Development of automatic and efficient immuno-separator of foodborne pathogenic bacteria using magnetophoresis and magnetic mixing

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Abstract: In recent years, the outbreaks of foodborne diseases caused by pathogenic bacteria have made considerable economic losses and shown a threat to public health. The key to prevent and control these diseases is fast screening of pathogenic bacteria, which is usually performed with three procedures: sample collection, bacteria separation and bacteria detection. For sample collection, the national standard methods are often employed. For bacteria detection, currently available methods such as Polymerase Chain Reaction and Enzyme Linked Immuno-Sorbent Assay are often used. For bacteria separation, traditional methods such as filtration and centrifugation are not capable to specifically separate the target bacteria. However, food samples are very complicated and require efficient pretreatment for bacteria separation and concentration to achieve accurate and reliable results. The conventional immune magnetic separation method can be used to specifically separate the bacteria, but it still cannot meet the requirements for food sample pretreatment due to very low concentration of target bacteria in food. Therefore, this study developed an automatic and efficient immuno-separator of foodborne bacteria based on magnetophoresis and magnetic mixing, and *E. coli* O157:H7 was used as research model. A magnetic mixer was applied to facilitate the immunoreaction between the immune magnetic nanoparticles and the target bacteria cells, and a magnetophoretic separation tubing was utilized for magnetophoretic separation of the magnetic bacteria. Under the optimal mixing time of 20 min and the optimal flow rate of 50 μL/min, the separation efficiency of *E. coli* O157:H7 could be more than 90%, showing that the developed immuno-separator is promising to be applied for efficient separation of foodborne bacteria and can be easily extended for separation of other biological targets by using their specific antibodies.

Keywords: foodborne bacteria, immuno-separator, bacteria separation, magnetophoresis, magnetic mixing, magnetic nanoparticles
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1 Introduction

In recent years, the outbreaks of foodborne diseases caused by pathogenic bacteria have made considerable economic losses and shown threaten to public health. Millions of people were infected every day globally and 230 000 people died of diarrhea diseases every year[1]. The US Center for Disease Control and Prevention (CDC) estimated that about 10-83 billion dollars were lost due to foodborne pathogens every year[2]. According to the statistics of the National Health and Family Planning Commission of China, pathogenic microorganisms, such as *Salmonella typhimurium*, *Bacillus cereus*, *Vibrio parahemolyticus* and *E. coli* O157:H7, were responsible for over 50% of foodborne illnesses in the past decade. The key to prevent and control these foodborne diseases is fast screening of these pathogenic bacteria. Therefore, it is of great importance to develop fast methods to detect the foodborne bacteria.

The procedure for detection of foodborne bacteria often includes sample collection, bacteria separation and bacteria detection. National standard methods are often employed to collect the bacterial sample. Polymerase Chain Reaction (PCR)[3-5] and Enzyme Linked Immuno-Sorbent Assay (ELISA)[6,7] are often used for bacteria detection. For bacteria separation, conventional methods such as filtration[8,9] and centrifugation[10,11] are often used. They are based on the physical parameters such as size or mass of the target bacteria, thus they are not capable to specifically separate the target. Due to the complex food samples with low bacteria concentrations, efficient pretreatments are required for bacteria separation to achieve accurate and reliable results prior to detection. The conventional immune separation method is often applied to specifically separate the bacteria using magnetic nanoparticles (MNPs) modified with the antibodies against the target bacteria to form the bacteria-MNP complexes (magnetic bacteria), followed by capturing these magnetic bacteria with a magnetic field to remove from the sample background and finally re-suspending the purified magnetic bacteria in a smaller volume of PBS solution[12,13]. The concentration of the conventional immune magnetic separation method is generally less than 10 due to its small sample handling capacity (generally less than 1 mL)[14]. At present, the conventional magnetic separation method can only separate very limited amount of bacteria, and

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available sensitive methods for rapid detection of bacteria, such as qPCR, often own a lower detection limit of 10^2 cfu/mL or more. However, many foodborne pathogenic bacteria should not be detectable in foods, indicating that the concentration of the bacteria in foods is required to be 0 cfu/mL. Therefore, efficient and specific separation methods for isolating more target bacteria from a large volume of food sample are required for sensitive detection of foodborne bacteria.

