Bacteria repelling on highly-ordered alumina-nanopore structures

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Bacteria introduce diseases and infections to humans by their adherence to biomaterials, such as implants and surgical tools. Cell desorption is an effective step to reduce such damage. Here, we report mechanisms of bacteria desorption. An alumina nanopore structure (ANS) with pore size of 35 nm, 55 nm, 70 nm, and 80 nm was used as substrate to grow *Escherichia coli* (*E. coli*) cells. A bacteria repelling experimental method was developed to quantitatively evaluate the area percentage of adherent bacterial cells that represent the nature of cell adhesion as well as desorption. Results showed that there were two crucial parameters: contact angle and contact area that affect the adhesion/desorption. The cells were found to be more easily repelled when the contact angle increased. The area percentage of adherent bacterial cells decreased with the decrease in the contact area of a cell on ANS. This means that cell accessibility on ANS depends on the contact area. This research reveals the effectiveness of the nanopored structures in repelling cells. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4918305>]

INTRODUCTION

Bacterial cells persist in numerous environments where humans live, including our food and bodies, among many others. They attach to various kinds of surfaces and grow well on them. Having bacterial free surfaces is an important component of not only keeping our daily life healthy and clean^{1,2} but also providing better medical treatments.^{3,4} Infection is often due to bacterial adhesion on medical implant materials, such as orthopedic joint prosthetics and artificial dental implants. Minimizing the risk of infection is a hot issue for medical professionals.^{5,6} In the case of surgery, bacterial adhesion is also serious. A surgeon can spread bacterial cells due to their adherence to surgical instruments and the patient during surgery.^{7,8}

To prevent surface contamination from the bacterial cells, repetitive sterilization and cleaning using chemicals and ultraviolet (UV) light is needed.^{9,10} These methods are simple ways to keep the surface clean from bacterial cells. However, chemicals can be harmful to humans and the process can be cumbersome to do repeatedly. Previous research has been focused on mechanisms of bacterial adhesion.^{11–13} Bacterial adhesion is affected by the contact area of a cell on a surface.¹⁴ Previous studies reported that more cells were observed where there were larger contact area.^{15–17} In addition to the contact area, the surface morphology should play a role. Surface texturing has been used for enhancing bacteria repelling because it reduces the contact. This means that surface texturing can manipulate the bacterial accessibility on surfaces. Many research groups state that surface texturing can manipulate the cell adhesion.^{16,18–22} Jiang *et al.*

reported that increasing surface area can assist cell contact and it can enhance the cell adhesion on the substrate.²³ Xu *et al.* demonstrated that a surface having a sub-bacterial size texturing can decrease prevalence of bacterial cells on the surface due to the decrease in the accessible surface area.²⁴ Previous studies have been focused on fabricating various types of surfaces but there is lack of design criteria for surface texturing.

In the present research, the crucial design factors for fabricating bacteria repelling surfaces have been studied with alumina nanopore structures (ANSs). Alumina has been widely used as a biomaterial to fabricate implants and medical instruments due to its favorable mechanical property, tribological performance, thermal resistance, and biocompatibility.²⁵ The ANS can be used as biomaterials because of its biocompatibility²⁶ and mechanical properties.²⁷ Alumina-based biomaterials can enhance their biocompatibility by surface texturing. The ANS was fabricated using a two-step aluminum anodizing method. In order to study the repelling performance, a centrifugal experiment was conducted. After the repelling experiment, the area percentages of adherent bacterial cells were measured. The initial existence of cultured bacterial cells on ANS and the strength of bacterial adhesion on ANS have been examined with various pore sizes. The ANS fabrication process is simple and cost effective. The bacterial cells were cultured on ANS with different pore sizes. Contact angles were measured and the contact area between a bacterial cell and the ANS was calculated for analyzing the mechanism of bacterial adhesion. It was found that ANS can reduce the initial existence of cultured bacterial cells and the strength of bacterial adhesion compared to a smooth surface. Based on Xu *et al.* study,²⁴ ANS can have a less accessible area than a smooth surface due to the pores. It means that the ANS has

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bacteria repelling behavior. This study suggests that ANS can be successfully designed for biocompatible material which can restrict bacterial adhesion, and can improve medical treatment as a biomaterial with discussed two critical parameters: contact angle and contact area.

