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• *BASIC RESEARCH* •

Preparation of immunomagnetic iron-dextran nanoparticles and application in rapid isolation of *E.coli* O157:H7 from foods

Hui-Li Duan, Zhi-Qiang Shen, Xin-Wei Wang, Fu-Huan Chao, Jun-Wen Li

Hui-Li Duan, Zhi-Qiang Shen, Xin-Wei Wang, Fu-Huan Chao, Jun-Wen Li, Department of Environment and Health, Institute of Hygiene and Environmental Medicine, Tianjin 300050, China
Supported by the National High-technology Research and Development Program of China (863 Program), No. 2003AA302260
Correspondence to: Jun-Wen Li, Institute of Hygiene and Environmental Medicine, 1 Dali Road, Tianjin 300050, China. junwenli@eyou.com
Telephone: +86-22-84655418 Fax: +86-22-23328809
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Abstract

AIM: To prepare a kind of magnetic iron-dextran nanoparticles that was coated with anti-*E.coli* O157:H7 IgG, analyze its application conditions, and try to use it to isolate *E.coli* O157:H7 from foods.

METHODS: Magnetic iron-dextran nanoparticles were prepared by the reaction of a mixture of ferric and ferrous ions with dextran polymers under alkaline conditions. The particles were coated with antiserum against *E.coli* O157:H7 by the periodate oxidation-borohydride reduction procedure. The oxidation time, amount of antibody coating the particles, amount of nanoparticles, incubation time and isolation time were varied to determine their effects on recovery of the organisms. Finally, the optimum conditions for isolating *E.coli* O157:H7 from food samples were established.

RESULTS: *E.coli* O157:H7 can be isolated from samples within 15 min with the sensitivity of 10^1 CFU/mL or even less. In the presence of 10^8 CFU/mL of other organisms, the sensitivity is 10^1-10^2 CFU/mL. Nonspecific binding of other bacteria to the particles was not observed. Two and a half hours of enrichment is enough for the particles to detect the target from the food samples inoculated with 1 CFU/g.

CONCLUSION: Isolation of target bacteria by immunomagnetic nanoparticles is an efficient method with high sensitivity and specificity. The technique is so simple that it can be operated in lab and field even by untrained personnel.

Key words: Magnetic iron; Dextran; Immunomagnetic nanoparticles; Isolation; *E.coli* O157:H7

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INTRODUCTION

The enterohemorrhagic *E.coli* (*EHEC*) O157:H7 is recognized as an important human pathogen. The outbreaks of *EHEC* infections have been attributed to a variety of sources, mainly raw meat and drinking water. Illnesses caused by *E.coli* O157:H7 can range from self-limited watery diarrhea to life-threatening manifestations such as hemolytic uremic syndrome or thrombotic thrombocytopenic purpura. The lack of efficient selective enrichment medium for this particular strain of *E.coli* reduces the sensitivity and specificity of the conventional cultural method. However, the technique immunomagnetic separation (IMS) presents a physical selective enrichment procedure. When magnetic particles are covalently coupled with antibody specific to *E.coli* O157:H7, it is possible to easily achieve the aim of rapid isolation of the target organisms. The objective of the research was to prepare a kind of immunomagnetic nanoparticles, analyze its application conditions and try to use it to isolate *E.coli* O157:H7 from foods.

MATERIALS AND METHODS

Materials

The polymer sample used in this study was commercial dextran with a molecular weight of 40 000 (T-40) obtained from Pharmacia, Uppsala, Sweden. Sephacryl S-300 gel was available from Sigma Corporation. Ferric chloride hexahydrate, ferrous chloride tetrahydrate and other chemicals were from local suppliers and were of analytical grade. All the aqueous solutions were prepared by distilled water and filtered through 0.22-μm membrane.

Anti-O157:H7-specific antibody was prepared and purified in our laboratory.

