Development of Metal Nanoparticle-Immobilized Microplate for High-Throughput and Highly Sensitive Fluorescence Analysis

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Abstract

Enzyme-linked immunosorbent assay (ELISA) is a widespread analytical biochemistry assay. In this work, a direct ELISA method using a metallic nanoparticle (NP)-immobilized 96-well plate was developed for high-throughput, highly sensitive fluorescence analysis. Immobilization of metallic NPs on a 96-well plate effectively amplified fluorescence signals of the assay. The silver (Ag) NP-immobilized plate showed the best fluorescence enhancement effect of all the metal-immobilized plates tested. We used the Ag NP-immobilized plate to detect biomolecules and bacteria and found that both the fluorescence intensity and the limit of detection (LOD) were strongly enhanced by more than 100 times compared with those of the unmodified 96-well plates. Quantitative and qualitative considerations for target bacteria regarding the impact of autofluorescence on detection were successfully obtained for several strains. Our results demonstrate the potential of applying Ag NPs for enhancing the efficiency of direct and indirect ELISA assays.

Keywords: ELISA, metal nanoparticle, bacterial detection, high-throughput, highly sensitive fluorescence analysis.
Introduction

Enzyme-linked immunosorbent assay (ELISA) has been applied for identification of various targets such as antigens, antibodies, toxic or functional proteins, bacteria, and viruses due to its high selectivity and quantitative/qualitative capability.\textsuperscript{1-6} ELISA is typically performed in a 96-well plate, which allows high-throughput detection of a variety of samples.\textsuperscript{7-9} However, the nature and composition of a target substance might necessitate a multistep complicated ELISA procedure to ensure its accurate and high-sensitive detection.\textsuperscript{10} In direct ELISA detection (the simplest detection format), an enzyme-labeled primary antibody adsorbed to the well in advance can help distinguish targets from other species and a detectable signal such as fluorescence or luminescence based on interactions between the enzyme and the targeted substrate is obtained.\textsuperscript{2} However, in most cases, the signal intensity is not strong enough,\textsuperscript{11} which leads to the involvement of conjugated secondary antibodies for amplifying the signal. The procedure utilizing secondary antibodies is called indirect ELISA detection.\textsuperscript{12} Although it has the advantages of high selectivity and easy availability of labeled secondary antibodies compared to the direct one, extra incubation steps as well as the elimination of non-specific signals are usually required.\textsuperscript{13,14} Therefore, an alternative method for enhancing the signal would be highly appreciated.

In our previous work, we demonstrated the fluorescence enhancement effect of gold nanoparticles (Au NPs).\textsuperscript{15} Briefly, the fluorescence intensity of dye molecules can be enhanced or quenched depending on their distance from and orientation relative to the surface of metallic NPs,\textsuperscript{16} which is caused by interactions between the dye molecules and the electric fields or localized surface plasmon resonance (LSPR) coupling generated from metallic NPs.\textsuperscript{17-23} Therefore, this effect might enable us to utilize metallic NPs on 96-well plates to amplify the fluorescence signal in ELISA. Recently, we found that the
Ag NP-immobilized plate showed the best fluorescence enhancement effect of all the metal-immobilized plates tested.\textsuperscript{24} In the previous report, various kinds of metallic NPs were immobilized inside the wells of a 96-well plate. As a result of comparing those fluorescence enhancements, it was found that the Ag NP-immobilized plate showed the maximum effect due to the inherent LSPR of metal NPs. In this study, Ag NP-immobilized plates were shown to be useful for detecting biomolecules and bacteria in terms of sensitivity and detection limits. A simple method was developed to enhance the fluorescent signal by modifying the microplate.