EXPERIMENTAL DETAILS

ANS fabrication

The self-assembled ANS was fabricated using a two-step aluminum anodizing method.²⁸ The pure aluminum (99.999%, thickness: 1 mm) foil was used as a substrate to fabricate ANS. The thickness of the aluminum foil was 1 mm. Electropolishing was performed with a mixture of ethanol and perchloric acid (C_2H_5OH : $HClO_4$ = 4:1 by volumetric ratio) to eliminate the oxidation film and surface defects by applying DC type 20 V and constant 7 °C temperature. As a pre-requisite process to get highly ordered nanopore structure, the electropolished aluminum substrate should be treated firstly by anodizing with applying 40 V (DC) in 0.3 M oxalic acid at 15 °C.

It was possible to get randomly formed alumina nanopore film having highly ordered nanodimple structure as a substrate after the first anodizing process. A mixture of chromic acid (1.8 wt. %) and phosphoric acid applied (6 wt. %) for 6 h at 65 °C dissolved the alumina nanopore film. The highly-ordered pores could not be obtained from the first anodizing process. The pore realms were originated at the bottom of the anodized layer, whereas the pores were created randomly at the top surface of anodized layer. Due to the volume expansion of the aluminum substrate during oxidation, the mechanical stress is generated.²⁹ This mechanical stress can produce repulsive force among each pore structure and lead to fabricate highly-ordered nanodimple arrays at the bottom of the layer. Using the aluminum substrate having nanodimple structures, the uniformly shaped highly ordered ANS was fabricated through the performing second aluminum anodizing process in an identical manner with first one. The initial pore diameter of ANS from the second anodizing process is about 35 nm. The pore diameter of ANS was controlled by 0.1 M phosphoric acid in rate of 0.6 nm/min at 30 °C. All samples were rinsed with deionized water and ethanol. They were placed in an oven before bacterial cell culturing to keep them dry.

Contact angle measurement

To study the surface energy of various pore sizes, the contact angles of deionized water droplets on the substrates were obtained. The contact angle was measured from a 1 μ l-deionized-water droplet on the surface of each ANS sample. A droplet shape measurement system, including a digital camera (PowerShot SD750, Canon), was used to take images of water droplets on the ANS.

Escherichia coli (E. coli) culturing on the ANS

The bacterial cells were cultured on ANS with various pore sizes. The examined bacterial cells are *E. coli* strain DH5 α . *E. coli* is widely studied and one of the most

understood cell systems.^{30–34} Previous understanding is the basis of the current work. The cells of *E. coli* were extracted from –80 °C storage and streaked on a Luria-Bertani (LB) based agar plate. The culture medium, liquid LB, was inoculated with individual colonies that were collected from the agar plate. With optical density at 600 nm (OD600) of 0.3, the bacterial cell culturing is inoculated. Cell culturing was continued with constant stirring for 24 h at 37.5 °C. For cell culturing on ANS, 40 ml of the LB media was poured into Petri dishes having different pore size ANSs with 1% bacterial cells addition from the inoculum. All ANS samples were rinsed using deionized water and ethanol and then put into the Petri dish with the nanopore surface facing up. Cultured *E. coli* was kept in an incubator for 18 h at 37.5 °C. After incubating, all ANS samples were taken out from the Petri dish and rinsed using deionized water five times. The bottom of each ANS was cleaned using ethanol. Remaining water was absorbed by carefully touching a Kimwipe on the side of ANS.

Atomic force microscope (AFM) characterization

The morphology of ANS and the shape of *E. coli* were characterized using an AFM (Nano-R, Pacific Nanotechnology, Inc.). To scan the surface of each sample, non-contact AFM mode was used with a silicon nitride (Si_3N_4) based cantilever tip. The tip diameter is about 30 nm and the normal spring constant of the tip is 0.2 N/m. The top surface of each ANS was scanned to characterize the pore size. Both the height and the phase image of the ANS, including *E. coli*, were obtained from non-contact AFM mode.

Bacterial repelling experiment

The behavior of bacterial adhesion was examined through a shear stress experiment.^{35,36} This method is an alternative approach to identify the effect of a textured surface on the strength of bacterial adhesion. The shear stress method is a simple step that provides an accurate estimate across a larger number of cells towards understanding the adhesion behavior of bacterial-networks on textured surfaces. Similarly, bacterial repelling experiment was conducted to understand the behavior of bacteria desorption in the room temperature with centrifugal force. The centrifugal force was applied to bacterial cells using the rotator (Model 616, EG&G PARC). The ANS with *E. coli* was attached on the Teflon plate. The rotational speed can be set by a magnetic motor. Figure S1 (supplemental material) shows that schematic diagram for bacterial repelling experimental set-up.⁴⁰ All attached ANS samples were rotated with 2000 rpm for 10 min at room temperature. After being rotated, each of the ANS was taken off from the Teflon plate and all were rinsed with deionized water three times. Extra water was carefully removed by using a Kimwipe.

