

Molecular Detection of Bacterial Contamination in Blood Components Using Magnetic-based Enrichment

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Research Article

Keywords: Bacterial Contamination, Blood Components, Molecular Detection, Magnetic-based Enrichment, Platelets

Posted Date: December 2nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1090812/v1>

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Abstract

Bacterial contamination of blood products is a major problem in transfusion medicine, in terms of both morbidity and mortality. Platelets (PLTs) are stored at room temperature (under constant agitation) for more than 5 days, and bacteria can thus grow significantly from a low level to high titers. However, conventional methods like blood culture and lateral flow assay have disadvantages such as long detection time, low sensitivity, and the need for a large volume of blood components. We used real-time polymerase chain reaction (PCR) assays with antibiotic-conjugated magnetic nanobeads (MNBs) to detect enriched Gram-positive and -negative bacteria. The MNBs were coated with polyethylene glycol (PEG) to prevent aggregation by blood components. Over 80% of all bacteria were captured by the MNBs, and the levels of detection were 10^1 colony forming unit [CFU]/mL and 10^2 CFU/mL for Gram-positive and -negative bacteria, respectively. The detection time is < 3 h using only small volumes of blood components. Thus, compared to conventional methods, real-time PCR using MNBs allows for rapid detection with high sensitivity using only a small volume of blood components.

Introduction

Bacterial contamination of blood products is a major problem in transfusion medicine [1–3]. Especially, transfusion of contaminated platelets (PLT) may cause serious infections and septic reactions [1, 4]. PLTs are stored at room temperature (under constant agitation) for 5 days; bacteria can thus easily multiply from low levels (< 1 colony forming unit [CFU]/mL) to high titers ($< 10^8$ CFU/mL) [5–7]. The US Food and Drug Administration (FDA) reported that transfusion of contaminated PLTs caused 51 deaths from 2001 to 2016 [8]. The European Commission reported that 43 transfusions were contaminated in the European Union from 2010 to 2013; 36 involved contaminated PLTs [9]. Although there have been a few cases, the morbidity and mortality rates are very high [10, 11]. To reduce mortality, accurate and rapid bacterial detection is required [7]. The gold standard for the detection is blood culture, which is one of the oldest clinical techniques [12, 13]. However, bacterial growth to detectable levels usually requires from 24 h [14] to several days [15]. Also, low bacterial titers and slow bacterial growth can cause false-negative results when automated blood culture systems are employed [16, 17]. Much effort has thus been devoted to the rapid and sensitive detection of bacterial pathogens in blood [18–21]. The enhanced bacterial detection (eBDS) system (Haemonetics Corporation, Braintree, MA, USA) indirectly detects bacteria by measuring decreases in oxygen concentration over 24 h, but cannot detect anaerobic bacteria [22]. The Platelet Pan-Genera Detection (PGD) test (Verax Biomedical, Marlborough, MA, USA, USA) is a lateral flow immunoassay detecting lipoteichoic acid (LTA) and lipopolysaccharide (LPS) in aerobic and anaerobic Gram-positive and -negative bacterial species, respectively, within 30 min [23]. However, the sensitivity is low (approximately 10^4 CFU/mL) and the false-positive rate is high [24].

Nucleic acid (NA) amplification via polymerase chain reaction (PCR) sensitively and specifically detects bacterial pathogens [25–29]. However, on the day of PLT production, the PCR sensitivity was only 12.8% that of the BacT/ALERT system (Organon Teknica Corp., Durham, NC, USA) because the bacterial loads were very low [30]. Also, PLTs contain many substances (such as immunoglobulin G) that interfere with NA amplification [31]. It is thus essential to enrich bacteria and prepare purified bacterial DNA for accurate and rapid detection. Many commercial kits are used to extract NA from blood products; most employ solid-phase extraction [32]. However, these cannot remove inhibitors or enrich pathogens and bacteria are not isolated. Immunomagnetic separation (IMS) using antibody-conjugated magnetic nanobeads (Ab-MNBs) is widely applied to isolate pathogens and thus eliminate inhibitory substances [33–36]. However, Ab-MNBs do not detect all bacteria that cause sepsis; the antibody is specific for 1 species but at least 10 species of bacteria cause sepsis. Thus, MNBs must be conjugated with materials that bind a broad spectrum of bacterial species. Potential candidates include antibiotics and certain lectin-based natural compounds, although these have not yet been validated clinically [37, 38].

Thus, we developed a method that enriches both Gram-positive and -negative bacteria using MNBs coated with two different materials; we then extracted bacterial DNA. Figure 1 shows two methods used for sample preparation (with and without specimen incubation). Performance was tested by spiking 2.5-mL amounts of apheresis plasma with *Escherichia coli* O157:H7 (*E. coli* O157:H7) and *Staphylococcus aureus* (*S. aureus*). Extracted bacterial DNAs were amplified via real-time PCR.