Considerable efforts have been made by scientists worldwide to develop new methods for efficient separation of foodborne bacteria or other biological targets based on the concept of magnetic separation. High gradient magnetic separation (HGMS) is an effective method for separating biological targets from a large volume of sample[15]. It often uses a separation channel filled with small iron balls or fine iron rods to capture the magnetic bacteria from the background under a magnetic field, which are released after the magnetic field is removed and re-suspended in a small volume of buffer solution. Compare to the conventional magnetic separation method, HGMS owns obvious advantages such as large sample handling capacity and short separation time, but it also has a big challenge for practical application since the complexity of food samples may results in the blocking and non-specific binding of the separation channel, and the residual magnetism of the balls or rods may result in only partial releasing of the magnetic bacteria after removing the magnetic field. Immune magnetophoresis, which is a novel method for separating biological targets from a large volume of sample, integrates the concepts of both magnetic separation and electrophoresis[16]. It often uses a magnetic field to attract the magnetic targets, which will flow horizontally at a fixed velocity in a magnetophoretic channel filled with buffer solution in laminar flow regime to yield a vertical shift, and finally achieve continuous-flow separation of the targets. Compared to the conventional magnetic separation and HGMS, it has stronger sample handle capacity, less blocking and increased automation. However, most studies on magnetophoretic separation were combined with Micro Total Analysis System (μTAS) and Micro-Electro-Mechanical System (MEMS) and used for separation of red blood cells, bacterial cells, and tumor cells, very few reports were found to use immune magnetophoresis for separation of foodborne bacteria.

In previous study, the authors have developed a 3D-printed magnetophoretic channel for separating avian influenza virus H5N1 using immune magnetic nanoparticles with the diameter of 150 nm and a gradient magnetic field with the gradient of 32 T/m and a maximum intensity of 0.65 T[17]. Under the optimal conditions, the separation efficiency of H5N1 viruses could reach 88%. However, this magnetophoretic system require a complete immune reaction between the immuno-MNPs and the target viruses to form magnetic viruses, and the magnetophoretic chip with a complex structure has potential leaking problems and needs very precise fluidic control. Therefore, a much simpler magnetophoretic tubing with an active magnetic mixer was investigated and developed for efficient and automatic separation of foodborne bacteria in this study, and *E. coli* O157:H7 was used as research model to evaluate this proposed magnetophoretic system.

2 Materials and methods

2.1 Materials and reagents

Phosphate buffered saline (PBS, 10 mM, pH 7.4) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Sigma Aldrich (St. Louis, MO) and used as buffer solution and carrier flow. Bovine serum albumin (BSA) was purchased from EM Science (Gibbstown, NJ) and used to block the channels and the unbound sites on the magnetic nanoparticles. The biotin labelled polyclonal antibodies against *E. coli* were developed by the State Key Laboratory of Food Science and Technology at Nanchang University and used to specifically react with the *E. coli* cells. Ethanol and isopropyl alcohol were purchased from Sinopharm Chemical (Shanghai, China). The deionized water was produced by Milli-pore Advantage 10 (18.2 MΩ cm, Billerica, MA) for preparation of all the solutions.

2.2 Principle of the immuno-separator

As shown in Figure 1, the bacterial sample was first mixed with the magnetic nanoparticles coated with the antibodies against *E. coli,* using an active magnetic mixer to form the magnetic bacteria. Then, the magnetic bacteria were pumped into the magnetophoretic tubing and the magnetic bacteria were captured against the bottom of the tubing at the presence of a strong magnetic field while the waste flowed out directly. Finally, the magnetic bacteria were flushed out and collected for bacteria test after the tubing was washed with buffer solution.

