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Original Article

Detection of *Salmonella typhimurium* by Antibody/ Enzyme-Conjugated Magnetic Nanoparticles

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Abstract A simple, highly sensitive method was developed to detect *Salmonella typhimurium* using 100-nm antibody- and horseradish peroxidase-conjugated magnetic nanoparticles (Ab-HRP-MNPs). The particles bound bacteria, while unbound particles were removed by filtration. The number of bacteria was determined based on the intensity of light emitted from the chemiluminescence reaction catalysed by bacterium-bound peroxidase in the filter. The light intensity was proportional to the cells in the range of 0-10^4 colony forming units (CFU), with a lower detection limit of 10 CFU.

Keywords: *Salmonella typhimurium*, Magnetic nanoparticle (MNP), Immunomagnetic separation, Filtration, Antibody, Horseradish peroxidase

Introduction

Immunomagnetic separation is an attractive approach for the rapid detection of bacteria, which are removed from complex sample matrices by antibody (Ab)-functionalized magnetic particles (MPs)[1] and detected by sandwich methods using antibodies conjugated with enzymes[2-6] or fluorescent dyes[7-9]. However, this method is relatively complex as it employs two different antibodies, one linked to the MP and the other to the enzyme or dye.

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A simplified assay method for detecting bacteria was developed using dually functionalized MPs containing both Ab and enzyme[10]. The method is based on the enzyme shadowing effect, in which the interaction between bacteria and MPs decreases enzymatic activity by blocking substrate access. However, this approach has an inherent limitation in terms of sensitivity, as it is difficult to distinguish a minute signal change from a blank value of 100% that is caused by a few bacteria.

Lab-on-a-chip (LOC) is a promising tool for the rapid detection of pathogens, enabling pretreatment, separation, and detection in a single integrated system[11]. In one study, *Escherichia coli* O157 : H7 was concentrated with Ab-coated glass beads on a microfluidic chip and ATP was measured by detecting bioluminescence; between 3.2×10^1 and 3.2×10^5 CFU of bacteria were detected in a 1-μL sample volume within 20 min[12]. The limit of detection for *E. coli* O157 : H7 was 10 CFU/mL using an immunoagglutination method with a microfluidic chip[13], but this approach required samples to be pretreated by filtration prior to chip application.

In the present study, bifunctional magnetic nanoparticles (MNPs) around 100 nm in size coupled with both Ab and enzyme were used to detect pathogenic bacteria. Free particles were separated from the MNP-bacteria complex by filtration after pretreatment based on their differences in size. The number of bacteria was then determined by measuring the enzymatic activity associated with the complex. This method may be suitable for LOC applications because the whole process can be easily carried out on a single chip containing a nanoporous filtration module[14,15]. Moreover, high levels of signal amplification can be achieved with this method, as a single MNP is conjugated with multiple