Results

Characterization of antibiotics conjugated MNBs. The synthesis of antibiotic-conjugated MNBs with PEG coatings is shown in Figures 2a and b. First, superparamagnetic Fe_3O_4 nanobeads with an average diameter of 150 nm were synthesized using a one-step hydrothermal method [39], coated with PEG, and conjugated with vancomycin (Van) or allantoin (Al) to yield MNBs@PEG-Van and MNBs@PEG-Al. Transmission electron microscopy (TEM) (JSM-3010, JEOL Ltd. Tokyo, Japan) (Figure 2c) showed that the MNBs had a typical core-shell structure, i.e., a 150-nm-diameter Fe_3O_4 core and 5.5-nm-thick PEG coating. The zeta potentials were derived using dynamic light scattering (DLS) (Nano ZS, Malvern Instruments, Malvern, UK). The zeta potential of unconjugated MNBs was -20.33 ± 0.67 mV, whereas those of MNBs@PEG, MNBs@PEG-Al, and MNBs@PEG-Van were -10.58 ± 0.86 , -12.40 ± 0.42 , and -6.93 ± 0.71 , respectively (Supplementary Table 1).

Efficiencies of bacterial enrichment by MNBs. As shown in Figure 3a, the MNBs@PEG-Al's capture efficiencies of *E. coli* spiked in phosphate-buffered saline (PBS) and apheresis plasma were 90% and 80.1%, respectively. The MNBs@PEG-Van's capture efficiencies of *S. aureus* spiked in PBS and apheresis plasma were 92.3% and 82.1%, respectively (Figure 3b). Field-effect scanning electron microscopy (FE-SEM) (JSM7500F, JEOL Ltd. Tokyo, Japan) (Figure 3c and d) showed *E. coli* and *S. aureus* bound specifically to MNBs@PEG-Al and MNBs@PEG-Van, respectively.

Real-time PCR for the detection of enriched bacteria. DNA was extracted from the enriched Gram-negative and -positive bacteria and subjected to real-time PCR. As shown in Figure 4a, *E. coli* at 10^2 CFU/mL was detected after 12 h of specimen incubation, and Figure 4b shows that *E. coli* not captured by MNBs were detected at only 10^4 CFU/mL. As shown in Figure 4c, 10^3 CFU/mL of *E. coli* was detected without prior specimen incubation, but DNA extracted from 10^1 to 10^2 CFU/mL could not be amplified. Figure 4d shows that the difference in cycle threshold (Ct) value was approximately 1.4 when apheresis plasma

containing *E. coli* captured (Ct; 30.54 ± 0.48) and not captured (Ct; 31.95 ± 0.95) by MNBs at a level of 10⁴ CFU/mL were compared, while MNBs@PEG-AI captured the bacteria effectively at a level of 10³ CFU/mL (Table 1). As shown in Figure 5a, *S. aureus* at 10¹ CFU/mL was detected after 12 h of specimen incubation. Figure 5b shows that *S. aureus* not captured by MNBs was detected at only 10⁴ CFU/mL. 10² CFU/mL of *S. aureus* was detected without prior specimen incubation, but DNA extracted from 10¹ CFU/mL could not be detected (Figure 5c). Figure 5d shows that MNBs@PEG-Van captured the bacteria effectively at a level of 10⁴ CFU/mL.

Discussion

When PLTs are stored for 5 days at RT with agitation, even if the initial suspension contains < 1 CFU/mL, the bacteria can proliferate [1, 4]. Current culture methods do not yield contamination data during PLT storage. Culture requires 1–5 days and data are available only after the PLTs have been released. Second, a large amount of blood (> 20 mL) is required for culture of both aerobic and anaerobic bacteria [23]. Molecular diagnostics requires small sample volumes (< 1 mL) and detects pathogens within 3 h. It is essential to reduce culture time by improving bacterial enrichment. Such enrichment by MNBs requires that they be dispersible in blood components, and that receptors such as vancomycin and allantoin bind to the bacterial pathogens. Plasma contains many proteins, clotting factors, and IgG [40]; these readily adsorb to non-PEG-coated MNBs. After a 20-min incubation, MNBs aggregated in plasma, but MNBs@PEG did not (Supplementary Figure 1). PEG prevented non-specific binding to the surfaces of MNBs. The MNBs (4 × 10⁹ to 4 × 10¹² beads/mL) were added to 1 mL amounts of apheresis plasma containing bacterial pathogens. Over 80% of all *E. coli* and *S. aureus* were captured at MNB levels of 4 × 10¹¹ to 4 × 10¹² /mL (Supplementary Table 2). Many bacterial species contaminate PLTs. IMS is not diagnostically useful; the species of bacteria present remain unknown until the results are confirmed. MNBs must be conjugated with receptors that bind to a broad spectrum of bacterial species. Vancomycin binds to peptides of Gram-positive bacteria that terminate in -Lys-D-Ala-D-Ala [41], and allantoin binds to the LPS of the outer membrane of Gram-negative bacteria [42]. The capture efficiencies and SEM images showed that these compounds were immobilized on MNBs@PEGs that bound *E. coli* and *S. aureus*, respectively.

