Fabrication of microtemplates for the control of bacterial immobilization

Yasuhiro Miyahara
Department of Materials, Physics and Energy Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa, Nagoya, 464-8603, Japan

Koji Mitamura
EcoTopia Science Research Institute, Nagoya University, Furo-cho, Chikusa, Nagoya, 464-8603, Japan

Nagahiro Saito[a)]
Department of Materials, Physics and Energy Engineering, Graduate School of Engineering, Nagoya University and Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa, Nagoya, 464-8603, Japan

Osamu Takai
Department of Materials, Physics and Energy Engineering, Graduate School of Engineering, Nagoya University and EcoTopia Science Research Institute, Nagoya University, Furo-cho, Chikusa, Nagoya, 464-8603, Japan

(Received 16 May 2008; accepted 22 June 2009; published 13 August 2009)

The authors described a region-selective immobilization methods of bacteria by using superhydrophobic/superhydrophilic and superhydrophobic/poly(ethylene glycol) (PEG) micropatterns for culture scaffold templates. In the case of superhydrophobic/superhydrophilic micropatterns, the superhydrophobic surface was prepared first by microwave-plasma enhanced chemical vapor deposition (MPECVD) from trimethylmethoxysilane. Then the superhydrophilic regions were fabricated by irradiating the superhydrophobic surface with vuv light through a stencil mask. In the case of the superhydrophobic/PEG micropatterned surfaces, PEG surfaces were fabricated first by chemical reaction of ester groups of *p*-nitrophenyl PEG with NH_2 group of NH_2-terminated self assembled monolayer from *n*-6-hexyl-3-aminopropyltrimethoxysilane. The superhydrophobic regions were fabricated by MPECVD thorough a stencil mask. In this study four bacteria were selected from viewpoint of peptidoglycan cell wall (*E. coli* versus *B. subtilis*), extracellular polysaccharide (*E.coli* versus *P. stutzeri*, *P. aeruginosa*), and growth rate (*P. stutzeri* versus *P. aeruginosa*). The former micropattern brought discrete adhesions of *E. coli* and *B. subtilis* specifically on the hydrophobic regions, Furthermore, using the superhydrophobic/PEG micropattern, adhesion of bacteria expanded for *E. coli*, *B. subtilis*, *P. stutzeri*, and *P. aeruginosa*. They observed a high bacterial adhesion onto superhydrophobic surfaces and the inhibitive effect of bacterial adhesion on PEG surfaces. © *2009 American Vacuum Society.* [DOI: 10.1116/1.3179158]

I. INTRODUCTION

Biodevices including biochips and biosensors have been developed by using biomolecules or living cells as constituent elements.[1] In these devices, bacteria,[2] eucaryotic organism,[3] organisms,[4] DNA,[5] polysaccharide,[6] enzymes,[7] and proteins has been tested.[8] These systems have the advantage of a high chemical and energetic conversion potential in room temperature and play an important role in development of bioindustry. Among them, in the bacterial systems, there are predominant features such as low-cost, high growth rate, high sensitivity against external stimuli in comparison with other systems.[9] Furthermore, in the point that more than 90% of bacteria living in nature are not well known, the bioindustry using bacteria is still under development and include a high potential for biodevice technology.[10]

In the fabrication of the biodevices containing bacteria, the well-controlled immobilization of bacteria on an intended substrate is crucial. A possible approach to control the bacteria immobilization is to utilize surfaces with different wettabilities. This property can have influences on chemical and physical interactions between a surface and bacteria.[11] However, the adhesive properties of bacteria to superhydrophobic surface and superhydrophilic surface, i.e., surfaces with extreme wettability, were not reported. As another route, the surface chemistry can be changed. It has been reported that poly(ethylene glycol) (PEG)-modified surface constrains cell adhesion.[12] The suppression effect is due to the fluctuation of PEG molecules. So, a micropatterned surface with a large different wettabilities and chemical composition regions influence the local cellular adhesion. Therefore, the wettability and the surface chemistry are supposed to be the key technique to control the local adhesion of bacteria.

