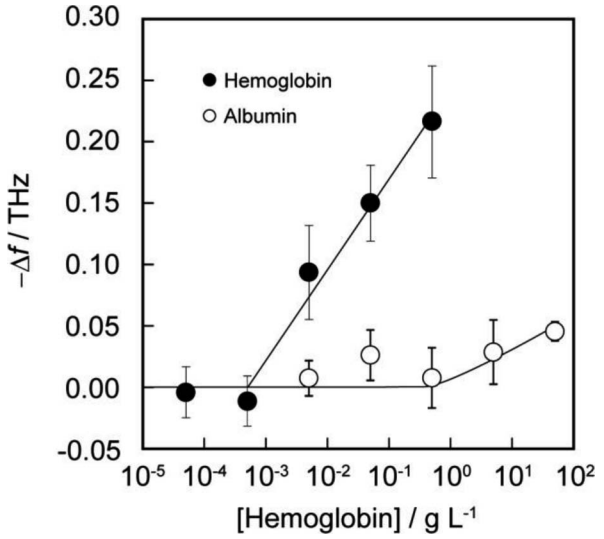

Fig. 2 Detection of hemoglobin and albumin using antihemoglobin-immobilized MMDs. The error bars were determined from six different measurements.

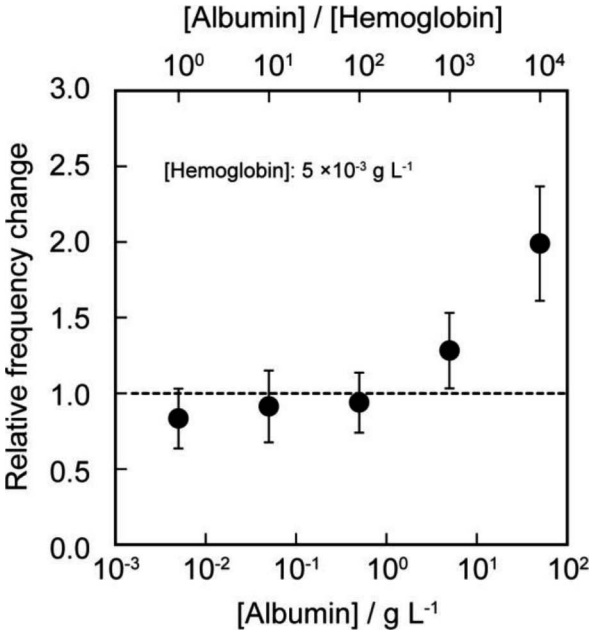

Fig. 3 Detection of hemoglobin in coexistence with albumin using antihemoglobin-immobilized MMDs. The error bars were determined from three different measurements.

IR region. The presence of poly(TMSMA-*r*-NAS) and antihemoglobin on the MMDs was confirmed using XPS (Fig. S2, Supporting Information). In addition, the atomic percentages of carbon, nitrogen, oxygen, and nickel on the MMDs are summarized in Table S1 (Supporting Information). The atomic percentages of carbon and nitrogen increased after the immobilization of poly(TMSMA-*r*-NAS) and antihemoglobin. The amount of the antibody on the poly(TMSMA-*r*-NAS)-immobilized surface was determined to be *ca.* 800 ng cm^{-2} (Supporting Information).

Detection of protein using antibody-immobilized MMD sensor

The detection of a protein was performed using the antibody-immobilized MMDs The measurement time for one sample was *ca.* 1 min. Rapid MMD sensing is applicable in screening and initial diagnosis. The $-\Delta f$ values of the MMD for hemoglobin and albumin detection are shown in Fig. 2. A well-defined change in the dipped frequency appeared at 5×10^{-3} g L^{-1} using the antihemoglobin-immobilized MMD, which is close to that achieved in the QCM experiment (Fig. S3, Supporting Information). We previously succeeded in detecting biotin-streptavidin, saccharide-lectin, and saccharide-bacteria interactions, using the MMDs, in which the detection limits of the MMD were comparable to those achieved using QCM and SPR sensors.[10,12] An antigen-antibody interaction was also detected by the MMD. There was little detection of albumin at the same concentration range to hemoglobin, using the antihemoglobin-immobilized MMDs. Albumin at over 5×10^{0} g L^{-1} was slightly detected by the MMDs.

Hemoglobin at a concentration of 5×10^{-3} g L^{-1} was detected in coexistence with albumin using the antihemoglobin-immobilized MMDs (Fig. 3). The $-\Delta f$ for hemoglobin in the presence of albumin with up to a concentration of 5×10^{-1} g L^{-1} was equal to that in the absence of albumin. The $-\Delta f$ for hemoglobin in the presence of albumin increased at over a concentration of 5×10^{0} g L^{-1} due to nonspecific adsorption of albumin on the MMD. Hemoglobin was detectable from the mixture solution containing albumin with a hundred-fold concentration using the antihemoglobin-immobilized MMDs. Because the standard concentrations of positive result for hemoglobin and total protein in urine is over 6×10^{-4} and 3×10^{-1} g L^{-1}, respectively, selective detection of hemoglobin in urine sample is possible.