Pathogen enrichment by MNBs prior to NA extraction reduced the levels of possible inhibitors and yielded more NA than commercial kits. The detection time was thus dramatically reduced (< 15 h). Both the *E. coli* and *S. aureus* enrichment rates were > 80% and levels of 10¹ and 10² CFU/mL, respectively, were detected after 12 h of specimen incubation. The levels of detection without incubation were 10² and 10³ CFU/mL, respectively. Thus, our method is at least 100-fold more sensitive than lateral flow assay kits such as the Platelet PGD test (limit of detection = 10⁴⁻⁵ CFU/mL) after 12 h of specimen incubation [24]. In addition, our method requires only small sample volumes (< 1 mL). However, we aim to further improve the sensitivity and devise a fully automated high-throughput system. Our current focus is on optimization of the sample preparation method.

Methods

Bacterial strains. *E. coli* (ATCC 25922; American Type Culture Collection, Bethesda, MD, USA) and *S. aureus* (ATCC 25923) were used. A single colony of either strain was transferred to 5 mL of Luria-Bertani (LB) broth (BD, Franklin Lakes, NJ, USA) and cultured for 18 h at 200 rpm and 37°C. The cultures were then diluted 100-fold with fresh LB broth and incubated at 200 rpm and 37°C until the optical density at 600 nm (OD₆₀₀) reached 1. Bacterial viability was measured using the standard colony counting method [43] and suspensions of 10⁸ CFU/mL in PBS (pH 7.4) were prepared.

Preparation of antibiotics conjugated MNBs. Figure 2a and b show the synthesis of antibiotic conjugated MNBs. Vancomycin and allantoin were obtained from Sigma-Aldrich (St. Louis, MO, USA). The MNBs (100 nm in diameter) were sonicated for about 40 s to prevent aggregation. MNBs (200 mg) were dispersed in 40 mL of 1 M HCl and stirred at room temperature (RT) for 1 h. The MNBs were separated over 2 min using a magnetic rack (Bioneer Co., Ltd, Daejeon, Korea), and residual HCl was removed by washing in 40 mL of PBS three times followed by dispersal in 10 mL PBS. PEG (25 mg) (Sigma-Aldrich) dissolved in 25 mL of Tris buffer (pH 8.5) was mixed with 50 mg MNBs overnight at RT. To conjugate Van to MNBs@PEG-COOH, 5 mg MNBs@PEG-COOH was added to 500 µL of 2-(N-morpholino) ethane sulfonic acid (MES) buffer (0.1 M, pH 6.0) (Sigma-Aldrich); 4 mg ethyl carbodiimide hydrochloride (EDC) (Sigma-Aldrich), 7 mg N-hydroxy succinimide (NHS) (Sigma-Aldrich), and 10 mg vancomycin dissolved in 1 mL of MES buffer were added and the mixture was stirred at RT for 15 min. Then, the MNBs@PEG-COOH suspension was dispersed in the vancomycin-EDC-NHS suspension via continuous stirring at RT for 2 h. MNBs@PEG-Van were separated using the magnetic rack and washed with 500 µL of PBS. Finally, MNBs@PEG-Van were resuspended in 1 mL of PBS and stored at 4°C. MNBs@PEG-AI were similarly prepared.

Characterization of antibiotics conjugated to MNBs. MNBs were dispersed in 99.5% (v/v) ethanol at 20 µg/mL. Copper grids bearing carbon films (Electron Microscopy Sciences, Hatfield, PA, USA) were immersed in 1-mL MNB dispersions for 10 min, removed using tweezers, dried at 70°C, and examined by TEM (JEOL Ltd.) operating at 200 kV.

Bacterial enrichment by MNBs. Apheresis plasma containing bacteria (*E. coli* O157:H7 and *S. aureus*) at 10¹-10⁴ CFU/mL were incubated at RT for 12 h. One milliliter of plasma was mixed with 200 µL of either MNBs@PEG-AI or MNBs@PEG-Van (4 × 10¹¹ particles/mL, final concentration) and the mixtures were incubated at RT for 20 min. Bacteria-MNB clusters were separated using a magnetic rack. The residues, which contained uncaptured bacteria, were transferred to 1.5-mL tubes. The MNBs were washed with 1 mL PBS twice and dispersed in 200 µL PBS.

FE-SEM of bacteria binding to antibiotics conjugated MNBs. Bacteria-enriched MNBs were washed twice with PBS and MNBs separated on a magnetic rack. Fixation [in 2% (w/v) glutaraldehyde] proceeded at RT for 1 h. MNB-bacteria complexes were washed three times with 1 mL PBS, incubated with 1% (w/v) osmium tetroxide for 1 h at 4°C in the dark, and washed three times with PBS followed by gradual dehydration in ethanol (30, 50, 70, 80, 90, and 99.5% [v/v]) for 10 min each time. Ten microliters of MNB-bacteria complexes were dropped onto copper grids covered with amorphous carbon and dried at RT for 2 h. FE-SEM (JEOL Ltd.) was used to image the complexes.