In this work, we developed micropatterns which potentially can immobilize the bacteria on specific microscopic regions by varying surface wettability and chemistry on a surface. Here we prepared two kinds of micropatterns: (1) superhydrophobic/superhydrophilic surfaces (different wetta-

[a)]Author to whom correspondence should be addressed; electronic mail: hiro@eco-t.esi.nagoya-u.ac.jp

bilities) and (2) superhydrophobic/PEG surfaces (different chemical surface compositions) for the well-defined immobilization of bacteria. Superhydrophobic or superhydrophilic surfaces were fabricated combining hydrophobic or hydrophilic groups on the surface.[13] Furthermore, we aimed to better immobilize bacteria with additional exclusive volume effect and hydration of PEG molecule using superhydrophobic/PEG micropatterns.

II. EXPERIMENT

A. Preparation of superhydrophobic/superhydrophilic micropatterns

Substrates (glass or silicon wafer) were cleaned by irradiation with vuv light at $\lambda = 172$ nm (Ushio Electric, UER20-172V). Fabrication of superhydrophobic films on the substrate was performed by microwave-plasma enhanced chemical vapor deposition (MPECVD) according to the previous reports.[14] In the MPECVD process, trimethylmethoxysilane (TMMOS) (Gelest Inc.) was introduced as precursor gas together with Ar gas (TMMOS=43.5 Pa, Ar=85 Pa). The superhydrophobic films were formed at a MW output power of 300 W for 20 min. The superhydrophobic/superhydrophilic micropatterns were obtained by exposure the superhydrophobic film with vuv light at $\lambda = 172$ nm through a meshed stencil mask (pore diameter $\phi = 1000$ μm) in air atmosphere at 10 Pa, for 30 min. After the irradiation, the exposed surfaces became hydrophilic ones.

B. Preparation of superhydrophobic/PEG micropatterns

Superhydrophobic/PEG micropatterns were fabricated by the following scheme. (1) As precursor of a PEG monolayer film, amino-terminated self-assembled monolayers (SAMs) were prepared by thermal CVD method of *n*-6-hexyl-3-aminopropyltrimethoxysilane (AHAPS) (Azmax Co. Ltd.).[15] The substrates treated by vuv irradiation and a 3.0 cm^3 glass vial containing 0.25 cm^3 of AHAPS dissolved in 1.75 cm^3 of toluene were set together in a PTFE vessel (300 cm^3). The sealed PTFE vessel was kept inside an oven at 100 °C for 1 h. (2) After the SAM formation, carboxyl-functionalized PEG molecules (*p*-nitrophenyl PEG, $M_w = 5000$) (Nichiyu Co.) were introduced on the amino-terminated SAM through esterification between amine groups on a substrate and carboxyl group of PEG molecules. (3) In the MPECVD system using a meshed stencil mask (pore diameter of $\phi = 1000$ μm), the superhydrophobic surfaces were formed on the PEG-modified film.

C. Evaluation of the micropatterns

The morphologies of the obtained surfaces were observed by atomic force microscope (AFM), Kelvin force microscope (KFM) [Seiko Instruments Inc. (SII), SPA-300HV SPI-3800N], and scanning electron microscope (SEM) (JEOL, JSM-6330F). Wettability test was carried out by water contact angle (WCA) measurements (KRÜSS, DSA10-Mk2).

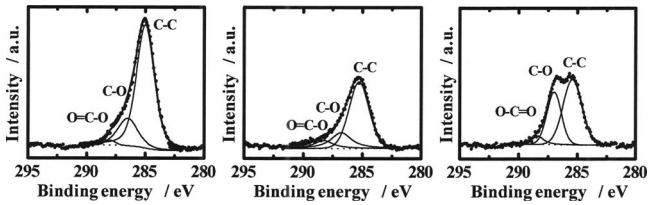

FIG. 1. XPS C1*s* spectra of superhydrophobic (left), superhydrophilic (middle), and PEG (right) surfaces.