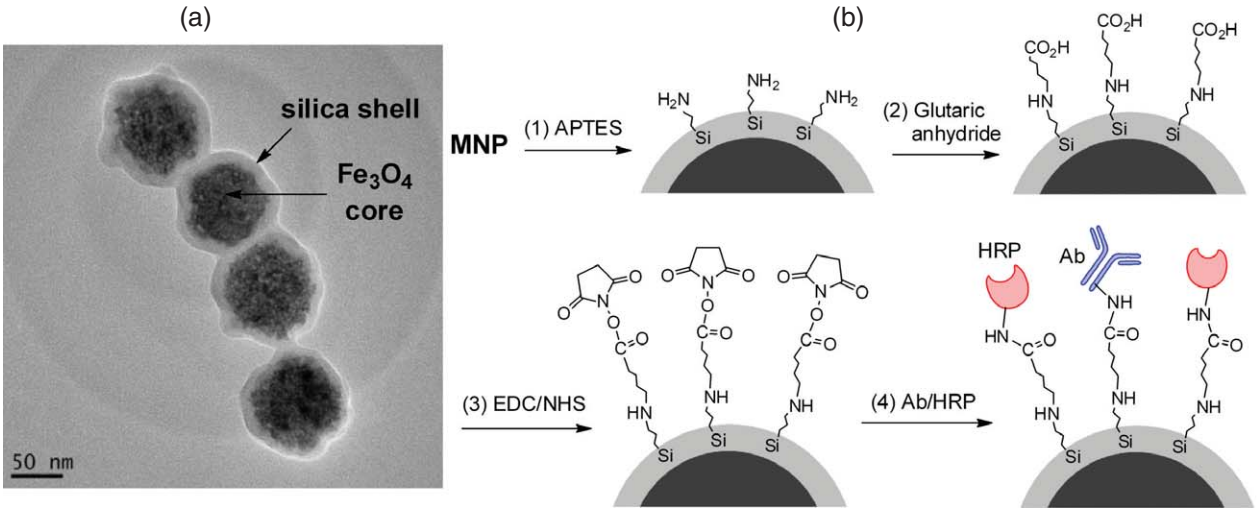

Figure 1. Transmission electron micrograph (a) and functionalization scheme (b) of MNPs.

enzyme molecules. As a proof of principle, 100-nm MNPs were functionalized with anti-*Salmonella typhimurium* Ab and horseradish peroxidase (HRP) to generate Ab-HRP-MNPs. After these had bound to *S. typhimurium*, free particles were removed by filtration using a 0.6-μm pore filter. Using a chemiluminescent method to estimate the HRP activity in the filter, as few as 10 CFU of *S. typhimurium* were detected in 0.1 mL suspension.

Results and Discussion

Functionalization and characterization of MNPs

MNPs with an 80-nm iron oxide core and 10-nm silica shell were used in this experiment (Figure 1a). The scheme for the MNP functionalization is shown in Figure 1b. Treatment of the MNP with (3-aminopropyl)triethoxysilane (APTES) produced a self-assembled monolayer containing surface amino groups that were converted to carboxyl groups by reaction with glutaric anhydride. The carboxyl groups were activated by sequential reactions with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The resulting NHS groups were used for chemical conjugation of anti-*S. typhimurium* Ab and HRP enzyme to generate Ab-HRP-MNP.

The Ab and HRP were conjugated at a molar ratio of 1 : 10 so as to maximize the number of enzyme molecules associated with each MNP. A 100-nm particle has the capacity to bind around 10^3 protein molecules with an average diameter of 6 nm; thus, each MNP would have a sufficient amount of Ab to bind target bacteria at this ratio. Successful conjugation of

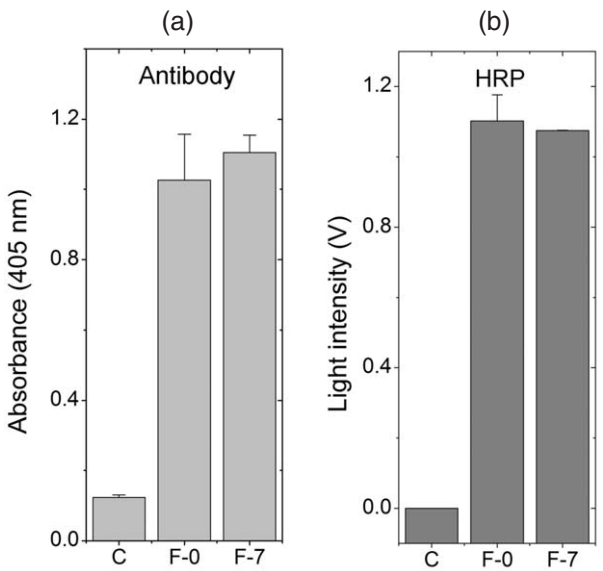

Figure 2. Activity of Ab (a) and HRP (b) immobilized on MNPs. The activity was estimated on days 0 (F-0) and 7 (F-7). Data represent the mean of triplicate measurements; error bars represent the standard deviation.

the Ab and HRP was evident by comparing the Ab-HRP-MNP with the control bare MNP (Figure 2a). Moreover, the immobilized Ab and HRP were stably maintained after 7 days owing to the covalent nature of the bonds.