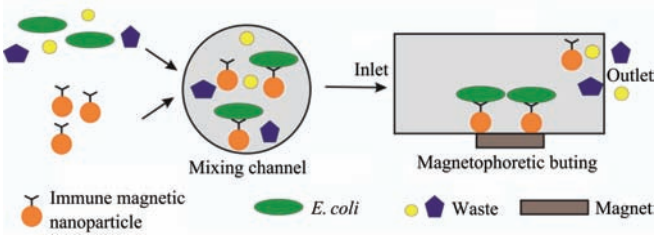


Figure 1 Principle of this proposed immuno-separator of foodborne bacteria

2.3 Culture and enumeration of the bacterial cells

The *Escherichia coli* O157:H7 cells (ATCC 43888) used as model were stored at –20°C with 15% glycerol and were revived by streaking on Luria-Bertani (LB) agar plates. They were firstly cultured in Brain Heart Infusion (BHI) at 37°C for 18-20 h. Then, the cultures were 10-fold diluted with sterile PBS to obtain the bacteria at the concentrations ranging from 10^1 to 10^9 CFU/mL.

For bacterial enumeration, the bacterial samples were gradient diluted with sterile PBS and 100 μL of the diluents were surface plated on the MacConkey agar plates. The plates were then incubated at 37°C for 22-24 h before the colonies were counted. The bacteria were enumerated in colony forming unit per milliliter (CFU/mL).

2.4 Preparation of the magnetic nanoparticles

The monodisperse magnetic nanoparticles (MNPs) with the diameter of 180 nm were functionalized with carboxyl group (~150 μM/g) and stored in deionized water at room temperature. The core of the MNPs is Fe_3O_4 with the Fe content of 10 mg/mL.

The anti-*E. coli* polyclonal antibodies (PAbs) were immobilized on the surface of the MNPs for specific separation of the *E. coli* cells based on previously-reported protocol with small modifications[18]. Briefly, the carboxylated MNPs were successively washed with HCl and deionized water using a magnetic separator prior to use. Firstly, EDC were freshly prepared and used to activate the carboxyl groups. After washing with deionized water to remove surplus EDC, the MNPs were immediately suspended in streptavidin. After the streptavidin modified MNPs were washed with PBS to remove surplus streptavidin, they were blocked with 1% BSA and washed with

PBS. Then, the MNPs were resuspended in the biotinylated PAbs and incubated at 15 r/min for 45 min. After washing with PBS to remove surplus antibodies, the PAb modified MNPs were finally suspended in PBS with 1% BSA at a final concentration of 1 mg/mL (Fe content) and stored at 4°C.

2.5 Fabrication of the magnetic mixer

The magnetic mixer was fabricated based on 3D printing and surface plasma bonding. First, the 3D drawing in .stl format was designed using SOLIDWORKS (Concord, MA, USA) and the mold was printed by the Objet30 3D printer (Stratasys, Eden Prairie, MN) with the Vero Whiteplus photosensitive resin. After the mold was immersed in 5% NaOH for 30 min to thoroughly remove the surplus support material, the silicone elastomer kit (SYLGARD 184, Dow Corning, Auburn, MI, US) was then used with the pre-polymer-to-curing agent ratio of 10:1 to obtain the PDMS channel. The PDMS channel and the PMMA slide were successively immersed in isopropyl alcohol for 10 min and absolute ethyl alcohol for 2 min, and then treated by surface plasma (Harrick Plasma, Ithaca, NY, US) for 50 s and finally bonded to form the on-chip mixer.

For the magnetic mixer, the cylinder chamber for magnetic mixing of the immuno-MNPs and the bacterial cells was designed with the diameter of 10 mm and the height of 4 mm, and a magnetic stirrer with the diameter of 3 mm and the length of 4.5 mm was embedded inside for the mixing of the MNPs and the bacteria using a DC motor to rotate a magnet.