**Experimental**

*Chemicals and materials*

All chemicals were reagent grade. Ultrapure water (\(>18 \text{ M}\Omega \text{ cm}\)) sterilized by UV light was used for all the experiments. Amino-functionalized 96-well plates were purchased from Sumitomo Bakelite Co., Japan. Fluorescein isothiocyanate (FITC)-conjugated streptavidin was purchased from Thermo Fisher Scientific Inc. FITC-labeled anti-*Escherichia coli* O157:H7 antibody was purchased from Kirkegaard & Perry Laboratories, Inc. Genetically modified verotoxin-nonproducing *E. coli* PV856 (O157:H7) and *E. coli* PV01-198 (O26:H11) were provided by Prof. M. Miyake, Department of Veterinary Science, Osaka Prefecture University, and Dr. K. Seto, Osaka Prefectural Institute of Public Health. *Salmonella enterica* (NBRC13245) was purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC). Chloroauric acid was purchased from Tanaka Kikinzoku Kogyo Inc., Japan. Silver nitrate, palladium chloride, ethylenediamine tetraacetic acid disodium salt (EDTA\(\cdot \)2Na), sodium borohydride, trisodium citrate dehydrate, and fluorescein were purchased from Fujifilm.
Wako Pure Chemical Co., Japan. Nutrient broth (NB) was obtained from Eiken Chemicals (E-MC35, Japan).

**Apparatus**

Size distributions and zeta potentials of metallic NPs were measured with a zeta potential and particle size analyzer (ELSZ-2Plus, Otsuka Electronics, Japan). Absorbance and fluorescence intensity of the 96-well plates were measured with a multi-label plate reader (ARVO X3, PerkinElmer). Bright-field microscopic images of the 96-well plates were obtained using optical microscopy (EVOS FL Auto, Life technologies).

**Preparation of Ag NP-immobilized 96-well plate**

A sodium hydroxide aqueous solution (1.0 M, 0.74 mL) was added to 100 mL of an aqueous solution containing 0.25 mM silver nitrate and 0.16 mM EDTA•2Na under vigorous stirring at 373 K. A yellowish Ag NP dispersion (0.0027 wt%) was obtained after stirring for 5 min. Absorption spectra of the Ag NPs dispersion in the well showed one peak at 406 nm, attributable to Ag NPs in the dispersed state. The pH of the dispersion was adjusted to 4.0 by the addition of hydrochloric acid (1.0 M, 0.65 mL) under vigorous stirring. The dispersion (0.45 mL) was added to a 96-well plate and incubated for 12 h. After the dispersion was removed, the 96-well plate was dried at 333 K for 1 h.

**Bacterial culture and purification**

All bacterial cultures and experiments were performed in a biosafety level 2 laboratory, developed and managed in accordance with safety regulations. Strains of *E. coli* O157:H7, *E. coli* O26:H11, and *S. enterica* were cultured in an NB agar plate at 303 K for 18 h. A
single colony was transferred to a liquid NB medium (30 mL) and incubated at 303 K for 18 h. A cultured suspension (10 mL) was centrifuged at 8,000 g for 15 min at 278 K, and the pellet was resuspended in a phosphate buffer (pH 7.0, 90 mM, 10 mL) by shaking for 1 min. This procedure was repeated 3 times.

Detection of fluorescently labeled streptavidin

A thiol-introduced biotin aqueous solution (0.25 mM, 0.40 mL) was added to both Ag NP- (30 nm) and Au NP- (30 nm) immobilized wells. After 2 h of incubation, the supernatant was removed and the wells were thoroughly washed with ultrapure water. After that, the predetermined concentration of a fluorescein-labeled streptavidin aqueous solution (0.40 mL) was added and incubated for another 2 h. Then, the supernatant was removed and the wells were thoroughly washed with ultrapure water again. Finally, the fluorescence intensity of the well at 535 nm was measured with a plate reader (Ex. 485 nm).