NA extraction. MagListo 5M Genomic NA extraction kits (Bioneer) were used to extract bacterial DNA as suggested by the manufacturer. Two hundred microliters of lysis buffer were added to a suspension of BE-MNBs followed by incubation for 10 min at RT. The elution volume was 100 μ L; the extracted DNA was stored at -80°C . DNA purity and yield were assessed by quantifying absorbance at 230, 260, and 280 nm using a Nano-200 spectrophotometer (Allsheng, Hangzhou, China). To detect Gram-positive bacteria, 10 μ g lysostaphin (Sigma-Aldrich) was added to the BE-MNBs followed by incubation for 10 min at 37°C prior to the addition of lysis buffer.

Real-time PCR assay. Real-time PCR was used to confirm the identities of the captured bacteria. The primers were designed using PrimerQuest (Integrated DNA Technologies Inc., Coralville, IA, USA): SA nuc_F (5'-TATGGACGTGGCTTAGCGTAT-3') and SA nuc_R (5'-GACCTGAATCAGCGTTGTCTT-3') for *S. aureus*; and EB tyrB_F (5'-AAGAGGATGCCTACGCCATT-3') and EB tyrB_R (5'-CTTGGCGGGCTGGAGTAGTT-3') for *E. coli*. Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) served as the PCR master mix; all primers were added to 0.2 μ M. QuantStudio 3 (Applied Biosystems) was used to perform PCR. Positive and negative amplification controls were included in every run. The positive controls contained DNA directly extracted from *S. aureus* and *E. coli*, and the negative control was RNase- and DNase-free water. A result was considered positive when the threshold cycle (Ct) was > 37.0 and the melting temperature (T_m) was appropriate ($76.0 \pm 0.5^{\circ}\text{C}$).

Declarations

Acknowledgments

This work was supported by the "Collabo R&D between Industry, Academy, and Research Institute" program funded by the Korean Ministry of SMEs and Startups in 2021 (project no. S3104763), and by research grant no. 2019-ER5402-01 from the Korean Disease Control and Prevention Agency.

Author contributions

J.L., K.U.P., and Y.J.H. conceived and planned the experiments. J.L., A.T.A., S.O., H.E.C., W.T., and D.L. performed the experiments. W.T. synthesized the nanobeads. H.E.C. performed real-time PCR. J.L., A.T.A., S.P., and K.U.P. analyzed the data and wrote the manuscript. J.L., A.T.A., K.U.P., and Y.J.H. interpreted the results, reviewed the data, and contributed to the final version of the manuscript.

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Table

Table 1. Results of real-time PCR analyses.

| Bacteria | Incubation | Bacteria captured by MNBs (CFU/mL) | | | | | | | | Uncaptured bacteria (CFU/mL) | | | | | | | |
|------------------|-----------------------------------|------------------------------------|------|-----------------|------|-----------------|------|-------|------|------------------------------|------|-----------------|----|-----------------|----|------|----|
| | | 10 ⁴ | | 10 ³ | | 10 ² | | 10 | | 10 ⁴ | | 10 ³ | | 10 ² | | 10 | |
| | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| <i>S. aureus</i> | with prior specimen incubation | 18.54 | 0.20 | 23.25 | 0.31 | 26.09 | 0.32 | 31.02 | 0.23 | 35.57 | 0.96 | NA | NA | NA | NA | NA | NA |
| | without prior specimen incubation | 28.21 | 0.71 | 34.52 | 1.10 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| <i>E. coli</i> | with prior specimen incubation | 29.07 | 0.18 | 33.56 | 1.09 | 35.60 | 0.51 | NA | NA | 32.37 | 0.59 | NA | NA | NA | NA | NA | NA |
| | without prior specimen incubation | 30.54 | 0.48 | 34.64 | 0.96 | NA | NA | NA | NA | 31.95 | 0.95 | NA | NA | NA | NA | NA | NA |

CFU; colony forming unit, SD; Standard deviation, NA; not applicable.

