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The Simple and Fast Isolation of *Escherichia coli* O157:H7 Using Magnet Nanoparticle Embedded Silica Nanotube for the Nucleic Acid Based Detection

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In this study, we developed a simple and fast isolation tool of *Escherichia coli* O157:H7 (*E. coli* O157:H7) using a magnet nanoparticle embedded silica nanotube (MNSNT) for the detection of *E. coli* O157:H7 in the sample with nucleic acid based amplification. This method does not require chaotropic salt and sophisticated equipment to isolate bacteria. The *E. coli* O157:H7 in the sample was effectively bound to the hydrophilic surface of MNSNT in low pH binding buffer containing divalent ions and PEG without the need for expensive biological reagents such as antibodies. This *E. coli* O157:H7 bound MNSNT was simply isolated by a magnet, prior to adding an amplification mixture to the same micro tube without transferring the sample to another tube. Using this novel method, the detection sensitivities of *E. coli* O157:H7 (10^2 cfu/1 g of seed sprout and 10^2 cfu/5 mL of water) were 80% and 100%, respectively, whereas that was 0% using the commercial method.

KEYWORDS: Bacteria Adsorption, Rt-PCR, *E. coli* O157:H7, Divalent Ions, SNT, Magnet Nano Particle.

INTRODUCTION

Soil is easily contaminated from the sheds of domesticated animals, industrial wastes, and wastewater from humans. It can also easily spread through the transportation of humans and animals, which can lead to water contamination.¹

Soil contains relatively high concentrations of bacteria, normally hundred thousands of bacteria, in the barren and fertilized lands. Normally, soil bacteria at these concentrations do not cause serious illnesses because long term exposure to soil bacteria has boosted the immune system.² However, super bacteria such as M- or V-SRA (Methicillin- or Vancomycin-Resistant *Staphylococcus Aureus*) have recently caused problems in Europe.

It has been reported that a massive and unprecedented outbreak of bacteria may be linked to contaminated vegetables such as cucumbers, lettuces, seed sprouts. Commonly, vegetables such as lettuces, cabbage, and seed sprouts are easily contaminated by any bacteria and is not limited to super bacteria because they grow close to the soil.³ *Listeria Salmonella* and *Escherichia coli* (*E. coli*) are often found on the surface of fruits and vegetables.

These bacteria cause food spoilage and result in food poisoning. Therefore, the detection of pathogenic bacteria from the food and environment is of great importance in order to prevent food poisoning.⁴

Various methods such as bacteria cultivation methods, nucleic based detection methods, and antibody based detection methods have been employed to detect and measure bacteria in environmental samples.⁵ The nucleic acid based detection method is one of the most sensitive methods to detect bacteria because of the development of powerful amplification tools such as PCR (Polymerase Chain

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Reaction). However, this approach requires sample preparation procedures to extract nucleic acids from the cell and concentrate/purify nucleic acids prior to the amplification process.⁶

Various solid state DNA isolation technologies such as CST (Charge Switch Technology) and SPRI (Solid Phase Reversible Immobilization) were developed in the past decades after “boom technology” using chaotropic salt and silica to improve the labor intensive and hazardous liquid based isolation protocol.⁷ These already well established and commercialized sample preparation methods have been optimized as bench top systems in the laboratory using high centrifugal force to wash out the unwanted materials such as cell debris and the high salts used as a NA adsorption-enhancing reagents, which include ethanol, chaotropic salts, or PEG. Despite the development of fully automated detection systems such as GeneXpert® (Cepheid, US), which combine sample preparation, amplification, and detection processes in a single chip or cartridge, a simple and fast procedure to concentrate the target analyte has not yet been developed. An integrated sample concentration step to detect bacteria was developed by our group.⁸ Silica or TEOS micro beads have been utilized to concentrate bacteria from a relatively large volume sample. However, this technology also requires pumps to load the samples in the system.

Nanotechnology is one of the most promising tools for the development of a simple and fast sample preparation method that does not require multiple steps and sophisticated systems for sample preparation because of the increased surface to volume ratio (SVR) of nanostructures.⁹ Moreover, nanostructures such as nanoparticle, nanowire, and nanotube can be multifunctional to include signal generation molecules, magnetic properties and contain a charged surface.¹⁰

In this study, we developed a simple and fast bacteria isolation method using MNSNT (Magnet Nanoparticle embedded Silica Nanotube). Under certain ionic conditions, bacteria in the sample were simply bound on the outside wall of MNSNT, which was collected with a magnet. The bacteria separated with MNSNT were then detected using PCR after heat induced cell lysis without the need for washing and elution steps. In addition, the sample did not need to be transferred between different tubes.