Specific detection of E. coli O157

A bacterial suspension of E. coli O157:H7 (0.50 mL) was mixed with a dispersion of FITC-labeled anti-E. coli O157:H7 antibody (1.0 mg/0.50 mL), and the mixture was stirred in a dark room for 30 min. The suspension was centrifuged at 8,000 g for 15 min at 278 K, and the pellet was resuspended in a phosphate buffer (1.0 mL). This procedure was repeated 3 times. The suspension (40 µL) was incubated in 96-well plates for 2 h. After removal of the suspension, the fluorescence intensity of the well at 535 nm was measured with a plate reader (Ex. 485 nm). This procedure was also performed for E. coli O26:H11, S. enterica, and a bacterial mixture.
Preparation of bacteria-contaminated real sample

Minced beef (25 g) bought from a local supermarket was incubated for 6 h at 303 K and then added into the phosphate buffer (225 mL). The beef juice was then extracted by stomaching. The suspension including some kinds of bacteria and residue of the beef was used as a real sample.

Results and Discussion

Immobilization of Ag NPs on a 96-well plate

The zeta potential of the Ag NPs was –35.4 mV since these coated by the negatively charged EDTA molecules. The wells in the 96-well plate were positively charged at pH 4.0 because the functionalized amine groups that had already modified the surface of each well were protonated. Consequently, Ag NPs were immobilized on the wells through electrostatic interaction.

Detection of biomolecules

We investigated the capability of the Ag NP-immobilized well to detect biomolecules. FITC-conjugated streptavidin dispersed in 0.40 mL of pH 7.0 phosphate buffer was incubated in the Ag NP-immobilized well modified with biotin for 2 h. After removal of the streptavidin dispersion, the fluorescence intensity of the well at 535 nm was measured with the plate reader (Ex. 485 nm). At first, the fluorescence intensity increased sharply with the increase of incubated streptavidin through the avidin–biotin interaction on the Ag NPs (Fig. 1A). As the amount of streptavidin increased to more than 25 pmol, the fluorescence enhancement effect of the Ag NPs well became weaker, owing to the decreased distance among the dye molecules.26,27 When the concentration was 0–25 pmol,
A linear relationship ($R^2 = 0.9956$) was observed (Fig. 1B). In comparison with the Au NP-immobilized well, the Ag NP well indicated a much higher sensitivity and excellent limit of detection (LOD) which was defined as 3 times the standard deviation of blank measurements.

The slope of $4.1 \times 10^3$ pmol$^{-1}$ noted for 0–25 pmol of streptavidin for the Ag NP well was 86 times higher than that for the Au NP well (47 pmol$^{-1}$). The LOD of the Ag NP well was calculated as 75 fmol. It was also 100 times better than that of the Au NP well (7.5 pmol). Therefore, the Ag NP-immobilized well possessed excellent detection capability for biomolecules, although it is necessary to select a binding method for each target.

**Detection of specific bacteria**

We applied the Ag NP-immobilized well for specific detection of *E. coli* O157:H7 cells. FITC-labeled anti-*E. coli* O157:H7 antibodies, which were utilized for the selectivity of the target bacteria and also as the fluorescent source, modified the surface of the bacteria as described in Scheme 1.

