Evaluation of Surface Structure of *Escherichia coli* Using Polypyrrole Matrix

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We propose a method to evaluate the surface structure of *Escherichia coli* focusing on the doping state of bacterial cells into polypyrrole (PPy) matrix. We found that the orientation of doping states of *E. coli* O rough was different from those of other serotypes of *E. coli* cells, which had O-antigen on their outer membrane. The results indicated that more than seventy percent of *E. coli* cells having O-antigen was horizontally doped into PPy matrix based on the chemical structure and the placement of O-antigen. On the other hand, the percentage for horizontal doping state of *E. coli* O rough cells was only approximately fifty percent. Moreover, the cells of each *E. coli* serotypes were specifically bound to their own shape-complementary cavities on the microspheres, but the binding affinity of *E. coli* O rough was a bit lower than that of other serotypes.

Keywords Cell-imprinting, *Escherichia coli*, polypyrrole, complementary cavity

(Received March 5, 2019; Accepted March 13, 2019; Advance Publication Released Online by J-STAGE March 22, 2019)

Introduction

Molecular imprinting is an efficient technique to create artificial receptors with complementary cavity which can be tailor-made by using small molecules, macromolecules, viruses and microorganisms as templates. Therefore, it has been utilized in a wide range of fields such as those related to medicine, food, and the environment.¹⁻⁶ Regardless of the template, the cavities with a precisely complementary structure of the template are formed on the polymer matrix. The formed cavity exhibits a specific chemical affinity to their original template through various chemical interactions such as hydrogen binding, electrostatics and van der Waals interactions.

We have previously reported that various gram-negative and gram-positive bacteria were directly immobilized as dopants into the conducting polymer matrix like polypyrrole (PPy) or poly(3,4-ethylenedioxythiophene) by electrochemical deposition.^{7,8} The doping state of the bacteria in the polymer film varied depending on the polymerization conditions, yet strongly reflected the surface structure of the bacteria. As the bacterial surface structure increases in specificity and is specifically transferred, their unique information will be left on the polymer matrix. Although the components of the cell wall are almost the same, their sequences and distributions are different depending on the bacterial species and strains. The outer membrane of *E. coli*, a gram-negative, is covered with lipopolysaccharides (LPS). There are many serotypes of *E. coli*, which are classified based on the O-antigen structure of a part of

the LPS.⁹ The O-antigen, composed of repeating units of saccharides, are also responsible for the significant differences in pathogenicity among *E. coli* serotypes.¹⁰⁻¹³ The recognition of a specific serotype focusing on the differences of the sugar chain makes it possible to prevent food poisoning and disease. In this paper, we discuss the doping states of several *E. coli* serotypes which have negative zeta potential on the PPy film to create a complementary platform indicating the slight differences on their bacterial surface.

Experimental

Chemicals

All chemicals were reagent grade. Ultrapure water (>18 MΩ cm) sterilized with UV light was used in all experiments. For safety reasons, experiments were conducted with genetically modified verotoxin-nonproducing *Escherichia coli* PV856 (O157:H7), *E. coli* PV276 (O157:HNM), *E. coli* PV01-198 (O26:H11), and *E. coli* PV03-017 (O26:HNM). These strains were provided by Prof. M. Miyake, Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, and by Dr. K. Seto, Osaka Prefectural Institute of Public Health. *E. coli* K-12 (O Rough:H48, NBRC3301).

SYTO9[®] was used as fluorescent dyes. Nafion[®]117 was purchased from Sigma-Aldrich, while gold-coated microbeads were purchased from Green Chem. Inc.^{14,15}

Bacterial culture

Bacterial cultures and experiments were executed in a biosafety level 2 laboratory designed and managed in accordance

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with safety regulations. Liquid cultures were grown at 303 K for 18 h in E-MC35 agar broth (Eiken Chemical Co. Japan). Similarly, colonies were suspended in 30 mL E-MC35, and cultured at 303 K for 18 h. Cells were then harvested by centrifugation at 7000g for 15 min and washed four times for 1 min each in freshly prepared phosphate buffer.

