Quantification of Enterohemorrhagic *Escherichia coli* via Optical Nanoantenna and Temperature-Responsive Artificial Antibodies

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Abstract

Enterohemorrhagic *Escherichia coli* are a dangerous bacterium known to be harmful to the human body, with some infections even resulting in death. Given this danger, food factories are required to perform a quick bacterial test to confirm the absence of this pathogen prior to shipping. We have developed a novel molecular imprinting polymer (MIP) particle that has encapsulated gold nanoparticles (AuNPs) and which can function as both a receptor and optical signal transmitter in biological systems. This MIP particle is artificially synthesized and can be engineered to specifically recognize and capture antigens on the bacterial cell membrane. In addition, MIP particles containing AuNPs generate strong scattered light signals and binding of the MIP particles improves the optical intensity of the target bacterial cells. This enables clear visualization under a darkfield microscope and quantification of the target bacteria using the scattering light intensity. Here we describe the successful quantification of *Escherichia coli* O157 cells in real meat samples using this technology in conjunction with a simple labelling step.
Introduction

Bacteria are critical components in most ecosystems and play an important role in our daily lives by supporting environmental purification, organic farming, and the production of functional foods. However, some of these microorganisms can cause infections and may have serious adverse effects on the human body. This is especially true for enterohemorrhagic *Escherichia coli* O157, which is a very dangerous bacterium that causes a large proportion of the world’s annual food poisonings. Infections with this pathogen can be lethal even in the presence of as few as 100 cells.¹⁻⁵ Therefore, food factories that provide food to many consumers are required to test their product for this pathogen prior to shipping. Currently, most sites rely on the colony counting method for identification and examination of this pathogen which is subsequently confirmed by the application of selective growth media.⁶,⁷ At present, bacterial tests including culture take time, often meaning that the test results are only available after the shipment has already left. Gram stain, which is a classic biological protocol for bacterial testing using optical microscopy and is still in active use today.⁸,⁹ However, this method can only identify the gram status of the cell, Gram-positive cells vs. Gram-negative cells, but it cannot determine the exact bacterial species detected in the evaluation. This means that it is not suitable in food factories. These issues mean that it is critical that we develop new methods that enable quick and simple bacterial evaluation and identification for use in these settings.

Metal nanoparticles have received considerable attention owing to their unique optical characteristics which are known to depend on their size, shape, and dispersion state.¹⁰,¹¹ Gold nanoparticles (AuNPs) are characterized by a high density of electrons and high chemical stability, which make them useful in numerous and diverse applications. The free electrons present on the AuNP surface produce a localized surface
plasmon resonance (LSPR) involving collective vibrations induced by interactions with visible light. Given this it should be possible to control the LSPR of a nanometer-scaled antenna structure using the careful assembly of AuNPs.\textsuperscript{12-15} The biomedical applications of AuNPs include immunogold-labeled antibodies.\textsuperscript{16} In this case, immune antibodies are generally introduced to the AuNP surface via covalent bonds to establish a binding site. We previously described the development of a novel detection method which relies on the production of a high-sensitivity optical antenna on the surface of a single bacterial cell using an immune antibody-enriched AuNP.\textsuperscript{17} However, antibody introduction induced agglutination of these AuNPs, and there was a problem in controlling the reaction.

Molecular imprinting polymers (MIP) are one of the smart materials known for their production of excellent target recognition sites following the removal of any templates introduced during polymerization. This removal process relies on the washing of the MIP with organic solvent or strong alkali medium for a long period, at least 24 h.\textsuperscript{18-22} Despite their potential there are several issues related to the application of MIPs, these include concerns around the MIP manufacturing process, such as complicated operation, multiple synthesis steps and wastewater treatment, and the deterioration of MIP performance in response to degradation of the matrix polymers during these processes. By developing an MIP, which relies on a temperature-sensitive material as the matrix, it has become possible to easily remove template molecules without degrading the MIP product.\textsuperscript{23} Moreover, it is expected that these MIPs will be used repeatedly and facilitate molecular recognition in different samples, since the target recognition of these MIPs can be controlled by changes in temperature.

In this paper, we investigate the construction of an optical antenna on the surface of \textit{E. coli} O157 cells using an artificial antibody on the surface of AuNPs conjugated using
MIP technology. This artificial antibody works by controlling temperature to alter the binding specificity. This manuscript also describes the development of a novel quantitative evaluation for enterohemorrhagic *E. coli* O157 in real meat samples using a combination of artificial antibody mediated optical antennas and dark field microscopy.

