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Affinity Capture Using Vancomycin-Bound Magnetic Nanoparticles for the MALDI-MS Analysis of Bacteria

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) provides a straightforward means to differentiate microorganism species based on mass spectral fingerprinting. The pathogen cell concentration in an infected sample, however, is generally lower than that capable of being detected directly by MALDI-MS. Furthermore, the presence of proteins or metabolites in biological fluids always causes unavoidable interference for the identification of microorganism species. Vancomycin, which binds to D-Ala-D-Ala moieties on the cell walls of Gram-positive bacteria and, therefore, inhibits peptidoglycan synthesis, is one of the most potent antibiotics. Thus, we have employed vancomycin-modified magnetic nanoparticles as affinity probes to selectively trap Gram-positive pathogens from sample solutions; i.e., these bacteria can be isolated from sample solutions by applying a magnetic field. The isolated cells could then be characterized by MALDI-MS. This approach effectively reduces the interference of protein and metabolite signals in the mass spectra of Gram-positive bacteria because vancomycin has such high specificity for the D-Ala-D-Ala units of the cell walls. The lowest cell concentration we detected for both *Staphylococcus saprophyticus* and *Staphylococcus aureus* in a urine sample (3 mL) was ~7 × 10^4 cfu/mL.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can be used to characterize microorganism species.[1−22] Generally, intact cells cultured from a medium are used directly for MALDI-MS analysis without requiring any tedious pretreatment. In the real world, however, such a preparation procedure may not be practical. A sample collected from a pathogen-infected patient always contains the pathogens within a complex biological matrix. The presence of this complex matrix frequently suppresses the signals of the ions generated from the microorganism cells during MALDI-MS analysis. Thus, the technique of affinity mass spectrometry was developed in an attempt to selectively concentrate trace amounts of bacteria from biological fluids.[20−22] Developing affinity probes that are specific for target bacteria is an approach that can effectively exclude the interference from the matrix samples. Fenselau and co-workers have proposed an affinity method to trap traces of bacterial cells from complex biological mixtures by the use of a lectin-immobilized substrate.[20−22] Additionally, Voorhees and co-workers employed immunomagnetic separation coupled with MALDI-MS to effectively characterize target bacteria from biological sample solutions.[18,19]

In this paper, we propose the use of vancomycin-immobilized magnetic nanoparticles as nanoscale probes to selectively trap traces of pathogens from biological fluids. Glycopeptide antibiotics inhibit the cell growth of Gram-positive bacteria by binding through hydrogen bonds to the terminal D-Ala-D-Ala moieties of

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the peptide units of the cell walls of these pathogens.[23] That is, these glycopeptide antibiotics are capable of recognizing the cell surfaces of bacteria. Vancomycin is one such glycopeptide antibiotic. Xu and co-workers proposed recently that vancomycin-modified Fe−Pt nanoparticles could be used as affinity probes to trap vancomycin-resistant Gram-positive or -negative bacteria;[24,25] these results were confirmed by analyzing electronic microscopy images. While impressive, this method has a drawback in that it is unlikely that the species of bacteria could be characterized this way because, for example, different *Staphylococcus* species have similar morphologies.

In this study, we immobilized vancomycin onto the surfaces of magnetic nanoparticles. The vancomycin-immobilized magnetic nanoparticles behave as affinity probes for pathogens and bind to the cell surfaces of target bacteria through hydrogen bonds; in essence, the target bacteria become magnetized and can be isolated readily from the sample solution by employing an external magnetic field. By applying MALDI-MS, which can be used as an analytical method to differentiate microorganism species based on their fingerprinting mass spectra,[1−22] we have demonstrated that the affinity probes have a high specificity toward their target bacteria, even in a complex biological fluid. We used Gram-positive bacteria, such as *Staphylococcus saprophyticus*, *Staphylococcus aureus*, and *Enterococcus faecalis*, as target bacteria to demonstrate the practical feasibility of using this approach.

EXPERIMENTAL SECTION

Reagents. $FeSO_4 \cdot 7H_2O$, trifluoroacetic acid, and acetonitrile were obtained from Merck (Seelze, Germany); $FeCl_3 \cdot 6H_2O$ was obtained from Riedel-de Haën (Seelze, Germany). Ammonium hydroxide was purchased from J. T. Baker (Phillipsburg, NJ). Sodium phosphate dibasic heptahydrate, 3,5-dimethoxy-4-hydroxycinnamic acid, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, and vancomycin were obtained from Sigma (St. Louis, MO). Sodium phosphate monobasic was purchased from Mallinckrodt.

Preparation of Magnetic Nanoparticles. Magnetic Fe_3O_4 nanoparticles were prepared by stirring $FeSO_4 \cdot 7H_2O$ (27.8 g), $FeCl_3 \cdot 6H_2O$ (54 g), and deionized water (50 mL) in a water bath maintained at 80 °C and then adding aqueous NH_4OH solution (8 M, 15 mL) into the $FeSO_4/FeCl_3$ mixture until a black precipitate appeared.[26] The mixture was stirred continuously for 30 min in the water bath at 80 °C. When the reaction was complete, the magnetic particles in the sample solution were aggregated onto the wall of the sample vial by positioning a magnet (magnetic field, ~5000 G) at the edge of the vial. The remaining solution was then removed by pipet. The isolated magnetic nanoparticles were washed repeatedly with hot water to remove any unreacted impurities. The magnetic particles were stored in a phosphate buffer solution (pH 6.3; 250 mL) before use. This phosphate buffer solution was prepared by mixing NaH_2PO_4 (10 mM, 350 mL) and Na_2HPO_4 (10 mM, 69 mL).