The elemental analysis was done by x-ray photoelectron spectroscopy (XPS) (SHIMADZU-KRATOS, AXIS).

D. Bacterial adhesion tests on micro-patterns

Four bacterial strains were used in this study: *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Pseudomonas stutzeri* (*P. stutzeri*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). *E. coli* and *B. subtilis* have been defined as a gram-negative model bacterium and gram-positive model bacterium, respectively.[15,16] *P. subtilis* and *P. aeruginosa* bacteria belong to gene Pseudomonas and have extracellular polysaccharide (EPS) composed by an algin acid.[17] The difference between *P. stutzeri* and *P. aeruginosa* consists in growth rate and gene arrangement.[18] Put it all together, the four bacteria were selected from viewpoint of peptidoglycan cell wall (*E.coli* versus *B. subtilis*), EPS (*E.coli* versus *P. stutzeri, P. aeruginosa*), and growth rate (*P. stutzeri* versus *P. aeruginosa*). Four kinds of bacteria were sowed on a superhydrophobic/superhydrophilic or superhydrophobic/PEG micropattern at 4.0×10^4 cell/dish and incubated under 5% of CO_2 concentration at 37 °C for 24 h. The culture medium was conformed to the condition of OXOID CM3.

After the incubation, the micropattern was rinsed with Dulbecco's phosphate buffered saline (Nacalai Tesque Inc.) and the number of bacteria adhered on the micropattern was counted by a phase-contrast microscope (Olympus, CKX41N—31PHP). The bacteria counting was made for at least ten fields of view for each sample.

III. RESULTS AND DISCUSSION

A. Surface characterization

In Figs. 1 and 2, XPS results (C1*s*) and AFM images of superhydrophobic, superhydrophilic, and PEG-modified surfaces were shown. The WCA, chemical state of carbon, and surface roughness were summarized in Table I. The contents of C–O and C=O bonds on the superhydrophobic surface were increased after the VUV irradiation (i.e., superhydrophilic surface). It is indicating oxidation of carbon atoms on the superhydrophobic surface, which caused decrease of WCA from 150° to 0°. However, the surface roughness (rms ~ 47.5 nm) did not change before and after vuv irradiation. On the PEG modified surface, the C–O bonds resulted from ethylene glycol groups were confirmed from the XPS measurement. However, the peak ratio between C–C and C–O bonds of PEG surfaces were not in agreement with the theoretical value of the sum of raw material component of

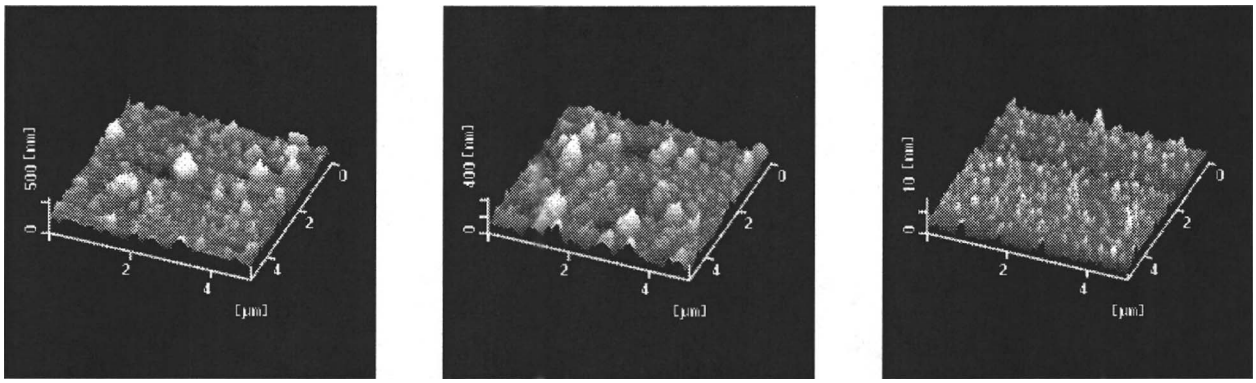

FIG. 2. AFM images of superhydrophobic (left), superhydrophilic (middle), and PEG (right) surfaces.