2.6 Development of the immune separator

The immune separator shown in Figure 2 consisted of a magnetic mixer with a rotary magnet for rapid reaction between the immuno-MNPs and the bacterial cells, a magnetophoretic tubing with a separating magnet for efficient separation of the continuous-flow magnetic bacterial cells, and a precise peristaltic pump for injecting the mixture of the immuno-MNPs and the bacterial cells and the washing solutions. The rotary magnetic field was generated by applying a voltage of 6 VDC on the DC motor to rotate the rotary magnet (NdFeB, Grade N45, 3 mm× 3 mm×10 mm) at a speed of approximate 1200 rpm. The magnetophoretic tubing was tightly adhere in parallel with the cuboid separating magnet (NdFeB, Grade N45, 5 mm×10 mm× 20 mm). The precise peristaltic pump was automatically operated using a micro controller to inject the solutions at the preset flow rate.

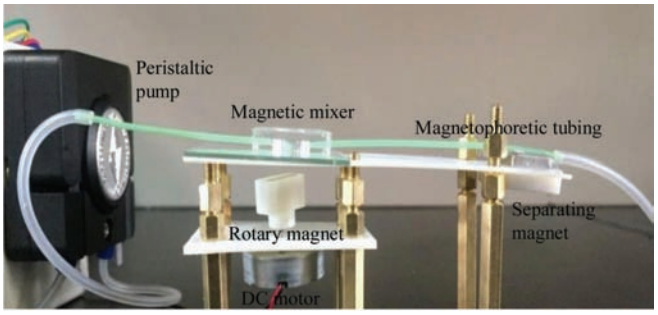


Figure 2 The proposed immuno-separator

2.7 Separation of the target bacteria

Prior to use, the magnetic mixer and the magnetophoretic tubing were blocked using 1% BSA for 30 min and washed with PBS to minimize the non-specific adsorption. 40 μL of the immuno-MNPs were magnetically separated to remove the background and then re-suspended with 400 μL of *E. coli* O157:H7 at the concentration of 1×10^4 cfu/mL. After vortexing, the mixture of the immuno-MNPs and the bacteria was equally divided into two parts, one of which was immediately pumped into the magnetic mixer for different time ranging from 10 min to 30 min and the other one was mixed using the conventional rotator for the same time as control. Then, the mixture, containing the magnetic bacteria and the surplus immuno-MNPs was pumped into the magnetophoretic tubing with different flow rates ranging from 50 μL/min to 150 μL/min. Both the magnetic bacteria and the immuno-MNPs were magnetophoretically separated and captured against the wall of the tubing at the presence of the separating magnet. After washing with PBS, the separating magnet was removed and the magnetic bacteria were then flushed out from the tubing with 100 μL of PBS. Besides, the control was treated using conventional magnetic separation method. Finally, the obtained magnetic bacteria using this proposed immuno-separator and the conventional magnetic separation method were both plated on MacConkey agar to obtain their concentrations for calculating the separation efficiency.

3 Result and discussion

3.1 Simulation of the magnetic mixing

The magnetic mixer plays an important role in this proposed immuno-separator since it has a great impact on the formation of the magnetic bacteria, which is the precondition of the bacteria separation. Since active mixing has often shown a better performance in limited space and time, a magnetic mixer was used to achieve active mixing. Moreover, due to the small size of the MNPs (around 180 nm), the magnetic force on the MNPs was much smaller than the drag force and thus the moving of the MNPs was dominated by the magnetic mixer.

To study the efficiency of the magnetic mixing, a 2D model of the magnetic mixer was simulated using COMSOL Multiphysics, and the RANS turbulent model and the *k*-ε model were used to simulate the magnetic mixing process. For better understanding of the mixing efficiency, the mixing index (M) was used to characterize the mixing efficiency and could be expressed as[19]:

$$M = \sqrt{\frac{1}{N}\sum_{i=0}^{i=N}(C_i - \bar{C})^2} \quad (1)$$

where, N is the total number of the elements; C_i is the concentration of the i-th element; and $\bar{C}$ is the mean concentration of all the elements.