The binding of Ab-HRP-MNPs to *S. typhimurium* was observed by scanning electron microscopy (SEM). *S. typhimurium* was mixed with Ab-HRP-MNPs, passed through a 0.6-μm pore polycarbonate track-etched (PCTE) filter, and observed by SEM after treatment

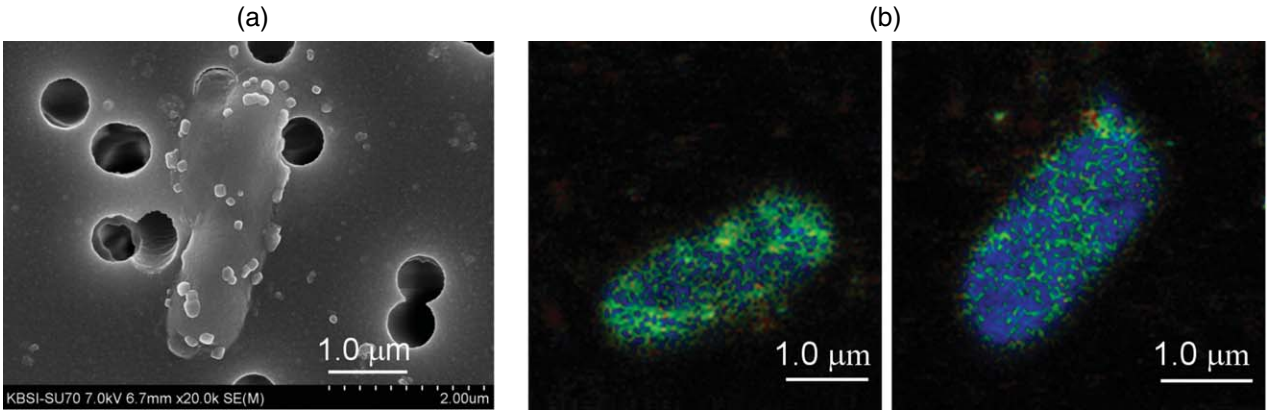

Figure 3. Binding of Ab-HRP-MNPs to *S. typhimurium*. (a) Scanning electron micrograph of an Ab-HRP-MNP-*S. typhimurium* complex in the 0.6-μm PCTE filter. (b) FLIM image of a mixture of *S. typhimurium* and Ab-F-MNP (left) or F-MNP (right). The green colour in the left panel shows Ab-F-MNP bound to bacteria.

with 10% dimethyl sulfoxide (DMSO) solution and drying. Bacteria were retained in the filter with Ab-HRP-MNPs that bound to the bacteria (Figure 3a). Ab-mediated binding of MNPs to *S. typhimurium* was also observed in the aqueous state by fluorescence-lifetime imaging microscopy (FLIM); for this experiment, particles were functionalized with Ab and fluorescein cadaverine instead of HRP. The Ab-conjugated fluorescent MNP (Ab-F-MNP) was added to *S. typhimurium*. Bacteria bound to Ab-F-MNP, but not to MNPs functionalized with fluorescein cadaverine only (F-MNP; Figure 3b).

Efficiency of filtration and the detection system

Efficient removal of unbound Ab-HRP-MNPs in the filtration step is essential for maximizing the sensitivity of this method. A PCTE filter was chosen because of its precise, uniform pore size relative to common web-type filters that have heterogeneous pores and may therefore not remove smaller particles. A filter pore size of 0.6 μm provided a compromise between efficient removal of unbound Ab-HRP-MNPs and retention of Ab-HRP-MNP-bacteria complexes.