Fifty-nine bacterial strains were used in this experiment. *E.coli* O157:H7 (strain 882364) was obtained from Institute of Epidemiology and Microbiology, Academy of Preventive Medical Sciences of China. Nineteen strains of other serotypes of *E.coli*, 6 strains of *S.aureus*, 16 strains of *Salmonella*, 14 strains of *Shigella* and 3 strains of *Y.enterocolitica* were all supplied by China Center for Type Culture Collection (CCTCC).

Synthesis of magnetic iron-dextran nanoparticles

Ferromagnetic iron-dextran particles were prepared by the

reaction of a mixture of ferric and ferrous ions with dextran polymers under alkaline conditions[1-4]. Routinely, under N_2 purging, 20 mL of 500 g/L dextran T-40 in distilled water was mixed with the iron solution containing 16 mL of 0.7 mol/L $FeCl_3 \cdot 6H_2O$ and 4 mL of 1.6 mol/L $FeCl_2 \cdot 4H_2O$, and kept at 60 ℃ for 15 min. With vigorous stirring, 40 mL of 5 mol/L ammonia solution was then added dropwise to the iron-polymer mixture, keeping at 60 ℃ for a further 15 min, with N_2 protecting from oxidation. The resulting suspension was neutralized with acetic acid. The aggregates in the outcome were removed by centrifugation at 600 r/min for 15 min. Unbound dextran was separated from iron-dextran nanoparticles by gel filtration chromatography on a Sephacryl S-300 column, which was equilibrated with 0.01 mol/L phosphate buffer at pH 7.4[5-7]. Particles collected had a concentration of 8 mg/mL as determined by dry weight analysis.

Oxidation of dextran

In order to couple the particles with antibody, oxidation of dextran must be under a mild condition, generally using $NaIO_4$ as the oxidant with a final concentration of about 5 mmol/L[8]. In this experiment, 0.25 mL of 25 mmol/L $NaIO_4$ was used to oxidize 1 mL of Fe_3O_4-dextran nanoparticles. The reaction was kept away from light and oxygen, with a regular rotation of 150 r/min for a period of time. After that, 0.2 mL of 2 mol/L ethylene glycol was added and rotated for another half an hour to terminate oxidation. The excess periodate was removed by dialyzing the suspension for 24 h against 0.01 mol/L phosphate-buffered saline (PBS) at 4 ℃.

Preparation of Fe_3O_4-dextran-antibody conjugates

After oxidation, part of hydroxyl groups had become aldehyde group, which displayed strong interactions with the amino groups of various compounds to form Schiff's bases[9-11]. A different amount of antibody was added to the particle suspension, mixed thoroughly, and placed in dark at 4 ℃ for 8 h. Reduction with 0.5 mol/L $NaBH_4$ for 30 min would contribute to form stable configuration. Uncoupled antibody was separated from the conjugates also by gel filtration chromatography on Sephacryl S-300 column as above.

Isolation and detection of E.coli O157:H7

E.coli O157:H7 suspension that was enriched in broth for 18 h followed by being diluted to 10^2 CFU/mL was chosen as the sample. Certain amount of magnetic nanoparticles were dispensed into 1.5 mL eppendorf tubes, and then 1 mL of the samples was added[12,13]. The tubes were closed and inverted several times. The samples were incubated for some time at room temperature, and rotation of about 20 r/min might bring the samples and the particles into closer contact. The particles were separated by placing a magnetic plate to the side wall of the tubes and the sample was kept for some time to concentrate the particles into a pellet on the side of the tubes. The tubes were opened carefully, aspirated and the sample supernatant as well as the remaining liquid in the cap was discarded. The magnetic plate was removed and 1 mL washing buffer (0.01 mol/L PBS pH 7.4 with the addition of 0.05% Tween-20) was added to remove nonspecific attachment. The particles were then separated again. As there existed the possibility that several bacteria could attach to one particle simultaneously and appear as one colony on the plate, the particle-bacteria complex in 50 μL of papain-PBS buffer (concentration of papain was 1.08 mg/mL) was resuspended and placed at 4 ℃ for 24 h, which helped antigen detach from antibody[14]. The suspension containing bacteria was transferred onto plates restrictive for *E.coli* O157:H7 (SMAC) to determine CFU (colony forming unit) by plate counting after an 18-h incubation at 37 ℃. At the same time, the initial number of the bacteria in 1 mL samples was counted by standard procedure. Recovery could be obtained from their ratio. For further confirmation of the isolated micro-organism, biochemical or molecular biological method can be used regularly[15-20].