Bacteria imaging and analysis

Using a digital optical microscope (VHX-2000, Keyence), images of bacteria before and after being

FIG. 1. AFM images of ANS with various pore diameters: The pore diameters are about (a) 35 nm, (b) 55 nm, (c) 70 nm, and (d) 80 nm.

repelled were acquired. Software ImageJ (NIH, Bethesda, MD) was used to compute the area percentage of adherent bacterial cells on surfaces. To calculate the area percentage of adherent bacterial cells, the total area covered by bacterial cells was divided by the sampling area of the ANS, i.e., $150 \mu\text{m} \times 150 \mu\text{m}$. On the cultured-sample surface ($3 \text{ cm} \times 3 \text{ cm}$), six different sampling regions were randomly chosen and evaluated to obtain the percentage of adherent bacterial cells.

RESULTS AND DISCUSSION

Morphological characteristics of ANS

Surface images of ANS were taken using an AFM with non-contact mode. The average pore diameters of ANS were measured by using the software for AFM image analysis “NanoRule (Pacific Nanotechnology, Inc.).” Figure 1 shows the AFM height images of the controlled ANS sizes: 35 nm, 55 nm, 70 nm, and 80 nm in terms of average pore diameter. The statistical data of pore size are presented in Table I. For all samples, the distance from pore to pore is about 100 nm. The images show that the ANS is hexagonally highly-ordered nanopore structure.

TABLE I. Average pore diameter of ANS and the corresponding contact area with unit area ratio.

Average pore diameter (nm)	Average pore diameter (stdv)	Unit area ratio	Contact area (μm^2)
0 (no pore)	0	1.00	0.79
35	2.8	0.89	0.7
55	2.7	0.73	0.57
70	2.7	0.56	0.44
80	2.7	0.42	0.33

Effects of pore size on contact angle

Figure S2 (supplemental material) shows the result of contact angle measurement.⁴⁰ The tread line shown in figure follows a proportional relation between pore size and contact angle. The relation can be expressed by curve fitting

$$A(d) = -11.6 \times 10^{-4} d^2 + 53.4 \times 10^{-2} d + 66.5, \quad (1)$$

where $A(d)$ is contact angle and d is the pore diameter. The value of contact angle is increased with increasing pore diameter from 0 nm (no pore) to 80 nm. As shown in the figure, the reference sample that has no pore has the lowest contact angle, i.e., 66.6° . The samples of pore size of 35 nm, 55 nm, 70 nm, and 80 nm display the contact angles of 90.4° , 91.7° , 98.7° , and 101.8° , respectively. These results correlate with published data.³⁷

Area percentage of adherent bacterial cells on ANS

Table II shows the statistical data results for adherent bacterial cells on the ANSs. Figure 2 shows the represented optical microscope images (color converted: black and white) of bacterial cells on ANS. The black color represents adherent bacterial cells on ANS. The top images are taken before conducting repelling experiment with various ANS’s pore size and the bottom images are taken after performed repelling experiment. As shown in this figure, the adherent bacterial cells are decreased with the increasing the pore size in case of “before repelling.” The other interesting result is that the adherent bacterial cells are mostly removed by the repelling experiment from all ANS samples, but there is slightly reduction in the area percentage of adherent bacterial cells after the repelling experiment on the samples without pores. In order to understand the adhesion mechanism of bacterial cells on the ANS both in case of “before repelling”

TABLE II. Area percentage of adherent bacterial cells before and after repelling experiment on various pore size of the ANS.

Pore size (nm)	Before repelling experiment		After repelling experiment	
	Average percentage of adhered bacteria cells (%)	Stdv	Average percentage of adhered bacteria cells (%)	Stdv
0 (no pore)	25.1	6.1	23.6	5.3
30	17.4	3.3	2.6	0.6
55	16.6	4.4	4.1	1.9
70	6.8	2.5	2.4	0.9
80	5.6	1.7	2.9	1.8

and “after repelling,” the contact condition between bacterial cells and ANS needs to be considered.