Figures

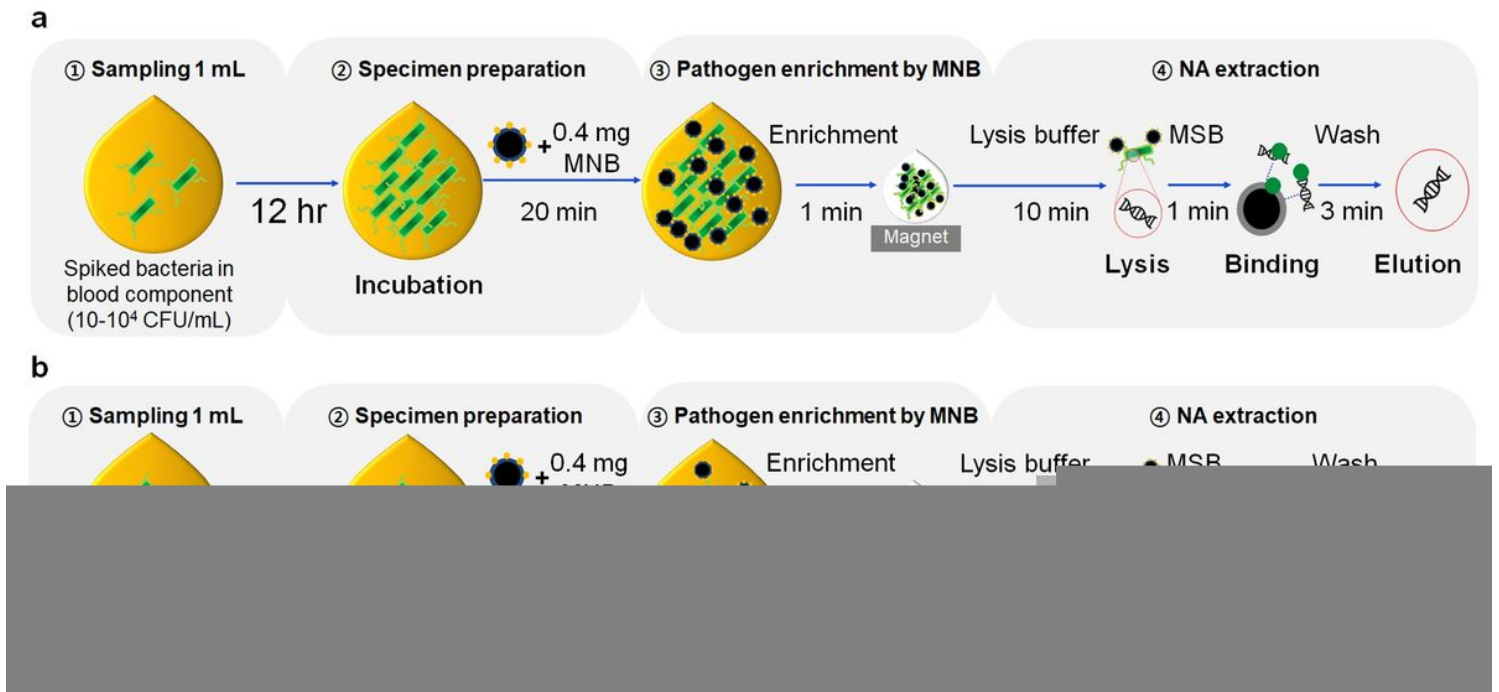


Figure 1

Schematic illustration of pathogen enrichment by MNBs (magnetic nanobeads) and NA (nucleic acid) extraction from 1-mL samples of blood components spiked with bacteria (a) after 12 h of specimen incubation at RT (room temperature), and (b) without prior specimen incubation. The procedural steps were as follows: sampling, specimen preparation, pathogen enrichment by MNBs, and NA extraction.