RESULTS

Properties of magnetic iron-dextran nanoparticles

Magnetic iron-dextran nanoparticles were black suspension. They were roughly spherical in shape with a diameter ranging from 40 to 60 nm when observed under laser scattering system, and contained an electron dense core of 5 nm which enabled them to be seen by transmission electron microscopy (Figure 1). After purification, particle suspension with a concentration of 8 mg/mL was obtained. They were stable in physiological buffers and suspension over a pH range of 4-10 was maintained. The particles could be aggregated in the presence of a magnetic plate, and had no magnetic moment in the absence of magnetostatic field, that is, they possessed superparamagnetic properties.

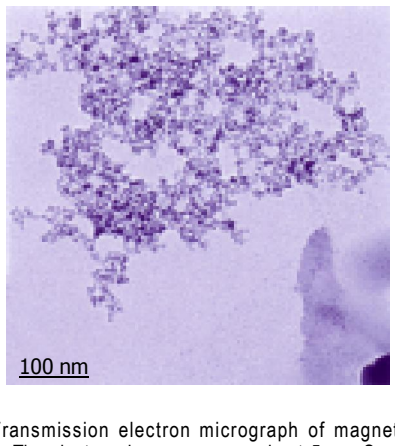

Figure 1 Transmission electron micrograph of magnetic iron-dextran nanoparticles. The electron dense core was about 5 nm. Scale bar = 100 nm.

Determination of oxidation time

Oxidation time is one of the important factors to determine the efficiency of linkage of antibody. In this paper, the nanoparticles were oxidized for 0.5-12 h (i.e., 0.5, 1, 2, 4, 6, 8, 10, 12 h), then connected with enough antibody (surplus antibody was removed by filtration). Now the particles could be used for detection (added enough particles into the sample, incubated for 30 min and magnetically separated for 5 min). Proper oxidation time could be determined by

comparison of the recovery (Table 1).

The results indicated that relatively high recovery could be obtained when the particles were oxidized for 4-12 h, with the concentration of particles of 8 mg/mL, and oxidation for 6 h could recover the most colonies.

Table 1 Effect of oxidation time on recovery (n = 5, mean±SD)

Oxidation time (h)	Colony forming unit (CFU/mL)	Recovery[1] (%)
0.5	9.60±1.82	9.80±1.85
1.0	17.80±2.68	18.16±2.74
2.0	26.60±3.78	27.14±3.86
4.0	61.60±6.47	62.86±6.60
6.0[b]	89.60±4.93	91.43±5.03
8.0	70.20±5.76	71.63±5.88
10.0	65.00±5.39	66.33±5.50
12.0	60.20±4.32	62.24±4.41

[b]P<0.01 *vs* other oxidation time. [1]There were 98 CFU/mL in the dilution (mean of five samples).

Determination of amount of antibody

When the particles were oxidized for 6 h, different amount of antibody (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 μg immunoglobulin per mg of magnetic nanoparticles) were added into the particle suspension (this time the excess antibody was not removed). The proper amount of antibody was defined also by recovery (Table 2). It could be seen from the results that the recovery increased followed by decrease, and had the highest recovery at the antibody of 25 μg/mg nanoparticles.