PEG and AHAPS (theoretical value is C–O : C=O nearly equals 1 : 2). XPS measurements and AFM images showed that the PEG molecules were not uniformly covered on a substrate but spread in domainlike. From AFM images of PEG modified surface, the estimated value of surface coverage was about 0.31 molecules/nm^2.

B. Observation of bacterial adhesion

In order to estimate the bacterial adhesion on superhydrophobic, superhydrophilic, and PEG surfaces, the number of bacteria on each surface after the incubation was counted using a phase-contrast microscope. Figure 3 represents the adhesion densities for all four bacteria on their surfaces besides results on a CH_3-terminated SAM [octadecyltrichlorosilane (OTS)-SAM] and Si–OH (bare glass) surfaces as references.

For all studied bacteria, the adhesion densities on superhydrophobic surfaces were the largest among the other surfaces and three or four times larger than on OTS-SAM. Although the superhydrophobic surface was terminated by CH_3 groups similar with the OTS-SAM surface,[19] the superhydrophobic surface grants a highly adhesive surface due to the larger contact area with bacteria, resulted from the high surface roughness. For the fabrication of bacterial biodevices, the local selective immobilization of bacteria is the key technique. In particular, in the local selective immobilization with high S/N ratio, the surface that enhances the bacterial adhesion to the specific region is as important as the surface that inhibits the bacterial adhesion as a background region. In this case a superhydrophobic surface is suitable as a high sensitive biosensors because the surface can enhance bacterial adhesion.

In the case of a superhydrophilic surface, the adhesion tendencies of *E. coli, B. subtilis* bacteria were different from that of *P. stutzeri, P. aeruginosa* bacteria. Adhesion densities of *E. coli* and *B. subtilis* to superhydrophilic surfaces were lower than hydrophilic Si–OH surfaces. One of the possible reasons is the increase in the exclusive effect of the hydrophilic groups. However, in the case of *P. stutzeri* and *P. aeruginosa*, there were opposite tendencies between superhydrophilic surfaces and hydrophilic Si–OH surfaces. The differences in bacterial adhesion would be resulted from the differences of the physical or chemical interactions of specific EPS. This is because there were no differences of adhesion between *E. coli* as gram-negative model bacteria and *B. subtilis* as gram-positive model bacteria. *P. stutzeri* and *P. aeruginosa* belong to gene *Pseudomonas* and have a characteristic EPS composed of the algin acid. This adhesion behavior excludes the effect of the hydrophilic groups on the surface and could be explained by surface roughness.

On the other hand, Fig. 3 reveals that the PEG surface suppressed the bacterial adhesion the most dominantly among the other surfaces. The result can come from an excluded-volume effect caused by the fluctuant free motion of PEG chain molecules. The results on bacterial adhesion on several surfaces give a guideline for the cell immobilization of specific regions. The combinations between

TABLE I. Characteristics of each surface.

Surface	WCA (Deg)	C–C (%)	C–O (%)	O–C=O (%)	RMS (nm)
Superhydrophobic	>150	80	20	0	47.6
Superhydrophilic	≒0	66	23	11	47.5
PEG	55	58	36	6	1.0

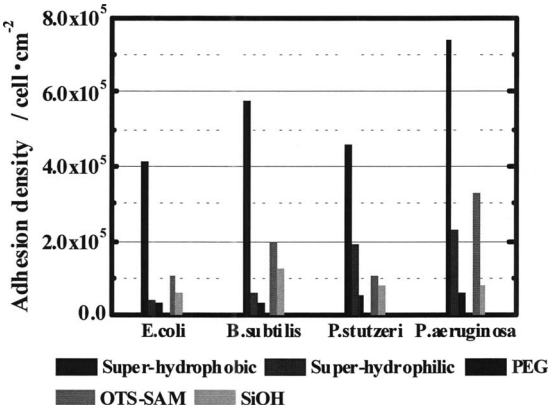

FIG. 3. Adhesion densities on specific surfaces.