Commercial syringe filter holders are typically composed of top and bottom pieces separated by a filter; liquid sample is applied from a syringe connected to the top piece. The filter is removed from the holder after filtration and transferred to a well to which HRP substrate solution is added. This process is cumbersome and error-prone, because filtered bacteria can be lost during the transfer step. We therefore prepared a tube assembled with the bottom piece as a well over the filter (Figure 4b) to which the substrate was directly added after filtration.

A luminometer system suitable for measuring light

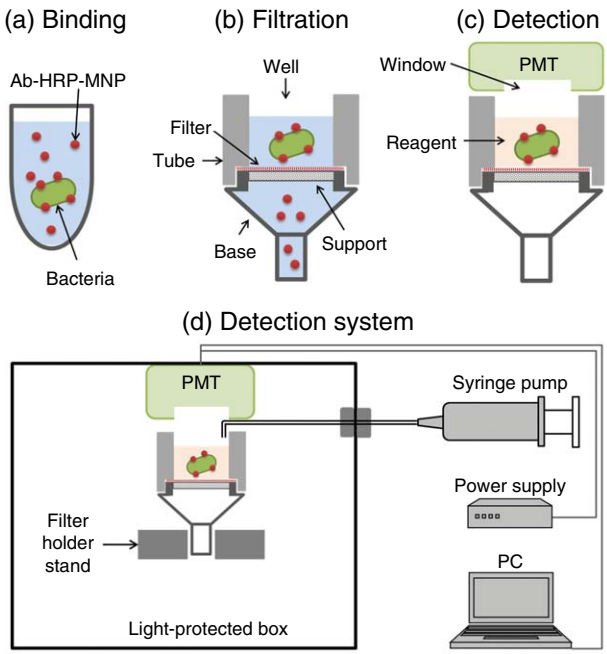

Figure 4. Schematic representation of the detection method and the detection system. (a) Binding of Ab-HRP-MNP to bacteria. (b) Separation of the Ab-HRP-MNP-bacteria complex from unbound Ab-HRP-MNPs. (c) Estimation of HRP activity in the filter by the chemiluminescence method. (d) Whole detection system.

emitted from the homemade filter holder was also constructed. By placing the holder below the photomultiplier tube (PMT) of the luminometer, the well was in close contact with the PMT window (Figure 4c), ensuring maximal capture of photons emitted from the well. The homemade luminometer and filter holder system detected in the order of 10^6 molecules of HRP enzyme,