**Experimental**

*Reagents and chemicals*

All chemicals used were reagent grade. Every experiment used ultrapure water (>18 MΩ cm) sterilized by UV light. Genetically modified verotoxin nonproducing *E. coli* PV856 (O157:H7) was provided by Prof. M. Miyake from the Department of Veterinary Science at the Osaka Prefecture University, and Dr. K. Seto from the Osaka Prefectural Institute of Public Health. Two different strains of *E. coli* (NBRC3301&3972) were purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC). Lipopolysaccharide (LPS) extracted from *E. coli* O157, chloroauric acid, *N*-isopropylacrylamide (NIPAm), acrylic acid (AAc), *N*-tertbutylacrylamide (TBAm), *N*,*N*'-metylenebis(acrylamide) (BIS), sodium dodecyl sulfate, ammonium persulfate, and *N*,*N*,*N*','*N*'-tetrametylethlenediamine (TMEED) were purchased from FUJIFILM Wako Pure Chemical Co., Japan.

*Bacterial culture*

All bacterial cultures and experiments were carried out in a biosafety level 2 (BSL-2) laboratory, and developed and managed in accordance with the appropriate safety regulations. All the *E. coli* strains were cultured in agar growth medium (E-MC35, Eiken Chemical Co., Japan) at 303 K for 18 h. A single colony was then selected and placed in liquid growth medium (30 mL) and incubated at 303 K for 18 h. These
suspension cultures (10 mL) were then centrifuged at 6,500 rpm (4,960 \times g) for 15 min at 278 K, and the precipitate was resuspended in phosphate buffer (10 mL) by shaking for 1 min. This procedure was repeated three times. The concentration of the resulting suspension was $2.5 \times 10^9$ cells mL$^{-1}$.

**Synthesis of nanocomposite**

The nanocomposite was synthesized following copolymerization of NIPAm (0.18 mmol), AAc (0.015 mmol), TBAm (0.13 mmol), and BIS (5.0 \mu mol) in a chloroaauric acid aqueous solution (0.14 mM, 50 mL) containing sodium dodecyl sulfate (35 \mu mol).\textsuperscript{23} TBAm was added to the solution after dissolution in ethanol (1.0 mL) and the comonomer solution was degassed in a sonication bath under vacuum for 10 min and then subjected to nitrogen bubbling for 30 min. After the LPS (0.20 mg) was added to the comonomer solution, copolymerization was induced in response to the addition of ammonium persulfate (0.13 mmol) and TMEED (0.10 mmol). This process was finalized by stirring the copolymerization solution for an additional 18 h at 298 K. The LPS, which acted as the template, and the unreacted monomers from the reaction solution were removed by dialysis in ultrapure water at 313 K for 4 days. LPS was simply removed by raising the temperature to 313K to prepare the MIP particle.

**Dark-field microscopy and light scattering evaluations**

A mixture of the nanocomposite dispersion (500 \mu L) and bacterial suspension (500 \mu L) was shaken at 298 K for 1 h. Drops (10 \mu L) of each bacterial suspension, and the nanocomposite dispersion were then pipetted onto a glass slide and dried at room temperature for 1 h. Dark-field observation was performed using an optical microscope (ECLIPSE Ni, Nikon, Japan) with a dark-field condenser, a 100 W halogen lamp, and a
charge-coupled device camera (DSRi1, Nikon, Japan). Light-scattering spectra were measured using a miniature grating spectrometer (USB4000, Ocean Optics), which was connected to the microscope using an optical fiber (core diameter, 400 μm).

**Beef sample preparation**

A piece of beef was purchased from a local supermarket and incubated at 303 K for 6 h. This rotten beef (25 g) was then mixed with 225 mL of phosphate buffer and then squeezed to extract the meat juice. Next, the beef particulates were removed by filtration and the meat juice in phosphate buffer was stored at 278 K. A 10 mL volume of each beef suspension was centrifuged at 8,000 rpm (7,000 × g) at 278 K for 10 min and the supernatant was removed. The precipitates were then resuspended in 10 mL of sterilized ultrapure water and this procedure was repeated three times to remove all of the small components (proteins and molecules) other than the bacteria from the beef sample. The bacterial species in the sample were identified by TechnoSuruga Laboratory Co, Ltd. (Shizuoka, Japan) and most samples were shown to include *Aeromonas salmonicida* and *Serratia liquefaciens*, and the total density of these cells was estimated at approximately 3.0×10⁶ cells mL⁻¹ without the addition of *E. coli* O157 suspension. Final evaluations were done following the addition of *E. coli* O157 at concentrations of 10¹ to 10⁸ cells mL⁻¹.