Fabrication of microspheres imprinted with bacterial cells

Gold-coated microbeads, the surface of which was modified with a self-assembled aminothiophenol monolayer, were dispersed in 20 mL 25 vol% aqueous ethanol containing 2.0 μL nafion.^{16,17} After ultrasonication for 20 min, the dispersion was immediately mixed with 30 μL pyrrole and 5.0 mL of *E. coli* O157:H7 suspension (3.8×10^9 cells mL^{-1}), and stirred for 40 min. Subsequently, polymerization was induced by oxidation with $(\text{NH}_4)_2\text{S}_2\text{O}_8$ for 12 h at room temperature. Polymer-coated microspheres were then collected by centrifugation at 1500g, and washed with ultrapure water. To remove bacterial cells, microspheres were re-dispersed in aqueous 0.10 M NaOH, stirred for 3 h, washed with ultrapure water, and dried in a vacuum chamber. Samples were observed by TM3030 (Hitachi, Japan) scanning electron microscope (SEM) operating at accelerating voltage 5 kV, and BX51 fluorescent microscope (Olympus, Japan). Fluorescent spectra were collected on FP-6300 spectrofluorometer (Jasco, Japan).

Dark-field microscopy

In dark-field microscopy, the scattered light is detected, while directly transmitted light is blocked with a dark-field condenser.^{18,19} Samples were thus imaged using an Eclipse 80i optical microscope (Nikon, Japan) equipped with a dark-field condenser, a 100-W halogen lamp, and a charge-coupled device camera. Light-scattering spectra were obtained using a USB4000 miniature grating spectrometer (Ocean Optics) coupled to the microscope via an optical fiber with core diameter 400 μm . Typical acquisition time was 400 ms. Spectra were corrected for spectral variations in system response, and white-light intensity distribution (main intensity 560 nm) through division by bright-field spectra was recorded through the sample. The collection volume was nearly diffraction-limited for the 100 $\times$ objective (NA 0.9)/fiber combination used, with cross-sectional area approximately $\sim 10 \mu\text{m}^2$. Samples were prepared by mounting on a glass slide 5 μL dispersion solution of microspheres, bacteria, and mixtures thereof, and air drying for 1 h.^{20,21}

Cell counting

Microspheres imprinted with *E. coli* O157:H7 (2.0 mg) were incubated with 2.0×10^7 cells mL^{-1} of SYTO9-stained *E. coli* O157:H7, O157:HNM, O26:H11, O26:HNM, and O rough. After incubation for 3 h, the supernatant cleared by gravity was added into the cuvette, and the fluorescence intensity of the supernatant was measured at 500 nm in the presence (*I*) or absence of microspheres (*I*₀).

Results and Discussion

PPy was formed on nafion-coated microspheres (mean diameter: 5.0 μm) by chemical oxidation in an aqueous medium containing *E. coli* cells.^{16,22} We supposed that cells adhered to the microspheres as the polymerization progressed and acted as dopant anions with a negative zeta potential (~ -30 mV). SEM and fluorescence microscopy confirmed that the cells were immobilized in the PPy film (Figs. 1A and 1B). Cell-imprinted

Fig. 1 (A) SEM and fluorescent microscope images of microsphere coated with *E. coli*-doped PPy film and (B) SEM images of *E. coli* cell-imprinted microspheres. *E. coli* (a) O157:H7, (b) O157:HNM, (c) O26:H11, (d) O26:HNM and (e) O rough.

microspheres were obtained through lysis and overoxidation reaction of PPy by immersing in 0.1 M NaOH aqueous solution.^{16,17} The overoxidation of PPy led to the removal of anionic species due to the loss of electrostatic interactions and curing the film.² As a result, the complementary cavities of *E. coli* cells' surface were successfully formed on the overoxidized PPy film by the cell imprinting technique. Figure 1C clearly indicated the formation of these cavities on the microspheres. Although there was no significant difference in the size of cells among O157:H7 ($2.1 \times 0.59 \mu\text{m}$), O157:HNM ($2.1 \times 0.72 \mu\text{m}$), and O rough ($2.0 \times 0.69 \mu\text{m}$), the number of round-shaped cavities on the microspheres imprinted with O rough was undoubtedly much more than that imprinted with other serotypes. We believe that there are two types of imprinting orientation: vertical and horizontal. The orientation of rod-like cells was statistically estimated by SEM observation of the cavities formed on the the top half of microspheres (Table 1). The percentage of bacilli-form cavities was only approximately 50% on the PPy film formed by the immobilization

of O rough while that of the microspheres immobilized with others was more than 70%. Although the whole surface of the bacteria is covered with sugar chains, it is expected that the existence of O-antigen will be closely involved in the doping state of *E. coli* cells during the polymerization process. On the other hand, there was no relationship among the number of imprinted cells, the orientation of the cavities, and the cell size.