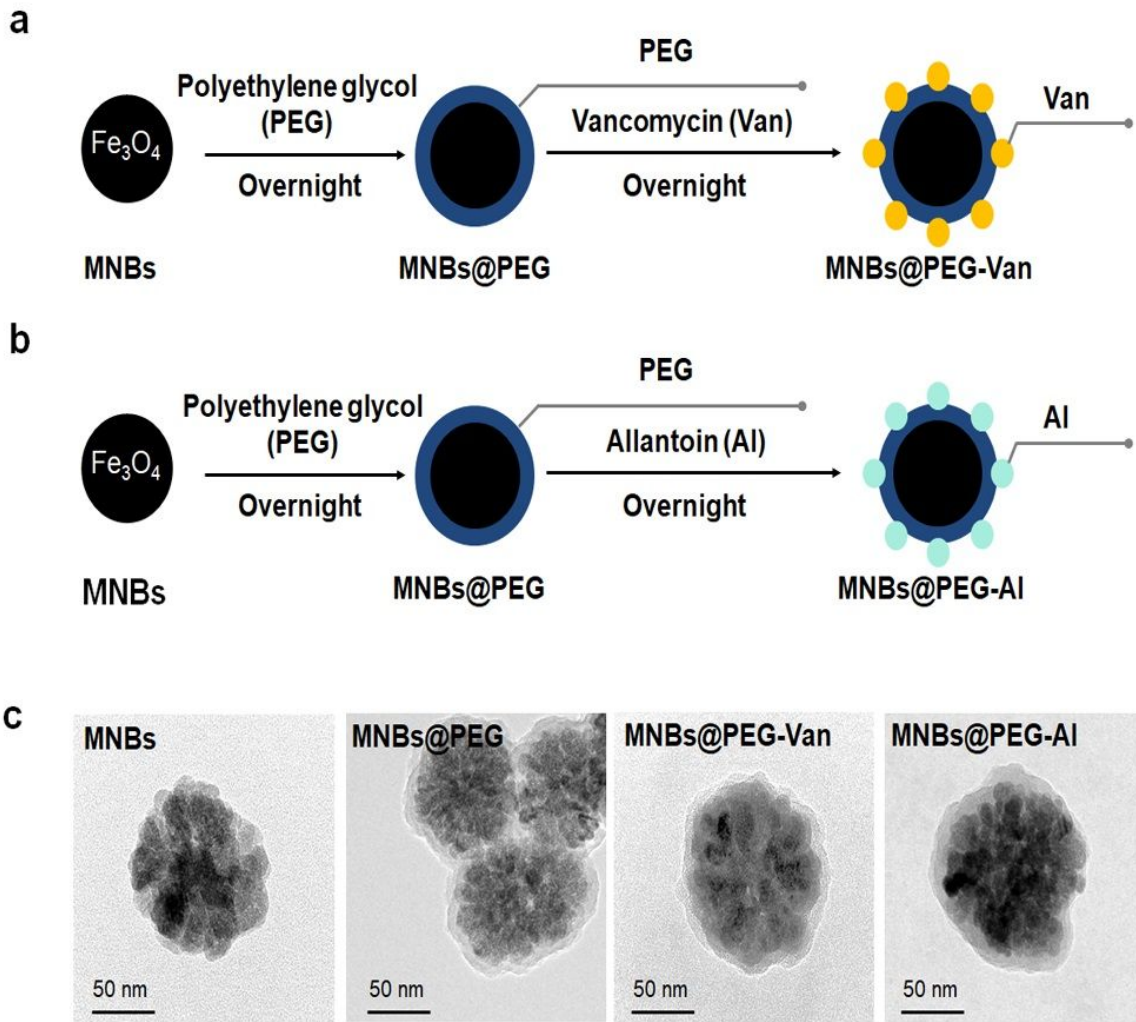


Figure 2
 Conjugation of materials to MNBs (magnetic nanobeads). (a) Schematic of the synthesis of MNBs@PEG-Van (polyethylene glycol-vancomycin). (b) Schematic of the synthesis of MNBs@PEG-AI (polyethylene glycol-allantoin). (c) TEM (Transmission electron microscopy) images of unconjugated MNBs, MNBs@PEG, MNBs@PEG-Van, and MNBs@PEG-AI.