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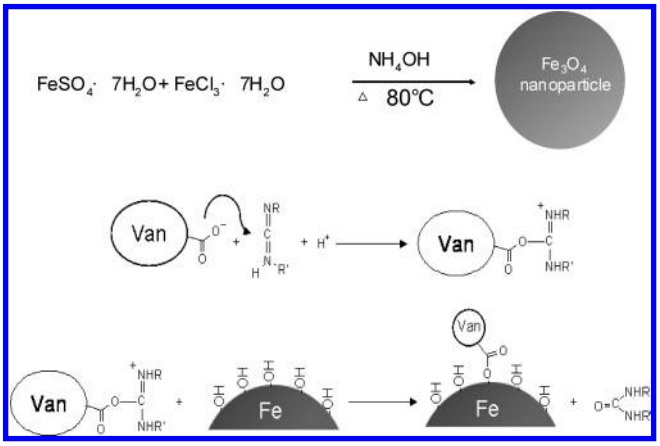
Scheme 1. Synthetic Route for Immobilizing Vancomycin onto the Surface of Magnetic Nanoparticles



Preparation of Vancomycin-Immobilized Magnetic Nanoparticles. Scheme 1 presents the synthetic route for immobilizing vancomycin onto the surface of Fe_3O_4 magnetic nanoparticles. A portion of the solution of magnetic nanoparticles (4.24 mg/mL, 1 mL) was mixed with solutions of vancomycin (1 mg/mL, 5 mL) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (1 mg/mL, 5 mL). The mixture was stirred for 24 h. After the reaction was complete, the product was aggregated onto the wall of the sample vial by applying a magnet externally to the vial. The remaining solution was discarded and the magnetic particles were washed with phosphate buffer (2 × 10 mL) and DI water (2 × 10 mL). The vancomycin-modified magnetic nanoparticles were then resuspended in a phosphate buffer solution (10 mL). From a TEM image, 500 nanoparticles were counted and averaged by using SigmaScan Pro5 (Systat Software Inc.); the average diameter of the particle was ~11.3 ± 1.7 nm. We estimated the number of vancomycin molecules bound to the surfaces of the magnetic nanoparticles by using UV−visible absorption spectroscopy. Based on the relative absorption at a wavelength of 280 nm between a stock solution of vancomycin (5 mg/mL) and the supernatant of the incubated Fe_3O_4−vancomycin sample after centrifugation, we estimated that ~9×10^{-8} mol (equivalent to 5.41×10^{16} molecules) of vancomycin was immobilized onto the surface of 1 mg of magnetic nanoparticles. We assumed that the density of the magnetic nanoparticles was 5.4 g/cm^3;[27] as a result, 1 mg of magnetic nanoparticles contains 2.48×10^{14} particles. A 10-μL van−Fe nanoparticle solution (0.424 mg/mL) contains 1.04×10^{12} van−Fe nanoparticles. Consequently, there are ~218 vancomycin molecules immobilized onto the surface of each magnetic nanoparticle. Jusuf et al. estimated the dimensions of a vancomycin were 28.81 Å × 28.81 Å × 66.13 Å.[28] Thus, we assumed that a vancomycin molecule was bond to the surfaces of magnetic nanoparticles via the surface area of 28.81 × 28.81 Å. The total surface area covering a magnetic particle by vancomycin molecules (218 molecules) is equivalent to 18094 Å^2. The total surface area for a magnetic particle is ~$4\pi r^2$ (= $4\pi(56.5$ Å $)^2 = 40095$ Å^2). The percentage covered by vancomycin is ~45%.

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Preparation of Bacterial Samples. *S. aureus*, *S. saprophyticus*, and *E. faecalis* were obtained from clinical specimens, while *Escherichia coli* JM 109 was purchased from the Culture Collection and Research Center (CCRC) in Taiwan. Samples of *S. aureus*, *S. saprophyticus*, and *E. faecalis* were collected from patients at the Buddhist Tzu Chi General Hospital, Hualien, Taiwan. Samples of *E. coli* JM 109, *S. saprophyticus*, and *S. aureus* were cultured in LB broth (25 g/L); *E. faecalis* was cultured in TSBY, which comprised trypticase soy broth (30 g/L) and yeast extract (5 g/L). After incubation overnight at 37 °C, the bacterial cells were centrifuged at 6000 rpm for 10 min. The bacteria were washed with sterilized water (2 × 10 mL). The bacteria were then heat-killed for 30 min in a water bath at 100 °C. The desired bacterial concentration was adjusted by measuring the optical density at 600 nm; it was checked by employing a serial dilution of the samples on potato dextrose agar and counting the colony forming unit (cfu) after incubation overnight at 37 °C.