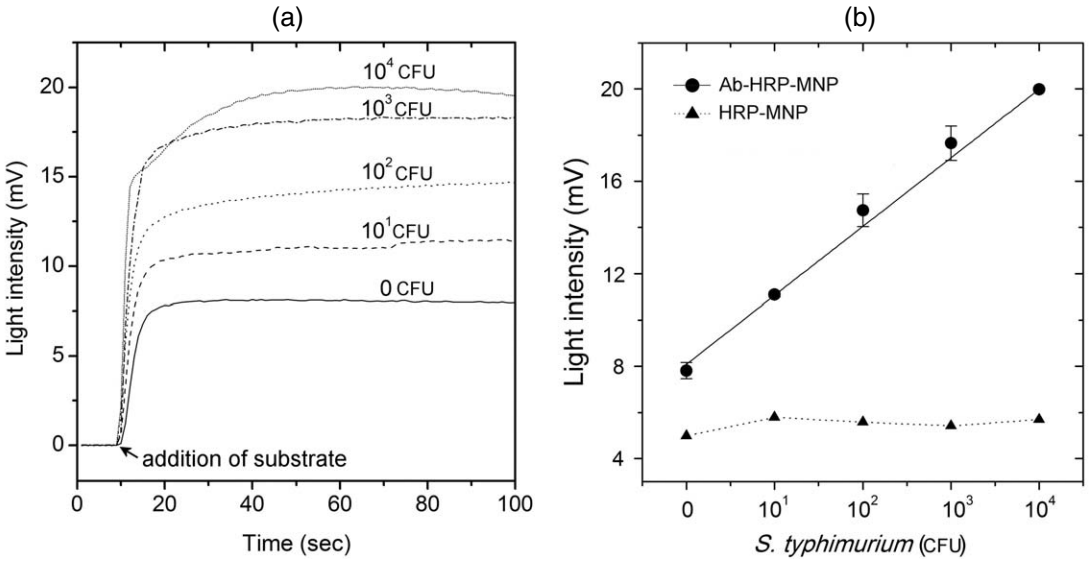

Figure 5. Detection of *S. typhimurium* by Ab-HRP-MNPs. (a) Time-dependent changes in light intensity in samples with different bacterial concentrations. (b) Relationship between light intensity and number of bacteria. Each data point represents the mean of duplicate measurements; error bars represent the standard deviation.

which was comparable to commercial luminometers (data not shown).

Detection of *S. typhimurium*

Samples containing 0, 10^1, 10^2, 10^3, and 10^4 CFU of *S. typhimurium* were prepared in 0.1 mL phosphate-buffered saline (PBS) and reacted with Ab-HRP-MNPs. The mixtures were passed through the filter holder and the HRP activity retained in the filter was estimated using the luminometer. The whole procedure, from filtration to measurement of light intensity, took less than 10 min. The light intensity increased rapidly and reached a plateau within 10 s of injecting the substrate solution (Figure 5a), showing a linear relationship to the logarithm of cell number with a small standard deviation (Figure 5b). This was not observed for MNPs functionalized with HRP only, demonstrating that the dose-dependent increase in light intensity was caused by Ab-mediated binding of particles to bacteria. The background light intensity of the blank sample was higher in the Ab-HRP-MNP than in the HRP-MNP. The difference can be explained by the preferential adsorption of immunoglobulin G proteins to self-assembled monolayer surfaces terminated with methyl or phenyl groups[16]. Nonspecific adsorption of Ab-HRP-MNPs to the PCTE filter would be higher than HRP-MNP because of the interactions between antibody molecules and the polycarbonate filter containing methyl and phenyl groups.

Conclusions

This study describes a simple, highly sensitive method for detecting bacteria, with a lower limit of detection of 10 cells. This method is also cost-effective, as capture and labelling are achieved by a single particle and only one type of Ab, either mono- or polyclonal, is needed. These features make this method suitable for commercial applications, provided that the HRP activity of individual Ab-HRP-MNPs is maintained at an optimal level for the reliable measurement of bacterial numbers and particle aggregation is prevented to avoid falsely positive signals.

Materials and Methods

Materials

MNPs were provided by Dr. Jin-Kyu Lee (Department of Chemistry, Seoul National University, Korea). Transmission electron micrographs were acquired at the Korea Basic Science Institute (KBSI, Gangneung, Korea). *S. typhimurium* (KCCM 11862) was provided by Dr. Il Sik Shin (Department of Marine Food Science and Technology, Gangneung-Wonju National University, Korea). Goat polyclonal anti-*S. typhimurium* Ab was from KPL (Gaithersburg, MD, USA). Other chemicals and proteins were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Lumigen PS-atto, the chemiluminescent substrate solution for

HRP, was from Lumigen (Southfield, MI, USA). The PCTE membrane filter (diameter, 13 mm; pore size, 0.6 μm) was from Millipore (Billerica, MA, USA).

Instruments

We prepared a new filter holder and luminometer for efficient coupling of the filtration and measurement steps. The filter holder was composed of the base piece − a Swinnex syringe filter holder (Millipore) − and an attached homemade tube (Figure 4b) separated by a filter to form a well. The luminometer was composed of PMT (Model H-9656, Hamamatsu Photonics, Hamamatsu, Japan), a stand for the filter holder, power supply, voltmeter, and syringe pump, all installed (except for the pump) in a light-protected box. The filter holder was positioned immediately below the window of the PMT on the luminometer stand (Figure 4c). The chemiluminescent substrate solution was supplied to the well of the filter holder via the syringe pump. Schematic representation of the whole detection system is shown in Figure 4d.