EXPERIMENTAL DETAILS

The Preparation of MNSNT

Hydrothermal reaction was conducted to fabricate Fe_3O_4 nanoparticles. The silica nanotubes still embedded in the Anodic aluminum oxide (AAO) templates were treated with argon plasma etching and refluxed with toluene solution to make the inside surface of the nanotube hydrophobic. The templates were then dipped in the solution of magnetic nanoparticles and refluxed under nitrogen for 30 minutes. The magnetic nanoparticles would slide

into the nanotube and exist the end of the tube according to a previously published protocol.¹¹

Bacteria Isolation with MNSNT

1 g of seed sprouts were prepared and spiked with 10^1 , 10^2 , or 10^3 CFU of *E. coli* O157:H7 (ATCC 43895) since the sprouts

In order to isolate bacteria from the sample, 1 ml of sample was mixed with 6 μm long MNSNT solution (ca. $10^7/50 \mu\text{l}$ of water) in a micro-tube for 5 min at room temperature. Bacteria bound MNSNT was separated from the solution using a magnet.

Direct PCR (Polymer Chain Reaction) with MNSNT

The bacteria bound MNSNT was re-suspended with 20 μl of PCR mixture including primer set specific to *E. coli* O157:H7. Bacteria were lysed for 5 min at 95 °C, prior to the conventional PCR process (35 cycles). All experiments were conducted in quintuplicated.

RESULTS AND DISCUSSION

The Direct Adsorption of Bacteria on the MNSNT

Figure 1 shows the TEM (Transmission Electron Microscope) image of the MNSNT. The length and diameter of the MNSNT were 6 μm and 60 nm, respectively. As shown in the inset of Figure 1, up to a third of the height of the MNSNT (2 μm) was packed with the magnet nanoparticles (MNPs) (15 nm in diameter) and the number of MNPs/SNT was calculated to be 2,000.

Bacteria adsorption on silica surface under acidic conditions was already demonstrated in previous studies.⁸ Bacteria adsorption occurred through non-specific interactions between the bacteria surface and hydrophilic surface of the silica. This method has a great advantage in regards to simplicity and durability when compared to antibody-assisted bacteria capturing systems.

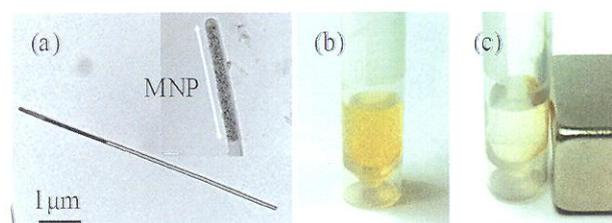


Figure 1. Magnet nanoparticle (15 nm diameter) embedded silica nanotube of 6 μm length and 60 nm diameter; (a) TEM image and (b–c) its separation capability by magnet. purchased from local market were not contaminated with bacteria. The *E. coli* spiked sprouts were soaked and mixed with 5ml of bacteria binding buffer (100 mM glycine, pH 3, 1% PEG, 10 mM MgCl_2) for 5 min.¹² This sprout rinsed solution was then utilized as the sample.

Figure 2 shows that the bacteria could directly bind to the surface of the SNT without the need for antibodies within a few minutes. As shown in Figure 2, the bacteria aggregated on the surface when a relatively high concentration (10^5 cfu/ml) of *E. coli* was applied, whereas no aggregation was observed at low *E. coli* concentration (10^1 cfu/ml) (See the inset). These results indicate that the aggregation phenomenon of *E. coli* enhanced the adsorption of *E. coli* on the hydrophilic surface; thus, the addition of chemicals that promote *E. coli* aggregation may help the bacteria adsorb on the silica surface.

$MgCl_2$ and PEG (Poly Ethylene Glycol) were used to enhance *E. coli* adsorption on SNT because it is known that $MgCl_2$ can promote fusion of bacteria and PEG is often used as a nucleic acid attractor.¹³ Figure 3 shows the effects of PEG and $MgCl_2$ on the *E. coli* adsorption rate on the MNSNT. At low pH, divalent cation, Mg^{2+} , had a positive effect on bacteria adsorption. It is likely that divalent cations could bridge the connection between the negative surfaces of *E. coli* and silica surface because the negative charge on the *E. coli* surface increases at lower pH values. Whereas, under high pH conditions, the cation effect on the bacteria adsorption decreases because the bacteria surface is neutral.⁸

As shown in Figure 3, the adsorption rate of *E. coli* on the MNSNT increased when 1% PEG was applied. The PEG bind to a large number of water molecules and effectively decrease the chemical activity of the water. This process dehydrates the lipid membrane of bacteria and promotes directly binding between the bacteria and surface.¹³ However, a high PEG concentration prevented adsorption of the bacteria on the surface of SNT and inhibited the PCR. Therefore, the optimal bacteria binding buffer was determined to be glycine buffer (100 mM, pH 3) containing 10 mM Mg^{2+} and 1% PEG.