Using Vancomycin-Immobilized Magnetic Nanoparticles To Probe Bacteria. The sample volume and the mixing time of bacteria and vancomycin-immobilized magnetic particles were determined based on a series of experiments. Our results indicated that optimized results were obtained when 3 mL of the sample solution was mixed for 1 h with 10 μL of the sample of vancomycin-immobilized magnetic particles ($\sim 1.04 \times 10^{12}$ nanoparticles); thus, we performed all of the probing experiments presented in this paper by adding the vancomycin-immobilized magnetic nanoparticles (10 μL) into a bacterial solution (3 mL). After gentle vortex mixing for 1 h, the vancomycin-immobilized magnetic nanoparticles were separated from the solution by applying a magnet externally to the sample vial such that the magnetic nanoparticles aggregated on the wall of the vial. The supernatant was removed by pipet. The isolated nanoparticles were rinsed with phosphate buffer solution (2 × 200 μL) and deionized water (1 × 200 μL) to remove any unbound impurities. Most of the rinsing solution was removed by pipet, but some water ($\sim$3 μL) remained around the magnetic particles because it was almost impossible to dry the sample completely. Subsequently, a MALDI matrix solution, sinapinic acid (15 mg/mL, 1 μL) dissolved in a deionized water/acetonitrile (2/1, v/v), was added to the remaining nanoparticles. After incubation for 20 min in a capped vial, a portion of this MALDI matrix solution (0.5 μL) was applied onto a sample target. After the solvents had evaporated, the sample was placed into a MALDI mass spectrometer for analysis.

On the basis of the method described by Demirev et al.,[13] we searched for the ions observed in the MALDI mass spectra in the SwissProt/TrEMBL database using the Sequence Retrieval System (SRS). We chose the average protein MW as the primary classifier within a ±3 Da mass tolerance and selected the bacterial protein subset of the protein database.

We employed UV absorption spectroscopic analysis to further investigate the trapping capacity of the vancomycin-immobilized magnetic nanoparticles toward Gram-positive and -negative bacteria. The bacterial solutions were prepared (0.1 mg/mL) and their optical densities measured directly using absorption spectroscopy before conducting nanoparticle trapping experiments. A portion of the vancomycin-immobilized magnetic nanoparticle solution (20 μL, 0.424 mg/mL) was then added into the bacterial solution (3 mL, 0.1 mg/mL). The solution was incubated for 1 h by gentle vortex mixing and then an external magnet was employed to isolate the magnetic particles conjugated with target bacteria from the sample solution. The remaining solution was then pipetted into a cell to measure its optical density using absorption spectroscopy.

Preparation of Transmission Electron Microscopy (TEM) Samples. A sample of the vancomycin-immobilized magnetic nanoparticles (10 μL) was added into an aqueous solution of *S. saprophyticus* (1 mL). After gentle vortex mixing for 1 h, the magnetic nanoparticles were aggregated on the sample vial's wall by placing a magnet next to the vial. The supernatant, which might contain bacterial cells, was removed by pipet. The isolated nanoparticles were rinsed with DI water and then suspended in DI water (1 mL). A portion of the suspension (0.5 μL) was deposited on a TEM copper holder. After drying, the sample was subjected to TEM analysis.

Instrumentation. All mass spectra were obtained using a Biflex III (Bruker Daltonics) time-of-flight mass spectrometer equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target having the capacity to load 384 samples simultaneously. The linear mode was operated during MALDI-MS analysis. The accelerating voltage was set to 19 kV, while the laser power was carefully adjusted during analysis to obtain the optimized mass resolution. Each mass spectrum was obtained by accumulating 100 laser shots. TEM images were obtained using a JEOL JEM-2000 FXII (Tokyo, Japan) instrument.

RESULTS AND DISCUSSION

Figure 1 displays a cartoon illustration of the proposed method of anchoring vancomycin-immobilized magnetic nanoparticles onto the cell surface of a Gram-positive bacterium. Vancomycin interacts with the terminal D-Ala-D-Ala peptide unit of the cell wall of the Gram-positive bacterium through the formation of five hydrogen bonds.[23] Figure 2a presents a TEM image of the magnetic nanoparticles we used in this study; their average size is ~11 nm. Figure 2b presents the TEM image obtained after employing the vancomycin-immobilized magnetic nanoparticles to probe target bacteria in an aqueous solution of *S. saprophyticus*. The vancomycin-immobilized magnetic nanoparticles cover the entire surface of the *S. saprophyticus* cells, which is not surprising when considering that it is a Gram-positive bacterium.[5] This result indicates clearly that it is possible to generate effective vancomycin-modified magnetic nanoscale probes that are capable of binding to the surface of *S. saprophyticus* cells.

We further characterized the bacteria labeled with the vancomycin-modified magnetic nanoparticles by using MALDI-MS. Generally, we observed no ions upon direct MALDI-MS analysis of *S. saprophyticus* at concentrations of $< 6 \times 10^6$ cfu/mL. Figure 3a displays the MALDI mass spectrum of *S. saprophyticus* obtained through direct analysis. The ions at m/z 3822, 4944, 4988, 6183, 6393, and 6616 appear in the mass spectrum and represent signals from *S. aureus*. Panels b and c in Figure 3 display the MALDI mass spectra of *S. saprophyticus* obtained after using the vancomycin-modified magnetic nanoparticles to probe aqueous solutions of *S. saprophyticus* (3 mL) having different concentrations. The ions at m/z 4944, 5198, 6037, 6183, and 6616 (Figure 3b) appear reproducibly in the MALDI mass spectrum obtained after using the nanoscale probes to trap the target bacteria from an aqueous solution of *S. saprophyticus* solution (6×10^6 cfu/mL, 3 mL). When