Morphology of bacteria

Understanding the contact condition between ANS and bacterial cells is important to study for bacterial adhesion. Figure 3 shows the AFM images of bacteria on ANS. The image was taken using the non-contact AFM mode. Before imaging, the sample was rinsed using deionized water and then air dried. As the figure shows, the bacterial cells are on the top surface of the ANS. From the AFM height image (Figure 3(a)), the parallel cross section shape with ANS surface of single bacterial cell looks like ellipse. A white circle highlighted a single bacterial cell was zoomed in at Figure 3(b). The bacterial cells are covering several nanopore of the ANS. The phase image of AFM (Figures 3(c) and 3(d)) clearly indicates that the elliptical shaped objects are bacterial cells due to them having a darker region which represents even lower stiffness. The surface of ANS which is made by ceramic (alumina) generally has a greater stiffness than bacterial cells. These AFM images show that a bacterial cell, which has elliptical shape, is attached on the ANS. According to this image, it is possible to simplify the contact model between bacterial cell and ANS.

Contact area calculation between bacteria and ANS

Based on the AFM images, a contact area between a bacterial cell and ANS can be calculated. A relative contact model,³⁸ which is about between AFM tip and ANS, was used to calculate the contact area. As illustrated in Figure 4, the contact area could be defined by the unit area of a hexagonal shape which is extracted from the AFM height image. The area is connected by six centers of pores in ANS. The contact area is determined by the following equations:

$$f(r) = \frac{\left(6 \times \frac{\sqrt{3}}{4} l^2 - 3\pi r^2\right)}{\left(6 \times \frac{\sqrt{3}}{4} l^2\right)} (@l = 100nm) = 1 - \frac{\pi r^2}{8660.25}, \quad (2)$$

$$F(r) = S \times f(r), \quad (3)$$

where $f(r)$ is a ratio of unit area, l is the pore to pore (inter-pore) distance, r is the radius of a pore, S is bottom area of bacteria, and $F(r)$ is the contact area. The inter-pore distance “ l ” is fixed with 100 nm among all ANS samples. The contact area is simply determined by multiplying “ $f(r)$ ” and “ S .” Based on the bacteria images taken on the ANS, the bottom area of bacterial cell “ S ” can be expressed by

$$S = \pi ab, \quad (4)$$

a measurement of semi-minor axis is expressed by “ a ” and a measurement of semi-major axis is expressed by “ b .” The average value of “ a ” and “ b ” are $0.25 \mu\text{m}$ and $1 \mu\text{m}$, respectively. The bottom area of single bacterial cell is about $0.79 \mu\text{m}^2$. The contact condition between the ANS and a bacterial cell is identified by AFM images and contact area calculations. As shown in Table I, the contact area between the ANS and bacterial cell decreased with the increasing pore size. This result clearly indicates that ANS having larger pore size is rarely in contact with bacterial cells.

Analysis of adhesion

As shown in Figure 2, the area percentage of adherent bacterial cells decreased with increasing pore size in the first case “before repelling.” The area percentage of adherent bacterial cells is smaller when the contact area of the bacterial cell decreases. In order to study the adhesion of bacterial

FIG. 2. Optical microscope images of bacterial cells on the ANS with various pore diameters. Black color represents bacterial cells. Top images stem from before repelling experiment and bottom images stem from after repelling experiment. The scale bar in each image represents $100 \mu\text{m}$.

FIG. 3. AFM images of bacterial cells on ANS (pore diameter: 55 nm): (a) two-dimensional height mode AFM image of bacterial cells on the ANS, (b) zoomed in three-dimensional height mode AFM image of single bacterial cell on the ANS, (c) two-dimensional phase mode AFM image of bacterial cells on the ANS, and (d) three-dimensional phase mode AFM image of bacterial cells on the ANS.

FIG. 4. Schematic diagram to define the contact area between a bacterial cell and ANS. The extracted AFM image of ANS (pore diameter: 80 nm) shows the hexagonal shape unit area. The inset graph shows a relation between the pore size and the contact area.