**Results and Discussion**

**Binding properties of the nanocomposite**

The nanocomposite was obtained as spherical particles with a temperature responsive copolymer as a protective layer. Transmission electron microscope (TEM) images of the nanocomposite particles revealed that this material adopted a sphere-like
structure with a mean diameter of 100 nm (Fig. 1a). Many AuNPs form inside the NIPAm copolymer, while no AuNPs were observed outside the copolymer particles. This suggests that the AuNPs automatically form in response to the ion exchange between the aurate and protonated amide segments in the NIPAm during the polymerization at 298 K. Therefore, the encapsulation of the AuNPs within this copolymer material was achieved in a single step.24 It is well-known that the NIPAm copolymer is a temperature-responsive material with a reversible phase transition.25-27 Below the lower critical solution temperature (LCST) of \( \sim 305 \) K, the copolymer forms a hydrophilic surface via the hydrogen bonds between the amide groups and water molecules. At room temperature, the sugar chains of the template LPS interact with the hydrophilic residues in the copolymer. Above the LCST, a phase transition occurs enhancing the hydrophobic properties of the copolymer. This change is a result of the inter- and intramolecular aggregation of the isopropyl groups within the copolymer itself. During dialysis at 313 K, LPS is released from the copolymer as the hydrophobic nature of the polymer at this temperature fails to interact with the hydrophilic sugar chains in the LPS.

The nanocomposite specifically bound to a single cell of \textit{E. coli} O157 (Fig. 1b) cells and does not bind other strains of \textit{E. coli} (NBRC3972, NBRC3301) with different LPS sugar chain sequences (Fig. 1c, d). When \textit{E. coli} O157 cells are mixed with the nanocomposite there are no unbound nanocomposite residues in any of the fields of view evaluated in this study. On the other hand, there are many unbound nanocomposite residues when we add other strains of \textit{E. coli}. This data confirms that these nanocomposites recognize the sugar chains in the LPS molecules and that this facilitates the specific recognition of \textit{E. coli} O157. The dark field microscopy revealed that it is possible to identify specific bacterial species following their by binding by specific
nanocomposite materials via the focused evaluation of the scattered light produced by the encapsulated AuNPs.\textsuperscript{10} Observations using a dark-field microscope also revealed that nanocomposites do not bind to different serotypes of enterohemorrhagic \textit{E. coli} and have specific binding properties against their target (O157). Moreover, we also evaluated the binding properties of these nanocomposites by focusing on the light scattering intensity of the bacterial cell interactions using this same type of microscopy. There was almost no difference in the light scattering spectra of single cells in different \textit{E. coli} when using unlabeled nanocomposites. However, when the nanocomposites were combined we were able to shown strong spots of light in response to interactions with \textit{E. coli} O157 cells (Fig. 2A). Nanocomposites binding increased the intensity of the light scattering by \textit{E. coli} O157 cells, with the spectral intensity of the \textit{E. coli} O157, which was clearly different from the intensity of other \textit{E. coli} strains, increasing by more than 4-fold following binding (Fig. 2B). This data confirmed the specificity of the nanocomposites and demonstrates that these materials function as a highly sensitive optical antenna on the surface of the target bacterium. Moreover, we were also able to confirm that the nanocomposite materials retained their LPS recognition patterns after dialysis. In addition, the increase in temperature to reach the mixed dispersion phase (to 313 K) induced a phase transition in the copolymer, and no binding between the nanocomposite and the target cells was observed. When the temperature of the mixed dispersion was dropped to 298 K, the nanocomposite was once again able to bind the target cells. This data reveals that the binding ability of the nanocomposite material is based on the availability of the shape-complementary cavities in the material and that the availability of these cavities can be controlled by temperature.