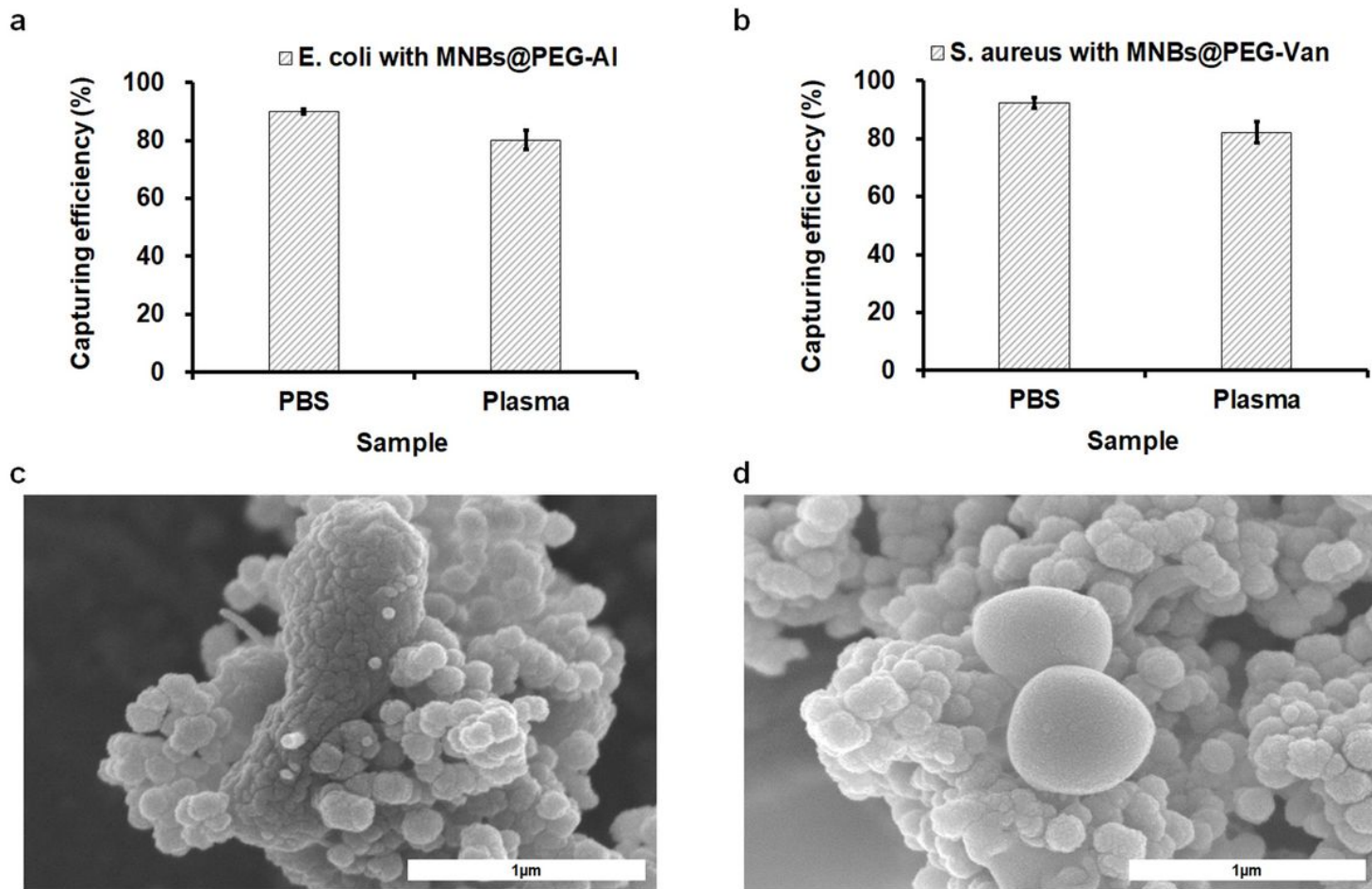


Figure 3

(a) Capture efficiencies of *E. coli* with MNBs@PEG-AI (polyethylene glycol-allantoin) from PBS (phosphate-buffered saline) and apheresis plasma. (b) Capture efficiencies of *S. aureus* with MNBs@PEG-Van (magnetic nanobeads@polyethylene glycol-vancomycin) from PBS and apheresis plasma. (c) FE-SEM (Field-effect scanning electron microscopy) images showing *E. coli* bound to MNBs@PEG-AI. (d) FE-SEM images showing *S. aureus* bound to MNBs@PEG-Van.

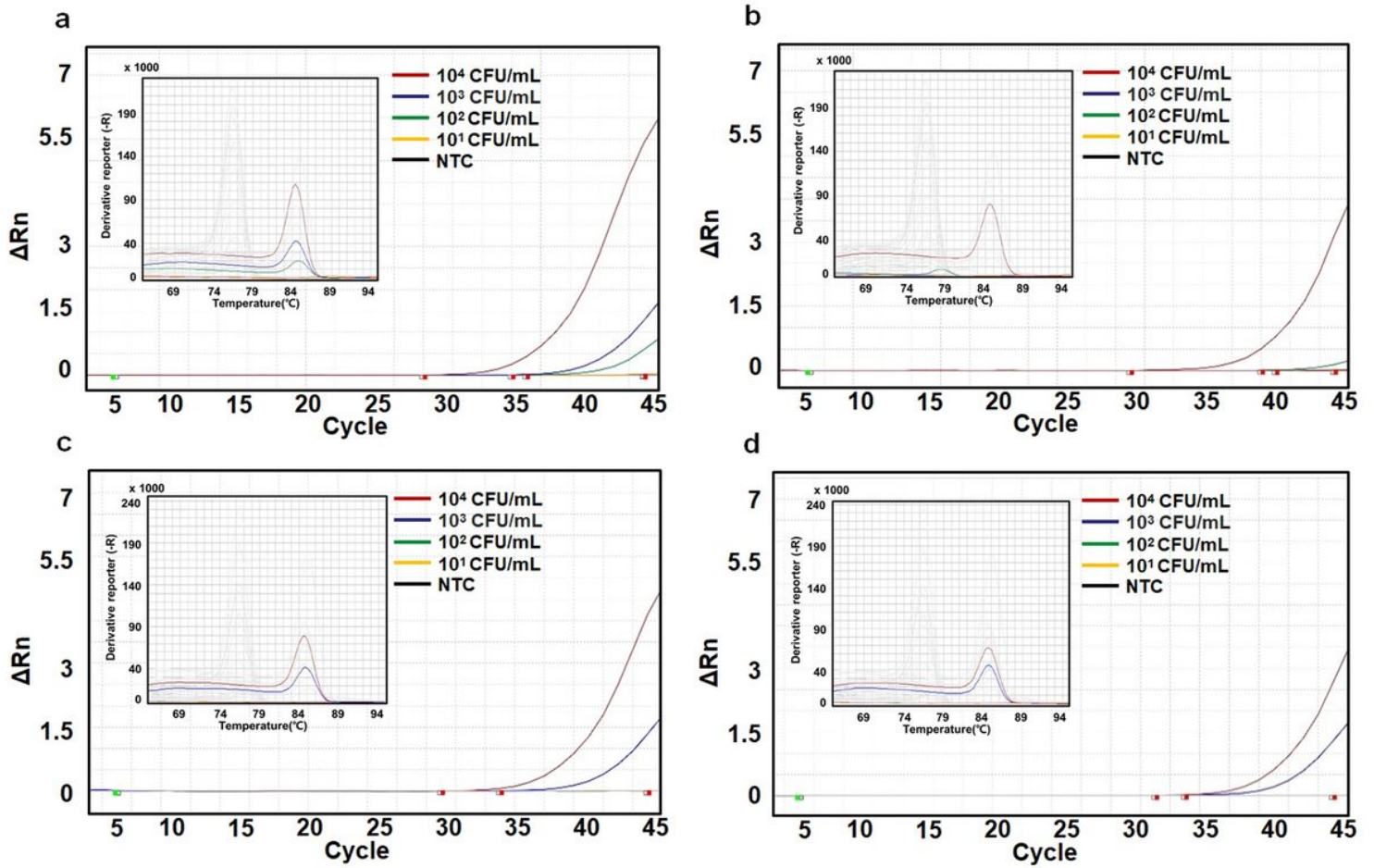


Figure 4

Results of real-time PCR for the detection of *E. coli*. Each shows amplification and melting curve. Figure 4a and 4b refer to the nucleic acid amplification performed with prior specimen incubation, whereas 4c and 4d without prior specimen incubation. Figure 4a and 4c show the results of bacteria captured by MNBs (magnetic nanobeads), while 4b and 4d of uncaptured bacteria. The positive controls are not shown.