cells to the ANS with various contact area, the area percentage of adherent bacterial cells on the ANS was measured by optical microscope with the “ImageJ” software. As stated above, the bacterial cells were represented as a black color, while the ANS surface was typified by white color in Figure 2. The total area of bacterial cells were figured out by summing all black color area (bacterial cells). The data shown in Table II were plotted in Figure 5. Figure 5 shows how many bacterial cells are on the ANS in terms of percentage (%) via pore size of ANS. The figure represents two main results: the rectangular shape data point represents the “before repelling” condition and the circular data point indicates the “after repelling.” The result of before repelling clearly shows that the area percentage of adherent bacterial cells decreased almost linearly with increase in the pore size. As the inset in Figure 4 shows, the contact area between the bacteria and the ANS decreases with increasing the pore size of ANS. This means that the adherent cells decreased with the decrease in the contact area. The $0.33 \mu\text{m}^2$, $0.44 \mu\text{m}^2$, $0.57 \mu\text{m}^2$, $0.7 \mu\text{m}^2$, and $0.79 \mu\text{m}^2$ contact area showed 5.6%, 6.8%, 16.6%, 17.4% and 25.1% cultured bacterial cell on

FIG. 5. Area percentage of adherent bacterial cells on ANS and the contact angle via pore size. Y-axis represents the result of bacteria repelling experiment. Error bars show standard deviation with one sigma.

ANS, respectively. This result confirms that a large contact area can lead to the increase in probability for bacteria to grab the ANS surface during cell culturing.

As a result of the “after repelling” condition, the area percentage of adherent bacterial cells is dramatically decreased between no pore and 35 nm pore size ANS, but there is no visible difference in the percentage of bacterial cells among the 35 nm, 55 nm, 70 nm, and 80 nm pore size ANSs. It has been reported that a substrate which has low surface energy will have a high contact angle in deionized water.³⁹ This means that the contact angle can represent the degree of surface energy of a substrate. From the contact angle measurement on the ANS (Figure S2), it is noted that the surface energy of the ANS decreased with increasing pore size. As discussed above, increasing the pore size represents decreasing the contact area. In other words, the contact angle decreased with increasing contact area. This contact angle trend shows opposing results on the area percentage of adherent bacterial cells. The contact angle increased significantly from 0 nm (no pore) to 35 nm in pore size. After that, the contact angle increased slightly with increasing the pore size. As mentioned, there is no significant difference in the area percentage of adherent bacterial cells between 35 nm and 80 nm pore size after the repelling experiment: the 66.6°, 90.4, 91.7, 98.7, and 101.8° contact angle condition showed 23.6%, 2.7%, 4.1%, 2.4%, and 2.9% bacterial cell on ANS, respectively. It appears that the bacterial adhesion, which is against the repelling force relates to the surface energy which is represented by the contact angle. A combination of a larger contact area and a smaller contact angle can make surface hold more bacterial cells. This is because of not only the capability for additional contact area between bacterial cells and the ANS, but also the high surface energy as represented by a low contact angle. The gap between the “before repelling” and “after repelling” experiment, in Figure 5, indicates how many bacterial cells are removed by the repelling experiment. The 0 nm (no pore), 35 nm, 55 nm, 70 nm, and 80 nm pore size shows that the area percentages of repelled bacterial cells are about 5.8%, 84.8%, 75.3%, 64.5%, and 48.3%, respectively.

According to the results of the area percentage of adherent bacterial cells, the adhesive strength of bacterial cells is the combination of the capability of the contact area and the surface energy. Specifically, the initial adherent bacterial cells can be mainly predicted by the contact area between the bacterial cell and the ANS, and the bacteria’s repelling performance can be primarily determined by the surface energy of the ANS. The strength of bacterial adhesion depends heavily on the surface energy of a substrate. A substrate that has low surface energy can easily repel the bacterial cells from its own surface.

CONCLUSIONS

This research investigated the mechanisms for adhesion of *E. coli* cells on a nanostructured alumina substrate. It was found that the cells were repelled due to the existence of pores. The no pore substrate showed 5.8% bacterial cell reduction, while the 35 nm pore diameter ANS produced

84.8% decrease. These results indicate that the mechanism of the bacteria adhesive strength depends on the cell-surface contact area and the water-substrate contact angle. One function of the contact area is that it makes the surface accessible to cells. The larger the contact area is, the more easily cells access is, and the larger the area of adherent bacterial cells. The contact angle, meanwhile, reflects the capacity of a surface to repel bacterial cells. The performance of the surface in repelling bacteria improves as the contact angle increases. More bacterial cells were repelled under a condition of higher contact angle (greater than 90°). ANS is able to achieve better bacteria repelling performance due to the combination of a small contact area and high contact angle. Using these two critical design parameters, it is possible to design and optimize highly biocompatible and bacteria repelling nanopore structures with ANS.

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