\textit{Real sample validation}
Given the success of our initial tests we went on to evaluate the ability of our nanocomposites to detect *E. coli* O157 cells in real sample suspensions extracted from rotten beef. The real sample suspensions obtained from the rotten beef were shown to contain both *Aeromonas salmonicida* and *Serratia liquefaciens*. Target *E. coli* O157 cells were added to the real sample suspension and used to prepare *E. coli* O157 evaluation solutions with a concentration of 0-100% of the total cell count. After adding nanocomposites to these suspensions, 10 µL mixture suspensions were dropped onto a glass slide and subject to dark field observation. In the samples without *E. coli* O157 all the bacteria in the field of view were observed as weak light spots (Fig. 3a). In the sample with *E. coli* O157, we saw some strong rod-shaped light scattering spots which coincided with the nanocomposite-labeled *E. coli* O157 cells (Fig. 3b). As the concentration of *E. coli* O157 cells within the suspension increased so too did the number of strong rod-shaped light-scattering spots (Fig. 3b-f). The number of light-scattering spots in each field of view corresponded to the proportion of *E. coli* O157 cells in the sample. So in the 50% solution which produced 42 cells per droplet, was shown to have 22 strong rod-shaped light-scattering spots (Fig. 3d). The ratios of the number of the strong (22 cells) and weak light scattering rod-shaped spots (20 cells) clearly corresponded to the ratio of the target bacteria (*E. coli* O157) and other bacteria in the sample. Table 1 shows that the number of strong light-scattering spots corresponds to the abundance of target bacteria in the sample with an accuracy of ± 4% standard deviation. Taken together this data confirms that nanocomposites produced using artificial antibodies against O157-LPS can specifically bind to targets in suspensions containing multiple types of bacteria and function as optical antennas.

To efficiently quantify the target bacteria within a certain microscopic field of view, the intensity of the scattering light over a wide range (0.13 mm²) on the glass slide was
measured. As the number of nanocomposite-labeled *E. coli* O157 cells increased in the real samples so the scattering light intensity of the optical spots increased with reliable correlation (Fig. 4). This confirms that *E. coli* O157 can be quantified based on the scattered light intensity generated from the nanoantenna on the target bacterial cells.

**Conclusions**

Nanocomposites molecularly imprinted using LPS derived from *E. coli* O157 show specific binding of *E. coli* O157. In addition, the nanocomposites formed by encapsulating AuNP with an NIPAm copolymer function as an optical antenna on the target bacterial surface based on their specific binding properties and light scattering characteristics, facilitating the identification and visualization of specific bacterial contamination. Furthermore, scattering light intensity was shown to have a solid correlation with the number of target bacteria in any given sample. Taken together this data supports the application of this method in the quick and easy identification of bacterial contamination in the food industry.

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References

1. F. R. Blattner, G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, Y. Shao, *Science*, 1997, 277, 1453.

2. J. P. Nataro and J. B. Kaper, *Clin. Microbiol. Rev.*, 1998, 11, 142.

3. T Hayashi, K. Makino, M. Ohnishi, K. Kurokawa, K. Ishii, K. Yokoyama, C. G. Han, E. Ohtsubo, N. Nakayama, T. Murata, M. Tanaka, T. Tobe, T. Iida, H. Takami, T. Honda, C. Sasakawa, N. Ogasawara, T. Yasunaga, S. Kuhara, T. Shiba, M. Hattori, H. Shinagawa, *DNA Res.*, 2001, 8, 11.

4. R. A. Welch, V. Burland, G. Plunkett III, P. Redford, P. Roesch, D. Rasko, E. L. Buckles, S.-R. Liou, A. Boutin, J. Hackett, D. Stroud, G. F. Mayhew, D. J. Rose, S. Zhou, D. C. Schwartz, N. T. Perna, H. L. T. Mobley, M. S. Donnenberg, and F. R. Blattner, *Proc. Natl. Acad. Sci. USA*, 2002, 99, 17020.

5. H. Shiigi, *Anal. Sci.*, 2019, 35, 235.

6. J. H. Warcup, *Trans. Brit. mycol. Soc.*, 1955, 38, 298.

7. S. Hameed, L. Xie, Y. Ying, *Trends in Food Science & Technology*, 2018, 81, 63.

8. H. C. Gram, *Fortschr. Med.*, 1884, 2, 185.

9. M. J. Wilhelm, J. B. Sheffield, M. Sharifian Gh., Y. Wu, C. Spahr, G. Gonella, B. Xu, and H.-L. Dai, *ACS Chem. Biol.*, 2015, 10, 1711.

10. H. Shiigi, M. Fukuda, T. Tono, K. Takada, T. Okada, L. Dung, Y. Hatsuoka, T. Kinoshita, M. Takai, S. Tokonami, H. Nakao, T. Nishino, Y. Yamamoto and T. Nagaoka, *Chem. Commun.*, 2014, 50, 6252.