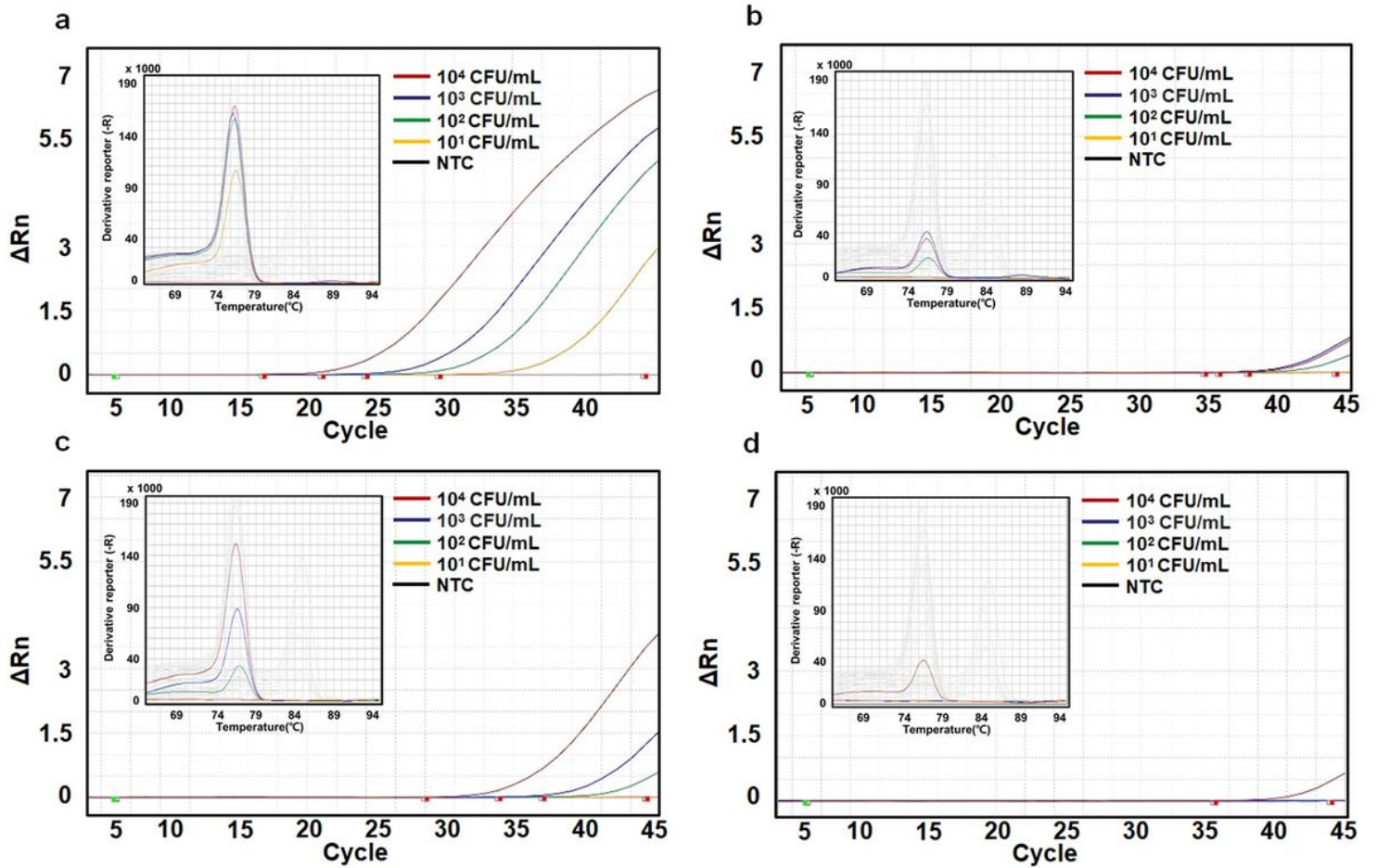


Figure 5

Results of real-time PCR for the detection of *S. aureus*. Each shows amplification and melting curve. Figure 5a and 5b refer to the nucleic acid amplification performed with prior specimen incubation, whereas 5c and 5d without prior specimen incubation. Figure 5a and 5c show the results of bacteria captured by MNBs (magnetic nanobeads), while 5b and 5d of uncaptured bacteria. The positive controls are not shown.

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