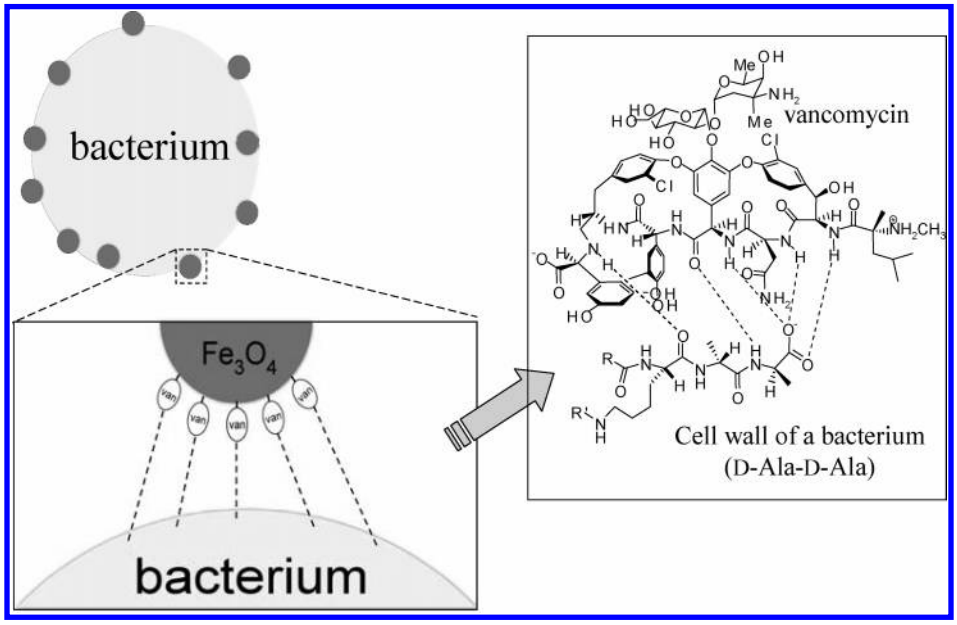

Figure 1. Cartoon illustrations of the proposed method for anchoring vancomycin-immobilized magnetic nanoparticles onto the surface of a Gram-positive bacterial cell and the binding of vancomycin to the terminal of D-Ala-D-Ala units of the peptides on the cell wall of a Gram-positive bacterium.

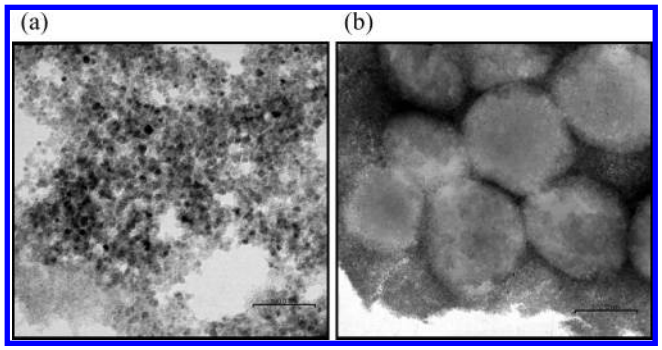

Figure 2. (a) TEM image of the synthesized magnetic nanoparticles. (b) TEM image obtained after employing the vancomycin-immobilized magnetic nanoparticles to probe target bacteria from an *S. saprophyticus* solution.

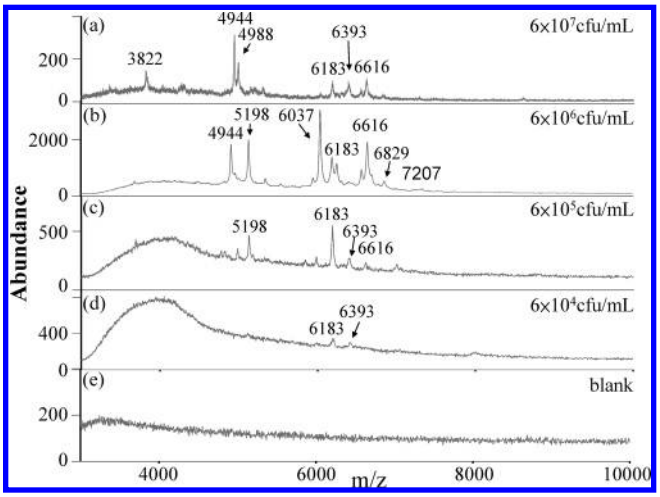

Figure 3. (a) Direct MALDI mass spectrum of *S. saprophyticus* (6×10^7 cfu/mL, 0.5 μL). MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria from aqueous sample solutions (3 mL) containing *S. saprophyticus* at concentrations of (b) 6×10^6, (c) 6×10^5, (d) 6×10^4, and (e) 0 cfu/mL.

the concentration of *S. saprophyticus* was lowered by 1 order of magnitude, the ions at m/z 5198, 6183, 6393, and 6616 appeared reproducibly in the corresponding MALDI mass spectrum (Figure 3c). Figure 3d presents the MALDI mass spectrum of *S. saprophyticus* obtained after using the vancomycin-modified magnetic nanoparticles to trap the target bacterial cells from an aqueous solution of *S. saprophyticus* (6×10^4 cfu/mL, 3 mL); two peaks, at m/z 6183 and 6393, are present in the mass spectrum at this low cell concentration. No ions appear in the mass spectrum when vancomycin-modified magnetic particles are used to probe a solution containing bacteria at 0 cfu/mL (Figure 3e). Gratifyingly, this approach allows traces of bacterial cells to be concentrated effectively from aqueous solutions of *S. saprophyticus*.