The detection ability of the antibody-immobilized MMD was compared with those of a frequently-used chemical method. Changes in the color of a test strip, based on peroxidase-like activity, is shown in Fig. S4 (Supporting Information). The color of the test strip was changed from white to blue, when hemoglobin was present; no color change was observed at a concentration of 5×10^{-4} g L^{-1}, and the color was clearly changed at a concentration of 5×10^{-3} g L^{-1}. However, it was difficult for us to judge the presence or absence of hemoglobin at 1×10^{-3} g L^{-1}. The detection limit using an antihemoglobin-immobilized MMD was similar to that using a hemoccult kit. Although test-strip, immunochromatography, and coagulation sedimentation methods for protein detection have the advantage of being easy to use, they are not quantitative or semi-quantitative. The MMDs have the advantages of quantitation, similar to QCM, and ease of use, similar to test strip.

Conclusions

An antibody-immobilized MMD was developed for protein detection. The antihemoglobin-immobilized MMDs selectively detected the target hemoglobin because of the antibody-antigen specificity. Hemoglobin was detectable in coexistence with hundredfold-concentrated albumin using the antihemoglobin-immobilized MMDs. The detection limit achieved using antihemoglobin-immobilized MMD was similar to that achieved using a commercially available hemoccult kit. The development of MMDs with smaller periodic sizes will lead to the detection limit for protein achieved using MMD being lower than those achieved using QCM and SPR. Because there are various antibodies for viruses, bacteria, toxins, and hormones as well as proteins, there is a wide range of antibody-immobilized MMDs. MMDs can be applied for screening and initial diagnosis, and

have the advantages of high throughput and quantitation.

Acknowledgements

This work was supported by a Grant-in-Aid for JSPS Fellows (25.5206), a Grant-in-Aid for Challenging Exploratory Research (26620106), and a Grant-in-Aid for Young Scientists (A) (23685027).

References

1. B. R. Eggins, "*Chemical Sensors and Biosensors*", **2002**, John Wiley & Sons, Chichester, West Sussex, UK.
2. R. Ulrich, *Infrared Phys.*, **1967**, *7*, 37.
3. S. Yoshida, K. Suizu, E. Kato, Y. Nakagomi, Y. Ogawa, and K. Kawase, *J. Mol. Spectrosc.*, **2009**, *256*, 146.
4. F. Miyamaru, S. Hayashi, C. Otani, K. Kawase, Y. Ogawa, H. Yoshida, and E. Kato, *Opt. Lett.*, **2006**, *31*, 1118.
5. H. Yoshida, Y. Ogawa, Y. Kawai, S. Hayashi, A. Hayashi, C. Otani, E. Kato, F. Miyamaru, and K. Kawase, *Appl. Phys. Lett.*, **2007**, *91*, 253901.
6. Y. Ogawa, S. Hayashi, C. Otani, and K. Kawase, *PIERS Online*, **2008**, *4*, 396.
7. S. Yoshida, E. Kato, K. Suizu, Y. Nakagomi, Y. Ogawa, and K. Kawase, *Appl. Phys. Express*, **2009**, *2*, 012301.
8. T. Kondo, S. Kamba, K. Takigawa, T. Suzuki, Y. Ogawa, and N. Kondo, *Procedia Eng.*, **2011**, *25*, 916.
9. T. Suzuki, T. Kondo, Y. Ogawa, S. Kamba, and N. Kondo, *IEEE Sens. J.*, **2013**, *13*, 4972.
10. H. Seto, C. Yamashita, S. Kamba, T. Kondo, M. Hasegawa, M. Matsuno, Y. Ogawa, Y. Hoshino, and Y. Miura, *Langmuir*, **2013**, *29*, 9457.
11. H. Seto, S. Kamba, T. Kondo, Y. Ogawa, Y. Hoshino, and Y. Miura, *Chem. Lett.*, **2014**, *43*, 408.
12. H. Seto, S. Kamba, T. Kondo, M. Hasegawa, S. Nashima, Y. Ehara, Y. Ogawa, Y. Hoshino, and Y. Miura, *ACS Appl. Mater. Interfaces,* **2014**, *6*, 13234.
13. P. D. Skottrup, M. Nicolaisen, and A. F. Justesen, *Biosens. Bioelectron.*, **2008**, *24*, 339.
14. B. Byrne, E. Stack, N. Gilmartin, and R. O'Kennedy, *Sensors*, **2009**, *9*, 4407.
15. T. R. J. Holford, F. Davis, and S. P. J. Higson, *Biosens. Bioelectron.*, **2012**, *34*, 12.
16. T. Endo, S. Ozawa, N. Okuda, Y. Yanagida, S. Tanaka, and T. Hatsuzawa, *Sens. Actuators, B*, **2010**, *148*, 269.
17. T. Endo, M. Sato, H. Kajita, N. Okuda, S. Tanaka, and H. Hisamoto, *Lab Chip*, **2012**, *12*, 1995.
18. Y. Terada, W. Hashimoto, T. Endo, H. Seto, T. Murakami, H. Hisamoto, Y. Hoshino, and Y. Miura, *J. Mater. Chem. B*, **2014**, *2*, 3324.
19. J. B. Pendry, L. Martin-Moreno, and F. J. Garcia-Vidal, *Science*, **2004**, *305*, 847.