As shown in Figure 3a, all the immuno-MNPs are assumed to be located at one point in the mixer and the bacterial cells are randomly distributed in the mixer before they are magnetically mixed. When the magnetic mixing time increases from 0 s to 1.5 s, the immuno-MNPs are diffused to more and more areas, and the mixing index decreases from 0.5 to 0, indicating that the immuno-MNPs and the bacterial cells are fully mixed when the mixing time is 1.5 s.

3.2 Verification of the magnetic mixer

Based on the simulation of the immuno-MNPs and the bacteria cells, they could be fully mixed in a very short time, however it still needs time for the antibody-antigen immuno-reaction. Therefore, three different time (10 min, 20 min and 30 min) were tested for the optimization of magnetic mixing. The separation efficiency (SE) is calculated by dividing the concentration of the separated magnetic bacteria by the concentration of the original bacteria, and used to evaluate the magnetic mixer.

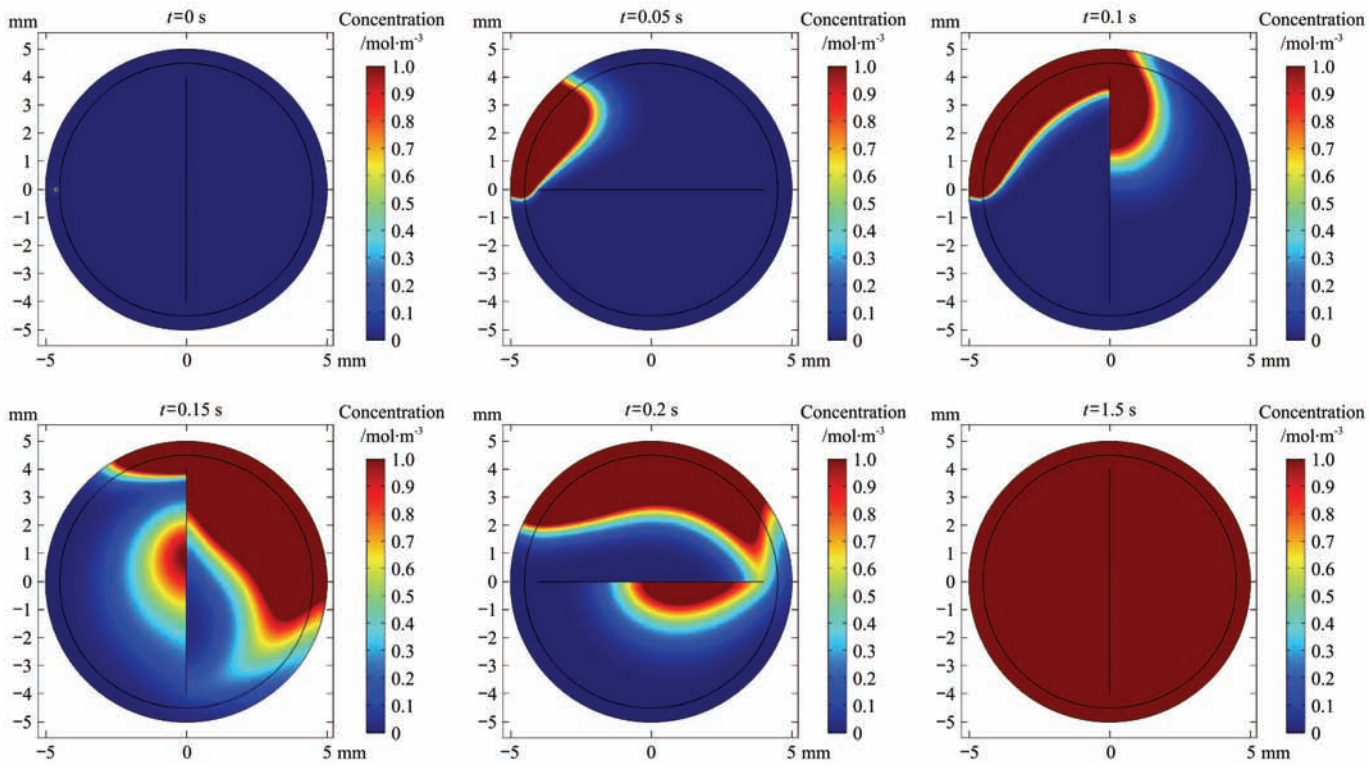


Figure 3 The concentration of bacteria cells in the magnetic mixer at different time