S. aureus is another example of a Gram-positive bacterium, and we employed our vancomycin-immobilized magnetic nanoparticles to trap it also from an aqueous sample solution (3 mL). Figure 4a displays the MALDI mass spectrum of *S. aureus* (4.3×10^7 cfu/mL) obtained through direct analysis. The ions at m/z 5522, 6884, and 7093 represent signals from *S. aureus*. Panels b–d in Figure 4 present the MALDI mass spectra of *S. aureus* obtained after using the vancomycin-modified magnetic nanoparticles to probe target bacteria. The cell concentrations in the samples used to obtain traces b–d in Figure 4 were 4.3×10^6, 4.3×10^5, and 4.3×10^4 cfu/mL, respectively. The ions at m/z 4081, 5028, 5522, 7093, 7564, and 9607 represent specific proteins of *S. aureus* that can be identified from the protein database.[13] Table 1 lists the corresponding proteins that we identified from the protein database. The ions at m/z 5522, 6569, 6884, and 7093 match those obtained in a previous study[29] to within a mass difference of ±4 Da. These spectra indicate that the vancomycin-modified magnetic nanoparticles are also capable of recognizing *S. aureus* in aqueous solutions and that these target bacteria, labeled by magnetic nanoparticles, can be readily isolated by the use of a magnet and

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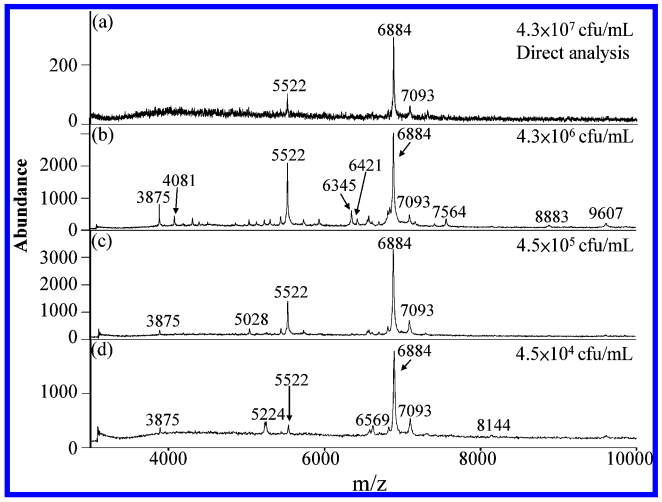

Figure 4. (a) Direct MALDI mass spectrum of *S. aureus* (4.3×10^7 cfu/mL, 0.5 μL). MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria from aqueous sample solutions (3 mL) containing *S. aureus* at concentrations of (b) 4.3×10^6, (c) 4.5×10^5, and (d) 4.5×10^4 cfu/mL.

Table 1. Peaks Obtained in the MALDI Mass Spectrum of *S. aureus* and Their Matched Proteins in the Protein Database

observed mass	protein in SwissPROT	mass (expt)	description
4081	P81684	4081	40 kDa vitronectin-binding cell surface protein [fragment]
5028	Q7A0F4	5029	hypothetical protein
5522	Q8VUL4	5524	BlAR1 [fragment]
7093	Q6GDA3	7096	putative membrane protein
7564	Q6GA49	7561	hypothetical protein
9607	Q8NWH8	9609	hypothetical protein MW 1420

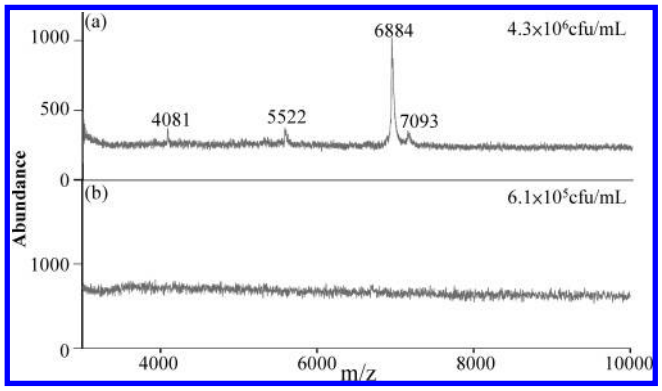

Figure 5. MALDI mass spectra obtained after using unmodified magnetic nanoparticles to probe target bacteria from aqueous sample solutions (3 mL) containing *S. aureus* at concentrations of (a) 4.3×10^6 and (b) 6.1×10^5 cfu/mL.

then characterized by MALDI-MS. In a control experiment, an *S. aureus* sample solution was examined by using unmodified magnetic nanoparticles as the affinity probes. Figure 5a displays the MALDI mass spectrum of *S. aureus* obtained after using unmodified magnetic nanoparticles to probe target bacteria (4.3×10^6 cfu/mL) from a sample solution (3 mL). Ions derived from *S. aureus* appear in the mass spectrum at m/z 4081, 5522, 6884, and 7093. When the concentration of *S. aureus* was lowered to 6.1×10^5 cfu/mL, however, we observed no ions in the mass