To clarify the difference in the function of the cavities formed by *E. coli* with and without O-antigen, we performed a rebinding test of the cell-imprinted microsphere. The *E. coli* O rough cell-imprinted microspheres were added into the SYTO9-labeled *E. coli* O rough suspensions (2.0×10^7 cells mL⁻¹) and then the mixture was incubated at 298 K.^{16,22} The fluorescence intensity of the supernatant (*I*) at 500 nm obtained was normalized by that of *E. coli* O rough suspension without microspheres (*I*₀). The cell-imprinted microspheres can capture the target cells spontaneously. The *I*/*I*₀ decreased drastically within the first 60 min and became nearly constant at 0.2 after incubating for 3 h (Fig. 2A). Similarly, *E. coli* O157:H7 cells bound to the cavities on its own cell-imprinted microspheres and its *I*/*I*₀ ratio also decreased drastically and reached a constant value of approximately 0.1. Statically, the number of the cavities on the microsphere prepared by *E. coli* O157:H7 was less than that by *E. coli* O rough, however, the binding capacity of *E. coli* O157:H7 cell-imprinted microsphere was about 10% better than that of *E. coli* O rough. Both SEM and fluorescent observations indicated that *E. coli* cells bound to the cavities without

unspecific adsorption on the surface of the microsphere (Fig. 2B). Moreover, we also found that *E. coli* O157:H7 cells were likely to bind horizontally to the corresponding cavities. This indicates that the imprinting cavity captured the target cell in exact orientation as it was formed, suggesting that the O-antigen placement on the surface of the cell was molecularly imprinted on the inner wall of the cavity. Previously, the binding constant, *K*_a, was reported as 1.1×10^5 M⁻¹, which is a good binding affinity between target cells and complementary cavities.²² Nevertheless, *E. coli* O rough cells having no O-antigen on their surface were captured in the vertical direction regardless of round-shaped or bacilliform cavities. Therefore, we conclude that the O-antigen-imprinted cavity worked as an artificial antibody which bound strongly to the O-antigen.

The dark-field image and light-scattering spectra of *E. coli* O rough cell-doped microspheres are shown in Fig. 3A. The microsphere was observed as a bright white light spot accompanied with strong light-scattering intensity at 560 nm, while the cell-imprinted microsphere itself did not scatter light with intensity $\cong 0$. This suggested that the scattering light attributed to the *E. coli* cells bound to the microsphere. The light-scattering intensity of the single microsphere depended linearly on the cell density of *E. coli* O rough and O157:H7 (Fig. 3B). The intensity of *E. coli* O157:H7 cell-imprinted microsphere was much stronger than that of *E. coli* O rough in the same conditions. Given that the light-scattering intensity of a single cell was unrelated to the species of *E. coli* and also there was no significant difference in the cell size between O157:H7 and O rough. We should consider that the light is strongly scattered due to a large difference in the refractive index between the PPy (>1.5) and water (1.3) inside a cell, compared to that between surrounding air (1.0) and water because the horizontally captured O157:H7 cells had more contact area with the PPy layer.²³ In other words, the contact area between the cells and PPy was more closely related to the light-scattering intensity rather than the number of *E. coli* cells. The contact area of the bacterial cells with PPy matrix depended on both the orientation of captured cells and the depth of capturing cavities. As observed in SEM images of cell-imprinted microsphere (Fig. 1C), because the diameter of the bare microsphere was 5 μ m, the thickness of the PPy layer was thin (<1 μ m). So, we supposed that the capturing orientation had a more significant effect on the contact area than the depth of

Table 1 Cavity on a single microsphere

Serotype	Number of cavities ^a / –	Horizontal orientation ^b , %
O157:H7	5.7	74
O157:HNM	7.7	85
O26:H11	3.1	79
O26:HNM	4.7	75
O rough	7.3	52

a. The average of number of cavities on the top hemisphere of a single microsphere was counted from the SEM images (*n* > 100).

b. The number of bacilliform cavity was divided by the total number of cavities including both bacilliform and round-shaped cavities.