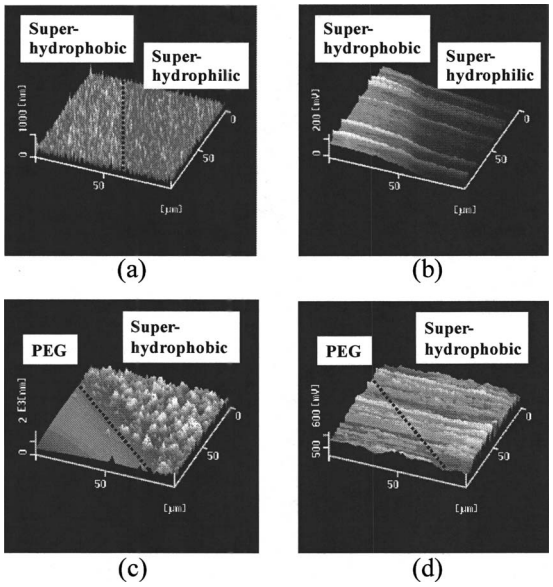

FIG. 4. AFM image of (a) superhydrophobic/superhydrophilic and (c) superhydrophobic/PEG micropatterns, and KFM image of (b) superhydrophobic/superhydrophilic, (d) superhydrophobic/PEG micropatterns.

superhydrophobic/superhydrophilic or superhydrophobic/PEG surfaces might realize such immobilization of the bacteria.

C. Observation of micropatterns

The formation of the micropatterns was confirmed by AFM, KFM, and SEM observations (as shown in Figs. 4 and 5, respectively). As indicated in Table I, the surface roughness, the morphologies of the superhydrophobic and superhydrophilic regions were almost the same, and the pattern formation cannot be confirmed by AFM and SEM images. However, by KFM observation, each region was clearly differentiated due to the electrostatic character, which resulted from the surface oxidation effect on superhydrophilic region by vuv irradiation. On the other hand, in the case of the superhydrophobic/PEG micropattern, the regions were morphologically discrete but not electrostatically.

These observations proved the formation of the superhydrophobic/superhydrophilic and superhydrophobic/PEG micropatterns.

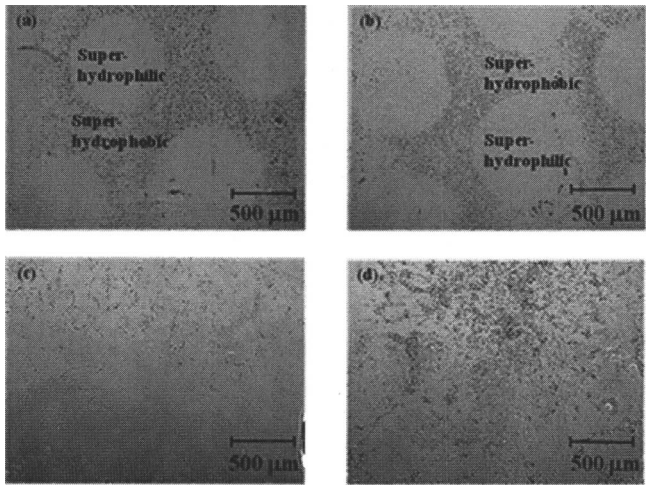

FIG. 6. Microscopic images of bacteria on superhydrophobic/superhydrophilic micropatters [(a) *E. coli*, (b)*B. subtilis*, (c) *P. stutzeri*, and (d) *P. aeruginosa*].

D. Estimation of bacterial adhesion on micropatterns

According to the prospective results from Figs. 4 and 5, the superhydrophobic/superhydrophilic and superhydrophobic/PEG micropatterns were examined for the bacterial adhesion test. Figures 6 and 7 shows visual microscopic images after the incubation on superhydrophobic/superhydrophilic and superhydrophobic/PEG micropatterns, respectively.