MNP functionalization

MNPs (5 mg in 1 mL ethanol) were chemically activated by adding APTES (0.5 μL) for 7-12 h at room temperature. Particles were washed thrice with 1 mL ethanol, resuspended in 0.9 mL ethanol, and dispersed by sonication for 20 s. Glutaric anhydride (0.1 mL 1 M solution in ethanol) was added for 7-12 h; after three washes with and resuspension in 1 mL ethanol, 1 mg particles was transferred to a new tube, washed once with 1 mL water and twice with 1 mL 0.1 M 2-(N-morpholino)ethanesulfonic acid, pH 6.0 (MES buffer), and resuspended in 0.9 mL MES buffer. EDC and NHS (50 μL 0.2 M solution for each) were added to the MNP and reacted for 30 min. After washing three times with MES buffer and once with PBS, MNPs were resuspended in PBS. Anti-*S. typhimurium* Ab (0.2 mg) and HRP (0.6 mg) were mixed in 0.5 mL PBS and added to 0.5 mL 1 mg/mL activated MNPs. Reactions were carried out at room temperature for 1 h. The resultant Ab-HRP-MNPs were washed 10 times with 1 mL PBS and passed through a PCTE filter. All washing steps were carried out by collecting the MNPs with a magnet.

Functionalized MNP characterization

Ab immobilization was evaluated with an alkaline phosphatase-linked secondary Ab (10 μg) incubated with 0.1 mL 1 mg/mL Ab-HRP-MNP at room temperature for 1 h. After 10 washes with PBS, Ab-HRP-MNPs were reacted with *p*-nitrophenyl phosphate solution (10 mM in 1 M diethanolamine, pH 9.8; 0.5 mM $MgCl_2$) for 5 min at room temperature. Absorbance was measured at 405 nm with a Synergy-HT microplate reader (BioTek, Winooski, VT, USA). HRP activity was estimated from the chemiluminescence reaction of 80 μL luminol (0.1 mM in DMSO), 40 μL 4-iodophenol (1.2 mM in 0.1 M Tris-HCl, pH 8.3; 0.002% Tween-20), and 80 μL of 3 mM H_2O_2 added to Ab-HRP-MNPs (0.1 mg in 20 μL of 0.1 M Tris-HCl, pH 8.3) with light intensity measured using the microplate reader.

Binding of *S. typhimurium* to Ab-HRP-MNPs

Particles (10 μg in 0.1 mL) were incubated with a bacterial sample (10^4 cells in 0.1 mL) at room temperature for 30 min. The mixture was filtered through a PCTE filter encased in a holder. The membrane was washed by successive injection of 3 mL wash buffer (0.05% Tween-20 in PBS) and 1 mL water into the holder. DMSO solution (0.1 mL 10% solution in water) was added to the top of the membrane, which was freeze-dried and visualized by SEM at the KBSI. To visualize MNP binding to *S. typhimurium* in an aqueous state, particles were functionalized with Ab and fluorescein cadaverine (Life Technologies, Carlsbad, CA, USA) by the same method used to prepare Ab-HRP-MNPs; next, they were added to *S. typhimurium* and visualized by FLIM at the KBSI.

S. typhimurium detection

Ab-HRP-MNPs (0.1 mL of 0.1 mg/mL) were dispersed in a bath sonicator for 20 s, added to a bacterial sample in 0.1 mL PBS, and incubated at room temperature for 30 min. The PCTE filter encased in the home-made filter holder was pretreated with 1 mL blocking buffer (0.25% bovine serum albumin in wash buffer). The Ab-HRP-MNP-bacteria mixture was passed through the filter, which was washed with 3 mL wash buffer. After adding 0.1 mL PBS to the top of the filter, the holder was placed in the home-made luminometer and voltage was recorded at 1-s intervals. After the voltage signal had stabilized, 0.1 mL Lumigen PS-atto was injected into the well using the syringe pump at a flow rate of 5 mL/min.

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