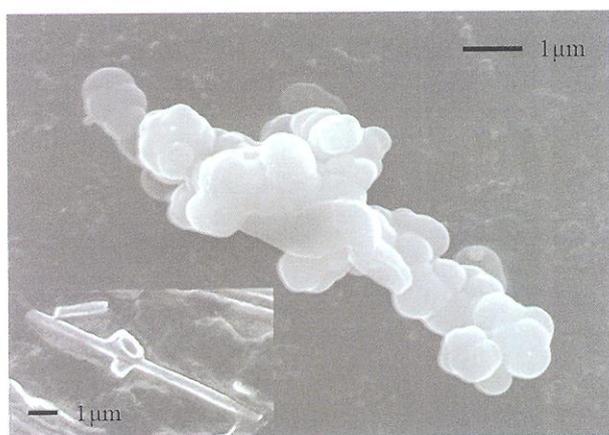


Figure 2. SEM (Scanning Electron Microscope) image of MNSNT bound by high concentration *E. coli* O157:H7 (10^5 cfu/ml applied) (inset: MNSNT bound by low concentration of *E. coli* O157:H7 (10^1 cfu/ml)).

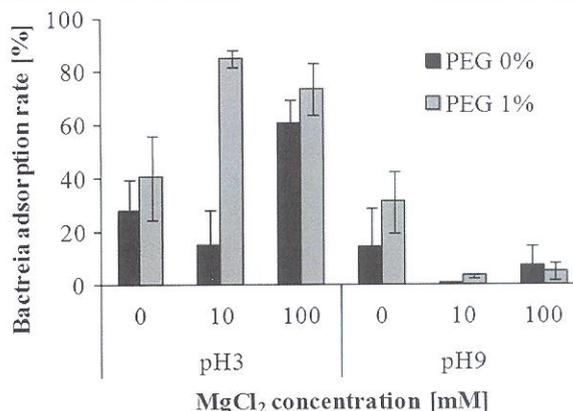


Figure 3. The effect of $MgCl_2$ and PEG on *E. coli* O157:H7 adsorption on MNSNTs under two different pH conditions.

Comparison of MNSNT and MNPs on the *E. coli* Isolation

MNSNT provides two different functions to isolate *E. coli* from the samples, hydrophilic outer surface on which bacteria can adsorb with chemical and physical force and magnet by which *E. coli* bound on MNSNT can be separated from solution. If these two functions could be provided by silica coated MNPs, MNSNT could be replaced by MNPs because the fabrication process of MNSNT is more sophisticated than that of MNPs.

MNSNTs (10^7 /ml) and MNPs (10^8 /ml) were prepared to investigate the ability of using this system to isolate *E. coli* from the sample. Figure 4(a) showed that the efficiency of *E. coli* adsorption with MNSNTs and MNPs decreased as the pH of the binding buffer increased (glycine buffer (10 mM, pH3) and Tris-HCl buffer (10 mM, pH9)). This result directly corresponded with a previous result using micro glass beads.⁸ Interestingly, MNSNTs were 3 fold more efficient than MNPs in regards to isolating *E. coli* at pH 3 (glycine 10 mM) and 9 (Tris-HCl 10 mM) even though the total surface area of MNSNTs and MNPs were same. It is likely that MNSNTs were more stable under these higher ionic conditions than MNPs because MNPs readily aggregate in the presence of 100 mM of $MgCl_2$ (data are not shown), whereas MNSNT, which have a high aspect ratio (100:1), do not aggregate under high salt conditions. This result demonstrates that SNTs, which have a high aspect ratio, are more useful than NPs since most biological solutions contain many salts. Surface modification of NPs with high negatively charged materials such as DNA and protein are often utilized; however, these functionalized NPs are still unstable when salt concentration is dramatically increased.