In the case of superhydrophobic/superhydrophilic micropattern, *E. coli* and *B. subtilis* showed discrete adhesion on superhydrophobic regions in comparison with superhydrophilic ones as shown in Fig. 6. For the other bacteria (*P. stutzeri* and *P. aeruginosa*), they adhered uniformly on both superhydrophobic and superhydrophilic regions. Reasons for the different behaviors between bacteria might be resulted from difference of physical or chemical interaction of the cellular surface with the each region and/or that of the adhesion mechanisms as previously noted.

As shown in Fig. 6, the immobilization on specific regions regulated by wettability was not achieved well for *P. stutzeri* and *P. aeruginosa*. Then, in order to improve their adhesions on specific regions, PEG modified region was introduced besides the superhydrophobic ones. The PEG regions are supposed to suppress bacterial adhesion more than superhydrophilic region as indicated Fig. 3. As expected, the

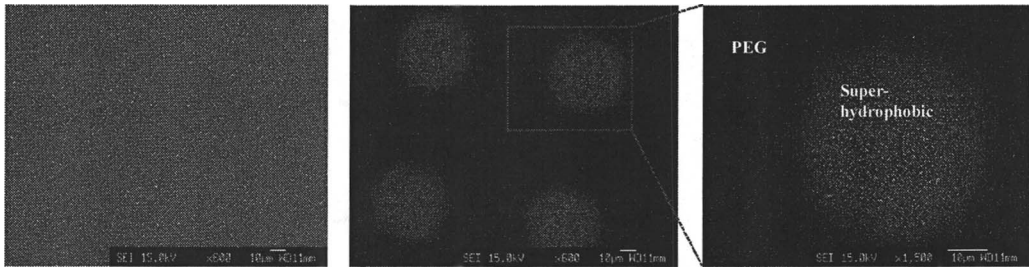

FIG. 5. SEM images of superhydrophobic/superhydrophilic (left) and superhyrophobic/PEG (right) micropatterns.

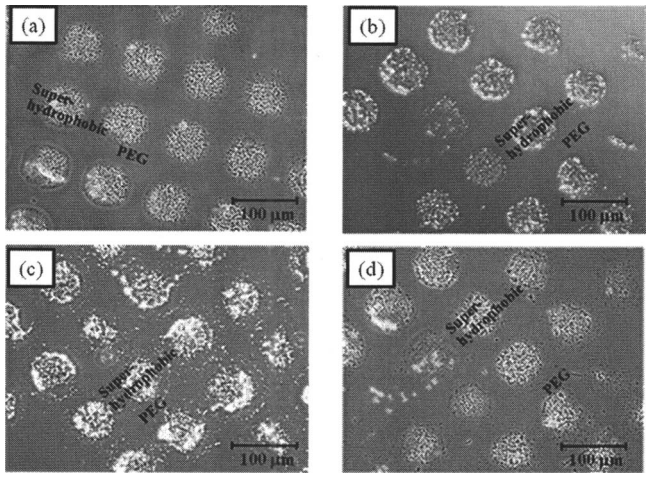

FIG. 7. Microscopic images of bacteria on superhydrophobic/PEG micropatterns [(a) *E. coli*, (b) *B. subtilis*, (c) *P. stutzeri*, and (d) *P. aeruginosa*].

more discrete adhesion on superhydrophobic regions was realized for all the bacteria used here by introduction of PEG regions instead of superhydrophilic regions (see in Fig. 7). This well-separated adhesion can be assisted by the excluded-volume effect of PEG molecules.

IV. CONCLUSION

In this study, we employed superhydrophobic/superhydrophilic and superhydrophobic/PEG micropatterns for immobilization of bacteria on specific microregion. The former micropattern brought the discrete adhesions of *E. coli* and *B. subtilis* specifically on superhydrophobic regions. Superhydrophobic surface enhanced bacterial adhesion more than CH_3-terminated SAM. For high sensitive (high S/N ratio) biosensors, in particular, the surface that enhances the bacterial adhesion to the specific region is as important as the surface that inhibits the bacterial adhesion to background region. Then superhydrophobic surface is suitable for the high sensitive biosensors.