The labeled bacteria were adsorbed on the Ag NP-immobilized well by incubating the suspension in the well for 2 h. After removal of the suspension, the fluorescence intensity increased as the amount of the bacteria increased (Fig. 2A). The adsorbed bacterial cells were counted by observation under bright-field microscopy after removal of the suspension (Fig. S1). It was found that $88 \pm 6\%$ of the incubated bacteria were adsorbed on the bottom of the well. In this case, the remaining bacterial cells were probably adsorbed on the well walls. The adsorption percentage was nearly constant as the number of incubated bacterial cells increased from $10^3$ to $10^7$ (Table S1). The bacterial cells,
which were covered by sugar chains, adsorbed through multiple hydrogen bonds onto the carboxy groups of EDTA covering the surface of the Ag NPs. Moreover, the bacterial cells with a negative zeta potential (−20 mV) were also electrostatically adsorbed to the positively charged well bottom of the unmodified well. There was no difference between the number of adsorbed cells in the unmodified and Ag NP-immobilized wells. Therefore, the increase in the fluorescence intensity depends on the amount of FITC-labeled cells. Moreover, the intensity obtained using the Ag NP-immobilized well was much greater than that obtained using an unmodified well in spite of the bacterial adsorption percentage being almost the same (87 ± 9%). However, compared with the previous results obtained for FITC-conjugated streptavidin, the fluorescence enhancement effect of Ag NPs on FITC molecules was much weaker on the surface of bacterial cells. This was because the best performance of the fluorescence enhancement effect was achieved when the dyes were located several nm away from the metallic NPs. We determined the area occupied by a single *E. coli* O157:H7 cell at the bottom layer by estimating the length (2.1 μm), width (0.65 μm), and height (0.59 μm) of the cells. Therefore, most of the FITC, which was located on the upper side of the adsorbed cells, did not contribute to fluorescence enhancement since they were more than 10 nm away from the Ag NPs at the bottom of the well. Only a part of the FITC molecules located between the bottom surface of the well and the lower part of the bacterial cells underwent fluorescence enhancement. Compared with that of the unmodified well, in which a slight increase in the intensity based on the adsorbed cells was obtained at over $3 \times 10^5$ cells, the LOD of the Ag NP-immobilized well still showed a significant improvement, and it was estimated at approximately 600 cells in the well (Fig. 2B).

The selectivity against other bacterial species was evaluated using *S. enterica* and *E. coli* O26 instead of *E. coli* O157. As the number of adsorbed cells increased up to $10^6$,
both bacteria showed a fluorescence intensity that was nearly the same as the intensity produced by the Ag NP-immobilized well without bacteria (Fig. S2). However, when the number of adsorbed cells was $10^7$, the fluorescence intensity showed by both bacteria became higher than that of the well without bacteria. A similar phenomenon was observed for the case of *E. coli* O157 without the FITC-labeled antibody, which was caused by the cellular autofluorescence phenomenon. A single bacterium intrinsically exhibits very weak fluorescence in the green region (500–560 nm), which may affect the fluorescence-related analysis to a certain degree. Yang et al. reported that the bacterial autofluorescence may vary from 80 to 1400 FITC equivalents per cell depending on the bacterial species.$^{31}$ When the number of cells was $10^7$, their fluorescence intensity was just up to $1.4 \times 10^{10}$ FITC equivalents (corresponding to 23 fmol of FITC), which is too small to have any significant effect on detection. We propose that the main reason for the observed fluorescence was the aggregation of adsorbed bacterial cells, which boosts the intensity of the bacterial autofluorescence. The inner surface area of each well was estimated to be $2.3 \times 10^8 \mu \text{m}^2$. Since the projected area of each *E. coli* cell was about 1.7 $\mu \text{m}^2$, one layer of adsorbed cells was theoretically fully filled by approximately $10^8$ cells. As indicated in the bright-field microscopic images (Fig. S1), the cells were uniformly distributed until their number in the well went up to $10^6$. When the number of adsorbed cells was $10^7$ or higher, aggregation occurred and led to an increase in the bacterial autofluorescence intensity per cross-sectional area.

The influence of other bacterial contamination on mixed samples with *E. coli* O157 was also investigated. The detection of *E. coli* O157 was carried out using suspensions including $10^5$, $10^6$, and $10^7$ cells of *E. coli* O26. Fig. 3 shows that the fluorescence intensity of the mixed sample contaminated by up to $10^6$ cells of *E. coli* O26 was consistent with that of the sample of only *E. coli* O157 (0 cells of *E. coli* O26). As the
number of contaminated *E. coli* O26 cells increased to $10^7$, the background intensity became higher until the number of targeted *E. coli* O157 was $10^3$ or lower, similar to the intensity noted for the sample containing $10^7$ cells of *E. coli* O26 as shown in Fig. S2. When the number of targeted *E. coli* O157 was $10^4$ or higher, the intensity again became consistent with that of the sample of only *E. coli* O157. We suggest that the rise in the background intensity is caused by bacterial autofluorescence as the amount of FITC molecules on the targeted cells is low. However, when the percentage of FITC-labeled *E. coli* O157 cells against the unlabeled ones is approximately 0.10% ($10^4$ cells of O157 against $10^7$ cells of O26) or higher, the fluorescence source is dominated by FITC. Therefore, it was assumed that detection of *E. coli* O157 using Ag NP wells could be directly achieved if the number of contaminating bacteria in the wells was less than $10^6$ cells. Samples with a higher concentration can be completely analyzed by dilution.