As shown in Figure 4, when the mixing time increases from 10 min to 30 min, the separation efficiency using the conventional rotator (in orange) and the proposed magnetic mixer (in blue) increases from 69.9% to 91.5% and from 87.6% to 94.8%, respectively. This might be attributed to the continuous magnetic mixing since it makes both the immuno-MNPs and the *E. coli* cells distribute more homogenously in the mixture and create more opportunities to react with each other, resulting in higher capture efficiency of the immuno-MNPs. Meanwhile, although the advantage is smaller with longer mixing time, the magnetic mixer has shown much higher separation efficiency. Compared to the reported conventional methods with the mixing time of at least 30 min for obtaining the separation efficiency of more than 90%[20,21], the separation efficiency of the magnetic mixer can reach more than 90% and remain the same level when the mixing time is more than 20 min. Therefore, the optimal magnetic mixing time of 20 min was used for the proposed immuno-separator.

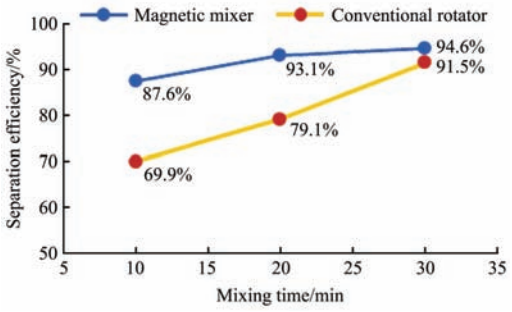


Figure 4 The separation efficiency for the conventional rotator and the magnetic mixer with different mixing time

3.3 Simulation of the magnetophoretic separation

During the magnetophoretic separation, there are gravity force, drag force, magnetic force, buoyancy force and inertia force acted on the MNPs[22]. The magnetic force (F_m) and the drag force (F_d) are much larger than the other forces, and they are considered to balance immediately[23], i.e.

$$F_d = F_m \tag{2}$$

Based on the Stokes' Law, the drag force for a MNP in a laminar fluid can be expressed as:

$$F_d = 3\pi\eta d v_m \tag{3}$$

where, η is the viscosity of the fluid; d and v_m are the diameter and the velocity of the MNP. The magnetic force on the MNP can be expressed as[24]:

$$F_m = mM\nabla B \tag{4}$$

where, m is the mass of the MNP; M is the magnetization of the MNP; and B is the intensity of the magnetic field. Therefore, the velocity of the magnetic nanoparticle can be expressed as:

$$v_m = \frac{mM\nabla B}{3\pi\eta d} \tag{5}$$

Because one bacteria cell can often react with multiple immuno-MNPs in this study, the velocity (v_{mb}) of the magnetic bacteria could be expressed as:

$$v_{mb} = \frac{NmM\nabla B}{3\pi\eta d} = v_f \tag{6}$$

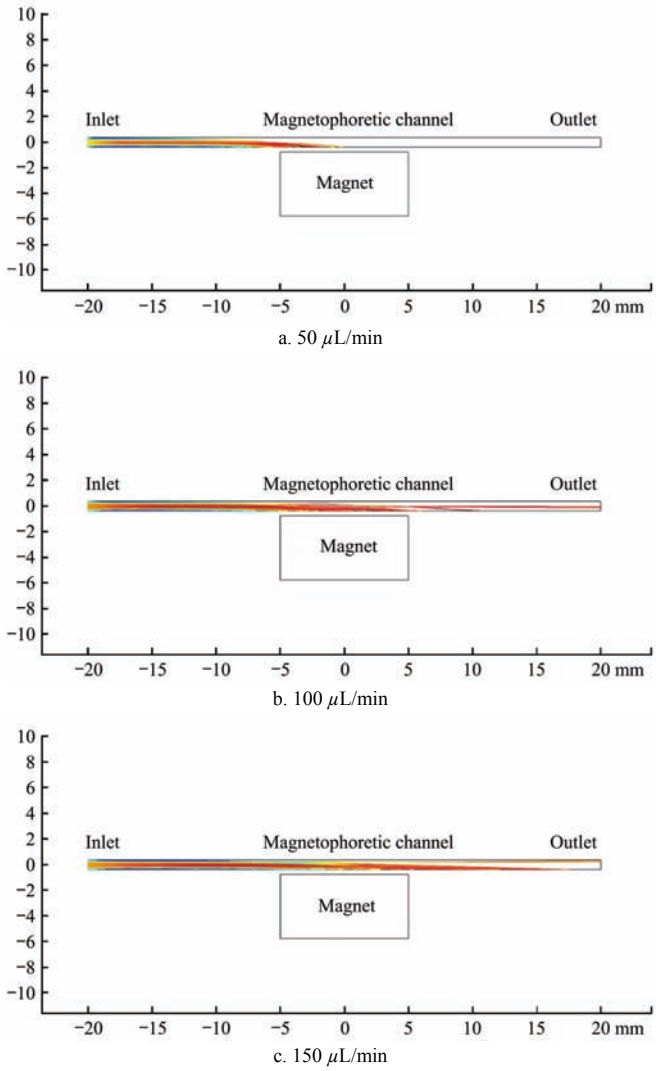
where, N is the number of the immuno-MNPs bound with one bacterial cell; and v_f is the velocity of the fluid. Besides, the *E. coli* cell is in a rod shape with a diameter of ~0.5 μm and a length of ~2 μm, its equivalent diameter could be expressed as[24]:

$$d = \frac{L}{\lambda}\sqrt{\frac{3}{2}\ln(2\lambda)} = 1.3\ \mu\text{m} \tag{7}$$

where, L is the length of the bacterium; and λ is the length to diameter ratio of the bacterium.

Based on the dynamic motion model of the magnetic bacteria, i.e. Equation (6), COMSOL was used to simulate the trajectory of the magnetic bacteria in the magnetophoretic tubing and optimize the flow rate of the PBS carrier. As shown in Figure 5, three different flow rates of 50 μL/min, 100 μL/min and 150 μL/min were used for simulation. When the flow rate is 50 μL/min, almost all the magnetic bacteria can reach the bottom of the tubing and probably be captured during the magnetophoretic separation at

the presence of the separating magnet. However, when the flow rate is 100 μL/min, parts of the magnetic bacteria cannot reach the bottom of the tubing before they flow through the magnetophoretic separation region, resulting in loss of some magnetic bacteria. When the flow rate increases to 150 μL/min, more magnetic bacteria are lost during the separation.



a. 50 μL/min

b. 100 μL/min

c. 150 μL/min

Figure 5 Simulation of the trajectory of the magnetic bacteria at different flow rates of the PBS carrier

3.4 Verification of the magnetophoretic tubing

To verify the simulation of magnetophoretic separation of the magnetic bacteria, three different flow rates (50 μL/min, 100 μL/min and 150 μL/min) were used for the optimization of carrier flow rate. The separation efficiency (SE) is used to evaluate this proposed magnetophoretic separation as well. As shown in Figure 6, the separation efficiency of the magnetic bacteria using the magnetophoretic tubing decreases from 95.7% to 44.9% when the flow rate changes from 50 μL/min to 150 μL/min. Therefore, the optimal flow rate of 50 μL/min was used for the proposed immuno-separator.

Compared to the reported magnetophoretic separator[24] which was the most similar separator with this developed one, the flow rate of the carrier in this immuno-separator is much higher, indicating that it can process the sample more rapidly. The magnetophoretic tubing applied in this study is much smaller than the reported magnetophoretic channel, which could be easier to attract and capture the magnetic bacteria using the high gradient magnetic field.