Table 2 Effect of amount of antibody on recovery (n = 5, mean±SD)

Amount of antibody (μg/mg particles)	Colony forming unit (CFU/mL)	Recovery[1] (%)
5	32.20±5.54	33.54±5.77
10	48.00±5.34	50.00±5.56
15	49.20±7.36	51.25±7.67
20	68.80±4.09	71.67±4.26
25[b]	89.60±4.16	93.33±4.33
30	75.00±6.75	78.13±7.03
35	64.80±4.87	67.50±5.07
40	54.00±4.64	56.25±4.83
45	47.80±6.06	49.79±6.31
50	33.80±6.83	35.21±7.12

[b]P<0.01 *vs* other amount of antibody. [1]There were 96 CFU/mL in the dilution (mean of five samples).

Table 3 Effect of amount of nanoparticles on recovery (n = 5, mean±SD)

Amount of nanoparticles (μL)	Colony forming unit (CFU/mL)	Recovery[1] (%)
5	88.60±4.93	92.29±5.13
10	87.60±4.04	91.25±4.21
15	88.20±6.06	91.88±6.31
20	88.40±4.04	92.08±4.21
25	90.20±4.32	93.96±4.50
30	89.00±5.61	92.71±5.85

[1]There were 96 CFU/mL in the dilution (mean of five samples).

Determination of amount of nanoparticles

Five, 10, 15, 20, 25, 30 μL of particle suspension was added into 1 mL samples separately, and then incubated for 30 min followed by 5 min of magnetic separation. The recovery showed that almost the same results could be gained though the amount of particles added was different (Table 3).

Determination of incubation time

Five microliters of particles was added to 1 mL samples, and the mixture was incubated for 5, 10, 15, 20, 30, 40, 50, 60 min. In order to detect the targeted bacteria as fast as possible, 10 min of incubation time was enough to obtain relatively high recovery (Table 4).

Table 4 Effect of incubation time on recovery (n = 5, mean±SD)

Incubation time (min)	Colony forming unit (CFU/mL)	Recovery[1] (%)
5	45.80±5.63	41.26±5.07
10[b]	96.80±7.26	87.21±6.54
15	101.40±9.94	91.35±8.95
20	98.00±8.37	88.29±7.54
30	98.40±8.05	88.65±7.25
40	101.00±7.14	90.99±6.43
50	99.80±8.61	89.91±7.76
60	98.80±7.29	89.01±6.57

[b]P<0.01 *vs* 5 min of incubation time. [1]There were 111 CFU/mL in the dilution (mean of five samples).

Magnetic separation time

The samples were magnetically separated for 1, 2, 3, 4, 5 min (Table 5) adopting the above results from the other factors. Two minutes of separation could draw almost all the particle-bacteria complex to the side wall of the tube, when 1.5 mL eppendorf tube was used and the intensity of magnetic field was 0.23 T.

Table 5 Effect of magnetic separation time on recovery (n = 5, mean±SD)

Magnetic separation time (min)	Colony forming unit (CFU/mL)	Recovery[1] (%)
1	40.80±6.91	42.50±7.19
2[b]	88.20±6.30	91.88±6.56
3	90.00±5.70	93.75±5.94
4	91.00±6.89	94.79±7.18
5	90.40±5.59	94.17±5.83

[b]P<0.01 *vs* 1 min of magnetic separation time. [1]There were 96 CFU/mL in the dilution (mean of five samples).

Sensitivity

From the above figures, we could see that the recovery remained at the level of more than 90% when the bacteria suspension was diluted to 10^2 CFU/mL in water. Further experiments tested that even if there were only 10 bacteria in the samples, the particles still could capture 7-8 organisms. The sensitivity was 10^1-10^2 CFU/mL in the presence of 10^8 CFU/mL of other organisms.

Specificity

Five microliters of immunomagnetic nanoparticles (specific to *E.coli* O157:H7) with 1 mL of other bacteria suspension was mixed separately, which included other serotypes of *E.coli*, *S.aureus*, *Salmonella*, *Shigella*, and *Y.enterocolitica*. After magnetic separation, the particle-bacteria complex was transferred onto selective plates specific to different strains. As a result, colony of other serotypes of *E.coli* were not found on the SMAC plates, and at the same time, there was no bacterial growth on their own selective plates. This indicated that the magnetic nanoparticles had high specificity and no cross reactivity and nonspecific binding were observed.