**Real sample analysis**

The suspension extracted from rotten beef was used for real sample analysis. Bacterial species of *Aeromonas salmonicida* and *Serratia liquefaciens* were found to contaminate the sample (identified by TechnoSuruga Laboratory Co., Ltd., Japan), and the total number of contaminated cells in the well was $1.2 \times 10^5$. *E. coli* O157 detection was carried out after adding $10^3$–$10^6$ cells of *E. coli* O157 to the sample solution (Table 1). Recovery of 100.6–102.4% were obtained, and the RSDs for all cases were less than 4.0%. These results reveal the feasibility of using Ag NP-immobilized plates in the detection of *E. coli* O157.

**Conclusions**
A direct ELISA method using Ag NP-immobilized 96-well plates was established for enhancing the fluorescence signal. In the detection of labeled targets, the fluorescence-enhanced plate was demonstrated to have fluorescence intensity and LOD greater than 100-fold compared with those of unmodified plates. Fluorescence-enhanced plates could not only be useful for detecting the molecules, proteins, and bacteria demonstrated in the paper, but also genes and viruses. The prospect of applying Ag NPs for signal enhancement in ELISA is very promising for specific binding properties, regardless of the target species. Urgent development of specific binding substances such as antibodies, nucleic acids, and molecular imprinted polymers are essential for detecting new threats with high throughput using the proposed procedure.

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Supporting Information

Further information regarding experimental details. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Table 1  Recovery of real sample analysis using Ag NP-immobilized well

| Sample | Added /cells | Measured /cells | Recovery /% | RSD /% |
|--------|-------------|----------------|-------------|--------|
| 1      | 1.00×10³    | 1.02×10³       | 102         | 4.0    |
| 2      | 1.00×10⁴    | 1.02×10⁴       | 102         | 3.0    |
| 3      | 1.00×10⁵    | 1.01×10⁵       | 101         | 2.4    |
| 4      | 1.00×10⁶    | 1.01×10⁶       | 101         | 1.0    |

Recovery is calculated by diving the measured number of cells by the number of added cells. (N = 10)
Figure Captions

**Fig. 1** Dependence of fluorescence intensity at 535 nm on amount of FITC-conjugated streptavidin in the range of (A) 0–250 pmol and (B) 0–25 pmol using Ag NP- (black dot) and Au NP- (white circle) immobilized wells (N = 10).

**Fig. 2** Dependence of fluorescence intensity at 535 nm on amount of *E. coli* O157 cells in the range of (A) 0–10^6 cells and (B) 0–2000 cells using an Ag NP-immobilized well (black dot) and unmodified well (open circle). The dashed line shows the intensity of the Ag NP-immobilized well without bacteria (N = 10).

**Fig. 3** Dependence of fluorescence intensity at 535 nm on amount of *E. coli* O157 in bacterial mixture dispersions including 0, 10^5, 10^6, and 10^7 cells of *E. coli* O26 obtained using a Ag NP-immobilized well. The dashed line shows the intensity without bacteria (N = 10).

**Scheme 1** Illustration of (A) procedure of bacterial detection using Ag NP-immobilized microplate and (B) fluorescence enhancement surface.
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Scheme 1  Illustration of (A) procedure of bacterial detection using Ag NP-immobilized microplate and (B) fluorescence enhancement surface.