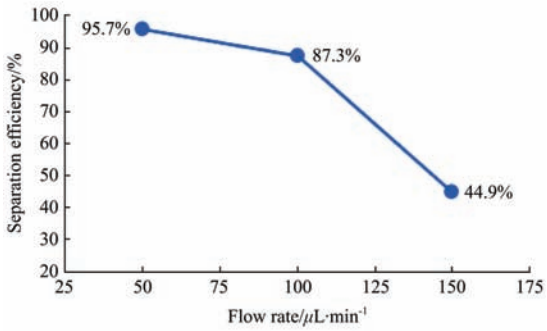


Figure 6 Separation efficiency of the magnetic bacteria with different flow rates

3.5 Separation of the target bacteria cells

To further evaluate the separation efficiency of the proposed immuno-separator, different concentrations of *E. coli* O157:H7 ranging from 10^3 cfu/mL to 10^5 cfu/mL were used with culture plate for bacteria enumeration under the optimal magnetic mixing time of 20 min and the optimal flow rate of 50 μL/min. Lower concentrations of the bacteria were not tested in this research because this study mainly discussed about the feasibility of the novel device and the space for mixing was limited. Separating the bacteria in the food matrix with lower concentrations would be continued in future work. As shown in Figure 7, the separation efficiency of *E. coli* O157:H7 almost remained at the same level (90%-95%), indicating that this proposed immuno-separator achieved a high separation efficiency and could be used for separation of different concentrations of *E. coli* with the same protocol.

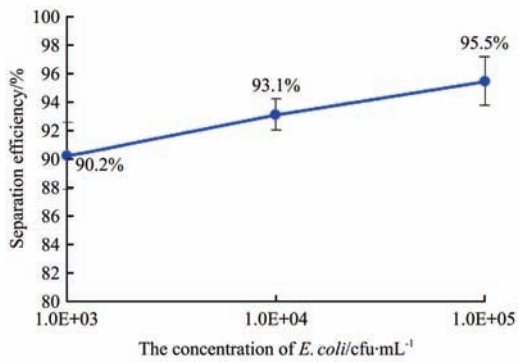


Figure 7 Separation efficiency of magnetic bacteria with different concentrations

Compared to the reported magnetophoretic device for separation of *E. coli* O157:H7[24], this developed immuno-separator has a comparable separation efficiency (93% for this developed separator vs. 97% for the reported separator), a faster sample separation speed (50 μL/min for this developed separator vs. 14.4 μL/min for the reported separator), and a much simpler setup (a common tubing and a small magnet for this developed separator vs. a self-designed flow chip and two big magnets for the reported separator). Compared to the conventional bacteria magnetic separation methods[20,21], this developed immuno-separator is able to achieve an automatic and efficient separation of the *E. coli* cells, which could greatly reduce the requirements of the well-trained technicians. Besides, compared with other microfluidic magnetophoretic systems[25,26], this proposed immuno-separator is featured with higher sample handling capacity and easier operation.

4 Conclusions

In this study, an automatic and efficient immuno-separator was

successfully developed and demonstrated to be capable of separating the target *E. coli* O157:H7 cells based on active magnetic mixing and magnetophoretic separation. Under the optimal magnetic mixing time of 20 min and the optimal carrier flow rate of 50 μL/min, the separation efficiency of the target bacteria was up to 95.7%.

The merit of this proposed immuno-separator is easily extended for specific separation of other foodborne pathogenic bacteria or biological targets by replacing the antibodies against *E. coli* with the antibodies or biological recognition elements against the targets. Besides, the proposed method holds the great potential to separate foodborne pathogenic bacteria in a large volume within a shorter time and an automatic operation, and is able to integrate with downstream detection methods to develop a sensitive, low-cost and automatic device for rapid screening of foodborne bacteria. More importantly, this immuno-separator can also be cooperated with either conventional culture methods or some rapid detection methods, such as biosensors[27] or PCR[28] for more sensitive detection of foodborne pathogens.

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