11. T. Kinoshita, K. Kiso, D. Q. Le, H. Shiigi, T. Nagaoka, *Anal. Sci.*, 2016, 32, 301.

12. G. P. Acuna, F. M. Möller, P. Holzmeister, S. Beater, B. Lalkens, P. Tinnefeld,
13. A. Moreau, C. Ciraci, J. J. Mock, R. T. Hill, Q. Wang, B. J. Wiley, A. Chilkoti, D. R. Smith, Nature, 2012, 492, 86.

14. K. Ishiki, K. Okada, D. Q. Le, H. Shiigi, T. Nagaoka, Anal. Sci., 2017, 33, 129.

15. K. Ishiki, H. Shiigi, T. Nagaoka, Anal. Sci., 2017, 33, 551.

16. W. P. Faulk and G. M. Taylor, Immunochemistry, 1971, 8, 1081.

17. H. Shiigi, T. Kinoshita, M. Fukuda, D. Q. Le, T. Nishino and T. Nagaoka, Anal. Chem., 2015, 87, 4042.

18. J. Yao, X. Li, W. Qin, Anal. Chim. Acta, 2008, 610, 282.

19. A. Beltran, R. M. Marcé, P. A. G Cormack, F. Borrell, J. Chromatogr. A, 2009, 1216, 2249.

20. X. Shan, T. Yamauchi, Y. Yamamoto, S. Niyomdecha, K. Ishiki, D. Q. Le, H. Shiigi and T. Nagaoka, Chem. Commun., 2017, 53, 3890.

21. X. Shan, T. Yamauchi, H. Shiigi, T. Nagaoka, Anal. Sci., 2018, 34, 483.

22. D. Q. Nguyen, X. Shan, M. Saito, K. Iwamoto, Z. Chen, H. Shiigi, Anal. Sci., 2019, 35, 763.

23. T. Kinoshita, D. Q. Nguyen, D. Q. Le, K. Ishiki, H. Shiigi, T. Nagaoka, Anal. Chem., 2017, 89, 4680.

24. H. Shiigi, Y. Yamamoto, N. Yoshi, H. Nakao, T. Nagaoka, Chem. Commun., 2006, 4288.

25. K. Narayanan, S. Chandel, N. Ghosh, P. De, Anal. Chem., 2015, 87, 9120.

26. N. Morimoto, Y. Sasaki, K. Mitsunushi, E. Korchagina, T. Wazawa, X.-P. Qiu, S. M. Nomura, M. Suzuki, F. M. Winnik, Chem. Commun., 2014, 50, 8350.

27. M. Heskins, J. E. Guillet, J. Macromol. Sci., Chem., 1968, 2, 1441.
| Rate of target identification in suspension<sup>a</sup> | Number of cells | Rate of target counting<sup>d</sup> | Standard deviation/% |
|----------------|-----------------|-----------------|------------------|
|                | Target<sup>b</sup> | Total<sup>c</sup> |                  |
| 0              | 0               | 20              | 0                | 0                |
| 0.10           | 5               | 35              | 0.14             | 4.0              |
| 0.30           | 12              | 44              | 0.27             | 3.0              |
| 0.50           | 22              | 42              | 0.52             | 2.0              |
| 0.70           | 35              | 52              | 0.71             | 1.0              |
| 0.90           | 34              | 40              | 0.86             | 4.0              |
| 1.0            | 44              | 46              | 0.96             | 4.0              |

<sup>a</sup> Ratio of target cells added to the suspension vs. the total number of cells.

<sup>b</sup> Number of events with strong light spots per dark field counted (N=6).

<sup>c</sup> Number of the strong and weak light spots per dark field counted (N=6).

<sup>d</sup> The ratio of strong light spots (target cells) in each dark field vs. the total number of cells.
Fig. 1 TEM images of the nanocomposite (a), *E. coli* O157 (b) *E. coli* (NBRC3972) (c) and *E. coli* (NBRC3301) with the nanocomposite (d).
Fig. 2 (A) Dark-field images and (B) light-scattering spectra for various type of *E. coli*. (a) *E. coli* O157, (b) *E. coli* (NBRC3972) and (c) *E. coli* (NBRC3301) with the nanocomposite. Acquisition time for all dark-field images was 400 ms.
Fig. 3 Dark-field images of real samples supplemented with *E. coli* O157 cells. Scale bars are 10 µm. Ratio of *E. coli* O157 cells were (a) 0, (b) 10, (c) 30, (d) 50, (e) 70 and (f) 90%. Acquisition time for all dark-field images was 400 ms.
Fig. 4 Correlation between light-scattering intensity in specific fields of view (0.13 mm²) and the percentage of the nanocomposite-labeled *E. coli* O157 cells within the suspension.
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