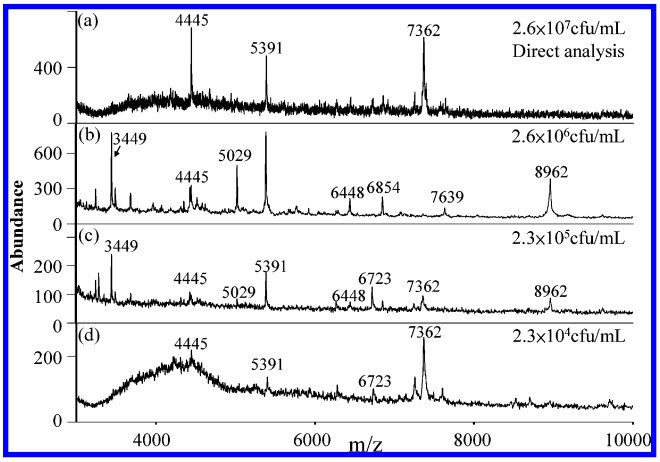

Figure 6. (a) Direct MALDI mass spectrum of *E. faecalis* (2.6×10^6 cfu/mL, 0.5 μL). MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria from aqueous sample solutions (3 mL) containing *E. faecalis* at concentrations of (b) 2.6×10^6, (c) 2.3×10^5, and (d) 2.3×10^4 cfu/mL.

Table 2. Peaks Obtained in the MALDI Mass Spectrum of *E. faecalis* and Their Matched Proteins in the Protein Database

observed mass	protein in SwissPROT	mass (expt)	description
3449	Q835H6	3449	hypothetical protein
5391	Q836Y5	5389	hypothetical protein
6448	Q834N8	6450	hypothetical protein
6723	Q7C3P5	6726	50S ribosomal protein L32−2
6854	Q835Q1	6852	hypothetical protein
6933	Q831T3	6930	ribosomal protein S21
7639	Q830F3	7639	hypothetical protein
8962	Q832F5	8960	hypothetical protein

spectrum (Figure 5b) after using unmodified magnetic nanoparticles to concentrate the bacteria. The results indicate that the unmodified magnetic nanoparticles have some ability to interact with the cell surfaces of *S. aureus*, but this trapping capacity is obviously much lower than that exhibited by the vancomycin-modified magnetic nanoparticles.

E. faecalis, a pathogen that commonly infects the human urinary tract, also belongs to the class of Gram-positive bacteria. Figure 6a presents the MALDI mass spectrum of *E. faecalis* (2.6×10^7 cfu/mL) obtained through direct analysis. The ions at m/z 4445, 5391, and 7362 are signals derived from *E. faecalis*. Panels b−d in Figure 6 present the MALDI mass spectra of *E. faecalis* obtained after using our vancomycin-immobilized magnetic particles to probe target bacteria from aqueous solutions of *E. faecalis* (3 mL). The cell concentrations used to obtain Figure 6b−d were 2.6×10^6, 2.3×10^5, and 2.3×10^4 cfu/mL, respectively. The ions at m/z 3449, 4445, 5029, 5391, 6448, 6854, 7639, and 8962 represent proteins from *E. faecalis*. The ions at m/z 3449, 5391, 6448, 6723, 6854, 7639, and 8962 match proteins present in the protein database (see Table 2). These results indicate that our vancomycin-immobilized magnetic nanoparticles are capable of probing the presence of *E. faecalis* in aqueous solutions and that the target bacteria trapped by the magnetic nanoparticles can be characterized by MALDI-MS.

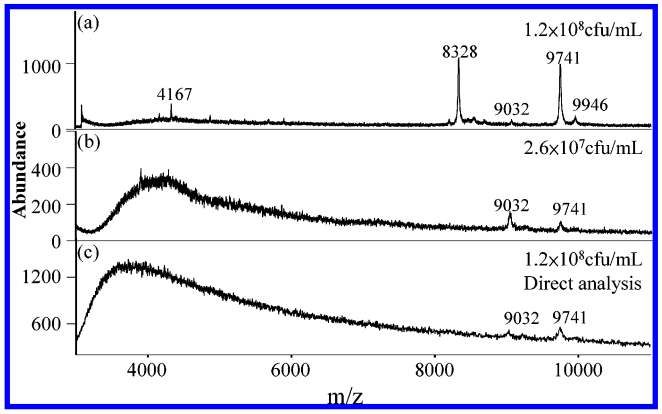

Figure 7. MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria from aqueous sample solutions (3 mL) containing *E. coli* JM 109 at concentrations of (a) 1.2×10^8 and (b) 2.6×10^7 cfu/mL. (c) MALDI mass spectrum recorded directly from *E. coli* JM 109 (1.2×10^8 cfu/mL, 0.5 μL).

Table 3. Peaks Obtained from the MALDI Mass Spectrum of *E. Coli* JM 109 and Their Matched Proteins in the Protein Database

observed mass	protein in SwissPROT	mass (expt)	description
8328	P05794	8328	dihydrofolate reductase type II
9032	Q93QL2	9034	CopB
9741	Q8XAC1	9742	excisionase
9946	P39296	9949	hypothetical protein