The MNPs were shown to aggregate when in the presence of $MgCl_2$ (Fig. 4(b)). *E. coli* adsorption on MNSNT increased

when $MgCl_2$ was added in glycine buffer (pH3, 10 mM), whereas adsorption on MNPs decreased with an increase in the $MgCl_2$ concentration. This result demonstrates that

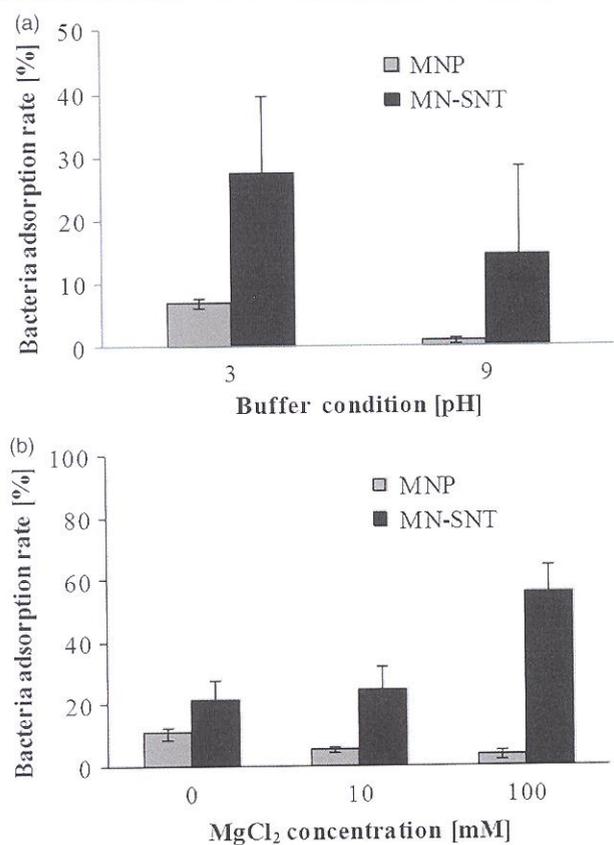


Figure 4. The comparison of MNPs and MNSNTs on the *E. coli* O157:H7 adsorption; (a) pH effect, (b) MgCl₂ effect.

MNSNT, which have a high aspect ratio, have unique characteristics relative to MNPs.

Detection of *E. coli* O157:H7 in Seed Sprouts

MNSNT-assisted detection of *E. coli* was demonstrated using seed sprouts contaminated (spiked) with *E. coli* O157:H7. In these experiments, 1 g of seed sprout (Fig. 5(a)) was soaked in the bacteria binding buffer. The MNSNT was then added to the sprout rinsed solution and mixed by mild vortexing for 5 min. The solution was then removed from the tube and MNSNT bound *E. coli* O157:H7 was remained in the tube using magnet. The MNSNT was then re-suspended in the PCR mixture, prior

(a) 

(b) <i>E. coli</i> [cfu]	Water [5ml]		Sprouts [1g]
	Qiagen	MNSNT	MNSNT
10 ³	2/5	5/5	5/5
10 ²	0/5	5/5	4/5
10 ¹	0/5	3/5	1/5
NC	0/5	0/5	0/5

Figure 5. Detection of *E. coli* O157:H7 in the (a) 1 g of seed sprouts and (b) its detection sensitivity results by PCR amplification.

to the heat lysis at 95 °C, and directly amplified by conventional PCR manual. As shown in Figure 5(b), the detection sensitivities of *E. coli* O157:H7 (10² cfu/1g of seed sprout and 10² cfu/5 mL of water) were 80% and 100% using this simple and fast sample preparation method, respectively. It is likely that unexpected materials, which might inhibit the PCR reaction, existed on the seed sprout samples because they grow on humic acid-rich soil. Therefore, this simple method was compared with a commercial sample preparation kit obtained from boom technology,⁷ which is known as the “gold standard”. As shown in Figure 5(b), the detection sensitivity of commercial kit combined PCR method was only 40% with sample (10³ cfu/5 mL of water), because the volume of elution water is usually 30~60 μl, in which only 2 μl was used for PCR. As a result, the simple and fast method using MNSNT to isolate *E. coli* on the seed sprout was 1~2 fold more sensitive than the “commercial kit” based on PCR result.

CONCLUSION

In this study, we developed an effective sample preparation tool for the detection of pathogens in vegetables samples such as seed sprouts by simply employing MNSNT. This bacteria isolation method does not require chaotropic salt (Guanidinium thiocyanate), and high concentrations of nonspecific salts (PEG, NaOH) and organic solvent (ethanol). This advantage leads to direct bacteria isolation and nucleic acid amplification without the need to transfer the sample to another tube. The main drawbacks associated with this technology are how to isolate the bacteria using a nano-material and how to amplify the mRNA in the tube using a solid substrate that has an enlarged surface. In this study, we isolated bacteria (*E. coli* O157:H7) by optimizing the aqua chemistry with divalent ions, PEG, and pH. In addition, amplification was performed under optimized conditions, which included the addition of BSA.

By conducting these processes in a single tube without the need to transfer the sample to another, no contaminants were present during the process. Thus, this tool holds promise for use in the development of a commercialized real time analysis system to quantify the amount of bacteria in various kinds of samples.

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