Furthermore, using the superhydrophobic/PEG micropattern, the adaptive range of bacteria open up and the more separated adhesions were obtained for *E. coli, B. subtilis, P. stutzeri*, and *P. aeruginosa*. The PEG molecules play an important role in suppression of bacterial adhesion.

Bacterial adhesions to these two micropatterns were varied differently between *E. coli, B. subtilis* group and *P. stutzeri, P. aeruginosa* group. The differences of bacterial adhesion were resulted from EPS of the bacteria.

We achieved the high sensitive immobilization of the bacteria on specific regions by using the micropatterns. The results obtained in this work enable a well-controlled bacterial immobilization and the construction of biodevices based on bacteria systems.

[1]H. Kaji, T. Kawashima, S. Sekine, and M. Nishizawa, J. Surf. Finish. Soc. Jpn. **59**, 371 (2008).
[2]K. Y. Suh, A. Khademhosseini, P. J. Yoo, and R. Langer, Biomed. Microdevices **6**, 223 (2004).
[3]W. G. Koh, A. Revzin, A. Simonian, T. Reeves, and M. Pishko, Biomed. Microdevices **5**, 11 (2003).
[4]K. Hobo, T. Shimizu, H. Sekine, T. Shin'oka, T. Okano, and H. Kurosawa, Arterioscler., Thromb., Vasc. Biol. **28**, 637 (2008).
[5]S. Szunerits, L. Bouffier, R. Calemczuk, B. Corso, M. Demeunynck, E. Descamps, Y. Defontaine, J. B. Fiche, E. Fortin, T. Livache, P. Mailley, A. Roget, and E. Vieil, Electroanalysis **17**, 2001 (2005).
[6]H. Sato, Y. Miura, T. Yamauchi, N. Saito, and O. Takai, Trans. Mater. Res. Soc. Jpn. **31**, 659 (2006).
[7]V. Combaret, C. Bergeron, S. Bréjon, I. Iacono, D. Perol, S. Négrier, and A. Puisieux, Cancer Lett. **228**, 91 (2005).
[8]L. Blanes, M. F. Mora, C. L. do Lago, A. Ayon, and C. D. Garcia, Electroanalysis **19**, 2451 (2007).
[9]I. Karube, *Handbook of Biosensors and Chemical Sensors* (2007), p. 194.
[10]F. Arai, T. Sakami, A. Ichikawa, and Fukuda, Jpn. Soc. Mech. Eng. **68**, 188 (2002).
[11]Y. H. An and R. J. Friedman, J. Biomed. Mater. Res. **43**, 338 (1997).
[12]N. Shirahata and A. Hozumi, J. Nanosci. Nanotechnol. **6**, 1 (2006).
[13]Y. Wu, M. Kouno, N. Saito, and O. Takai, Thin Solid Films **515**, 4203 (2007).
[14]Y. Wu, H. Sugimura, Y. Inoue, and O. Takai, Thin Solid Films **435**, 161 (2003).
[15]C. A. Fux, M. Shirtliff, P. Stoodleya, and J. W. Costerton, Trends Microbiol. **13**, 58 (2005).
[16]M. Morikawa, Y. Kawai, Y. Aihara, K. Washio, K. Takano, and S. Kanaya, Seibutsu-kogaku kaishi **84**, 488 (2006).
[17]H. Morisaki, Y. Kasahara, and T. Hattori, J. Gen. Appl. Microbiol. **39**, 65 (1993).
[18]J. Lalucat, A. Bennasar, R. Bosch, E. G. Valdes, and N. J. Palleroni, Microbiol. Mol. Biol. Rev. **70**, 510 (2006).
[19]P. Jiang, S. Y. Li, H. Sugimura, and O. Takai, Appl. Surf. Sci. **252**, 4230 (2006).