Vancomycin is believed to have a high affinity toward the cell surfaces of Gram-positive bacteria because of the five hydrogen bonds depicted in Figure 1. We were interested in examining the capacity of our nanoscale particles to selectively probe Gram-negative bacteria from a sample solution. *E. coli* JM 109, a Gram-negative bacterium, was used as the sample for examination. Figure 7a presents the MALDI mass spectrum obtained after employing our vancomycin-modified magnetic nanoparticles as affinity probes to concentrate target bacteria from an aqueous solution of *E. coli* JM 109 (1.2×10^8 cfu/mL, 3 mL). The ions that appear in the mass spectrum at m/z 4167, 8328, 9032, 9741, and 9946 represent *E. coli* JM 109; Table 3 lists the corresponding proteins identified from the protein database. When the cell concentration of *E. coli* JM 109 in the sample solution was lowered to 2.6×10^7 cfu/mL, we observed only the ions at m/z 9032 and 9741 in the corresponding mass spectrum (Figure 7b). In comparison, the MALDI mass spectrum obtained directly from an *E. coli* JM 109 solution (1.2×10^8 cfu/mL, 0.5 μL) is similar to that presented in Figure 7b. That is to say, although the vancomycin-immobilized magnetic nanoparticles are capable of trapping *E. coli* JM 109, a Gram-negative bacterium, from the sample solution, the trapping capacity is much lower than that exhibited toward Gram-positive bacteria.

Figure 8 presents the MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles in an effort to trap target bacteria selectively from sample solutions containing mixtures of *E. coli* JM 109 (Gram-negative) and *S. aureus* (Gram-positive) at various cell concentrations. When the cell concentration of *E. coli* JM 109 (1.2×10^8 cells/mL) in the sample solutions was 1 or 2 orders of magnitude higher than that

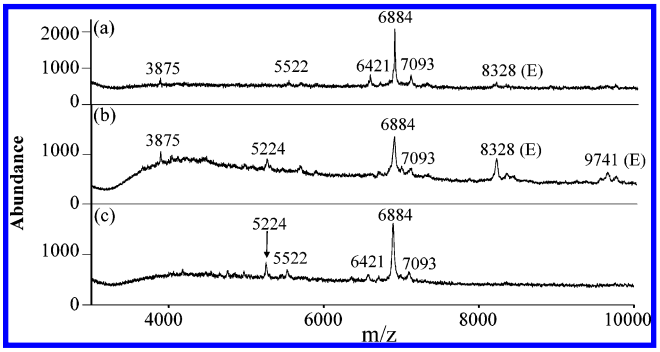

Figure 8. MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria from aqueous sample solutions (3 mL) containing (a) 1.2×10^8 cfu/mL *E. coli* JM 109 and 4.3×10^6 cfu/mL *S. aureus*, (b) 1.2×10^8 cfu/mL *E. coli* JM 109 and 4.5×10^5 cfu/mL *S. aureus*, and (c) 2.6×10^7 cfu/mL *E. coli* JM 109 and 4.5×10^5 cfu/mL *S. aureus*. The peaks marked with the letter "E" represent ions generated from *E. coli* JM 109.

of *S. aureus* (4.3×10^6 or 4.5×10^5 cells/mL, respectively), the MALDI mass spectra (Figure 8a and b, respectively) displayed ions at m/z 3875, 5522, 6421, 6884, and 7093 generated from *S. aureus*, but only two ions, at m/z 8328 and 9741 (marked with the letter "E"), that arose from *E. coli* JM 109 cells. That is to say, even at large excesses of *E. coli* JM 109, the nanoparticles seem to bind to the *S. aureus* cells selectively. When the cell concentration of *E. coli* JM 109 was lowered to 2.6×10^7 cfu/mL, while maintaining the cell concentration of *S. aureus* at 4.5×10^5 cfu/mL, we observed in the mass spectrum only the ions at m/z 5224, 5522, 6421, 6884, and 7093 that were generated from *S. aureus* (Figure 8c); i.e., no ions generated from *E. coli* JM 109 were present. The results indicate that the vancomycin-immobilized magnetic nanoparticles may have quite good selectivity toward *S. aureus* even when a high concentration of *E. coli* JM 109 cells is present in the sample solution. We must bear in mind, however, that it is also possible for *E. coli* JM 109 to have a lower ionization efficiency during MALDI-MS analysis than does *S. aureus*, which would lead to the observation of fewer peaks for the former species in the mass spectra. We employed absorption spectrometric analysis to further confirm whether the vancomycin-immobilized magnetic nanoparticles were more specific toward Gram-positive bacteria than Gram-negative ones. If the bacterial cells in a sample solution are selectively trapped by the vancomycin-immobilized magnetic nanoparticles, the solution that remains—after employing a magnetic field to remove the nanoparticles conjugated with target bacterial cells—should have a lower optical density at 600 nm than that of the original bacterial solution. Thus, the changes in the optical densities of sample solutions before and after extraction by the nanoprobes provide the relative capacities of vancomycin-immobilized magnetic nanoparticles toward the different bacteria. Furthermore, on the basis of the experimental results, we estimate that an optical density of 1 corresponds to an *S. aureus* concentration of 3.5×10^8 cfu/mL, *S. saprophyticus* at 3.8×10^8 cfu/mL, *E. faecalis* at 5.5×10^8 cfu/mL, and *E. coli* JM 109 at 1.1×10^8 cfu/mL. Panels a–d in Figure 9 display the UV absorption spectra of *S. aureus*, *E. faecalis*, *S. saprophyticus*, and *E. coli* JM 109, respectively, before and after extraction using the vancomycin-immobilized magnetic nanoparticles. The solid lines present the UV absorptions of the bacterial solutions before incubation with the vancomycin-immobilized