Fig. 2 (A) Time-dependency of the normalized intensity, *I*/*I*₀. (B) SEM and fluorescent images of the cell-imprinted microsphere after incubation in the stained target suspensions.

Fig. 3 (A) Light-scattering spectra of *E. coli* O rough cell-imprinted microsphere after incubation with the indicated concentration of *E. coli* O rough suspension. The inset shows dark-field image of the *E. coli* cell-doped microsphere. (B) Dependence of the light-scattering intensity of the cell-imprinted microsphere on the density of target cells.

cavity. In this case, the contact area of horizontally captured O157:H7 cells with their horizontal complementary cavities was much larger than that of the vertically captured O rough cells.

In order to further confirm the difference in surface structure among various species of *E. coli* cells, the specificity of the cell-imprinted microsphere was investigated. The cell-imprinted microspheres were added to the suspension of each *E. coli* serotype: O157:H7, O157:HNM, O26:H11, O26:HNM, and O rough ($OD_{600} = 0.4$ for all suspension). After 3 h of incubation, the microspheres were precipitated by gravity, the supernatant were removed and the light-scattering intensity of the single microsphere was measured (Fig. 4). The uptake (s_0) of the cell-imprinted microsphere added into the target cell suspension was calculated as $(I_0 - I)/I_0$. The uptake (s) for respective different serotypes of *E. coli* was normalized by dividing by s_0 of the target. The uptake ratio (s/s_0) of *E. coli* O157:H7 cell-imprinted microsphere for *E. coli* O157:H7 was the highest among all the strains, thus highlighting the specificity of the microspheres, with $s/s_0 < 0.1$ for others. The specificity of the imprinted microspheres resulted from the shape and size complementarity to *E. coli* O157:H7 as well as from the chemical structure of the cavity surface. Although the s/s_0 of *E. coli* O rough cell-imprinted microsphere for target was the highest among all the strains, the specificity was a bit lower than that in the case of *E. coli* O157:H7. This result again indicates that the complementary structure of “artificial antibody” formed on the inner wall of the cavity recognizes accurately the shape and the placement of O-antigen on the surface of target cells, and also suggests that the cavities interact with the target cells at multiple points.

In summary, the recognition ability and light scattering intensity of cell-imprinted microspheres depended strongly on the doping states of template cells. During the polymerization, *E. coli* cells with O-antigen interacted with PPy matrix at multiple points and were horizontally doped. The cavity recognized the target cells specifically based on their complementary surface structures formed on the inner wall. On the other hand, *E. coli* O rough cells doped irregularly on the PPy matrix. The results of binding affinity and specificity definitely indicated the role of O-antigen was not only as a classifying factor of different *E. coli* serotypes but also had a significant effect on the formation of cell-imprinting cavities.

Fig. 4 Uptake ratio (s/s_0) of *E. coli* O157:H7 and O rough cell-imprinted microsphere, where s is the light-scattering intensity for a test species, and s_0 is the light-scattering intensity for the target.

Although no obvious difference could be found among the species having O-antigen, we could at least distinguish the *E. coli* species with or without O-antigen by the platform of cell-imprinted microsphere. Further research for investigating the surface structure of bacterial cell based on the platform will be conducted.

Acknowledgements

This work was financially supported by the Ministry of Agriculture, Forestry and Fisheries through the Science and Technology Research Promotion Programme for the Agriculture, Forestry, Fisheries and Food Industries as well as by the Japan Society for the Promotion of Science through the Grants-in-Aid for Scientific Research (B) (KAKENHI 25288039, 16H04137) and Grant-in-Aid for Challenging Exploratory Research (KAKENHI 26620072).

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