Food sample preparation and immunomagnetic separation

We fetched some foods such as ham, liver, ground beef, rice, steamed bun and milk from local supermarkets. Food samples were prepared according to the reports of some documents[21,22]. A 1-g food samples were added to 0.1 mL of an O157 suspension (10^1 CFU/mL). After various hours of enrichment culture, 1 mL of samples were detected as above. We gladly found that the target micro-organisms could be isolated from food samples after 2.5 h of enrichment (but for milk, it had to take 4 h), much shorter than regular enrichment time, in the presence of at least 10^8 CFU/mL background bacteria. Food granules showed little interference with IMS.

DISCUSSION

Food safety has attracted increased attention of the government and general public. Some microbiological detection technique have been used to identify the infection of gastrointestinal tract[23,24]. Traditional methods for the detection of trace amount of bacteria require amplification or enrichment of the target bacteria in the sample[25-27]. These methods tend to be laborious and time-consuming because of the complicated assay procedures. Strategies based on molecular biology, such as PCR have been made to improve the sensitivity of detection, and gene chip to be adaptable to high-throughput bioanalysis for multiple pathogens. But these strategies are trapped by pre-enrichment and isolation of target bacteria from the samples. In order to shorten the process of enrichment, effective measure of concentration must be taken to sufficiently increase the cell density.

IMS was a technique emerged in the 1980s. Magnetic microspheres (MMS) were a kind of solid particles that had a core of metal ions covered with macromolecule polymer. Attracted by magnetic field, MMS could separate from other components in the liquid. When coupled with specific antibody, they got the ability to capture antigen even when there were a lot of background bacteria. Compared with other isolation methods, IMS was a physical separation that sublethally injured cells might be recovered more efficiently by IMS from foodstuffs and hence reduced the number of false negative results. Magnetic iron-dextran nanoparticles are an improvement over previously prepared MMS; in that they are extremely stable and do not aggregate during mild protein coupling reactions or bacteria isolating procedures. This is largely due to their small size and the substantial dextran coating which surround the iron oxide core.

In this paper, immunomagnetic nanoparticles were used to facilitate the separation and concentration of *E.coli* O157:H7 from other components in foods. The magnetic iron-dextran nanoparticles are easily synthesized within 30 min, and purified by conventional biochemical separation techniques. In order to isolate target bacteria, we have coupled specific antibody to the nanoparticles, which had been oxidized with periodate under mild conditions. The Schiff's base linkage produced between an aldehyde group of dextran and an amino group of antibody was further stabilized to a secondary amine by borohydride reduction. Appropriate oxidation time must be confirmed to ensure the final function. If it is short, the amount of the aldehyde group was too small to couple enough antibody. On the other hand, over oxidation would change aldehyde group into carboxyl, and also influence the linkage of antibody. Antibody specificity is also an important condition when using immunomagnetic nanoparticles in positive selection of bacteria. But the amount of antibody should not be too much, because the excess antibody would occupy the antigen site of the bacteria, and the competition between free antibody and combined antibody would occur. As several bacteria might get attached to one particle, papain was used to let antigen detach from antibody. But some particles and bacteria may have been lost in the washing procedure, reducing the number of CFU recovered.

In summary, we have developed a fast and sensitive immunological method for bacteria isolation that uses antibody-conjugated magnetic nanoparticles. The reliable detection of trace amounts of *E.coli* O157:H7 in inoculated food samples demonstrates the practical usefulness of this method. This technique could be adapted in research using antibodies specific for various bacterial pathogens for the detection of a wide variety of bacterial pathogens. This study clearly exhibits the excellent properties of bioconjugated nanomaterials in applications in bioanalysis and biodetection.

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