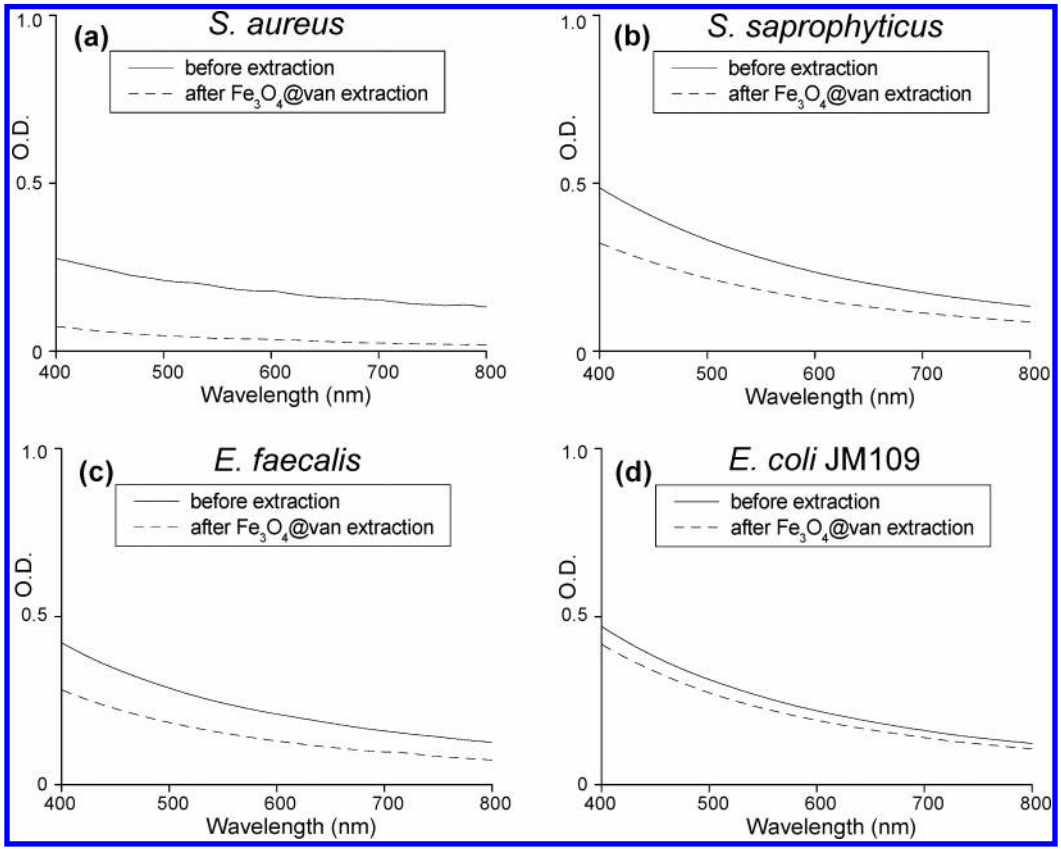

Figure 9. UV absorption spectra of (a) *S. aureus* (0.1 mg/mL, 3 mL), (b) *S. saprophyticus* (0.1 mg/mL, 3 mL), (c) *E. faecalis* (0.1 mg/mL, 3 mL), and (d) *E. coli* JM 109 (0.1 mg/mL, 3 mL) sample solutions recorded before (solid lines) and after (dotted lines) extraction for 1 h using the vancomycin-immobilized magnetic nanoparticles (20 μL, 0.424 mg/mL).

magnetic nanoparticles, while the dotted lines display those of the remaining solutions after incubation for 1 h and employing a magnet to remove the magnetic nanoparticles. Obviously, the absorption at 600 nm of the remaining solutions of *S. aureus*, *S. saprophyticus*, and *E. faecalis* decreased significantly after incubation with the vancomycin-immobilized magnetic nanoparticles (Figure 9a−c). In contrast, the optical density at 600 nm of the remaining solution of *E. coli* JM 109 decreased only slightly (Figure 9d). These results indicate that the vancomycin-immobilized magnetic nanoparticles are more specific toward *S. aureus*, *E. faecalis*, and *S. saprophyticus*, i.e., Gram-positive bacteria, than they are toward *E. coli* JM 109 (a Gram-negative bacterium).

Additionally, the vancomycin-immobilized magnetic particles can be employed to trap a mixture of Gram-positive bacteria. Figure 10 presents the MALDI mass spectrum obtained when vancomycin-immobilized magnetic particles were used to probe a mixture (3 mL) of *S. aureus* (3.4×10^6 cfu/mL) and *E. faecalis* (1.3×10^6 cfu/mL). The peaks at m/z 3875, 5028, 5522, 6345, 6421, 6884, 7564, and 8144 that are marked with the letter "S" represent *S. aureus*, while the peaks at m/z 3449, 4304, 4445, 5391, 7639, 8962, and 9708 that are marked with the letter "E" represent *E. faecalis* (cf. Figures 4 and 6). The results indicate that both of these Gram-positive bacteria can be concentrated in the same sample solution.

To simulate real conditions, we spiked *S. saprophyticus*, a pathogen that commonly infects the urinary tract of young women, into a sample of urine. Figure 11 presents the MALDI mass spectra obtained after employing the vancomycin-modified magnetic nanoparticles as affinity probes to concentrate the traces of *S. saprophyticus* bacteria from the urine samples. The cell concentrations of *S. saprophyticus* in the samples used to obtain the spectra in Figure 11a−c were 5.8×10^6, 7.4×10^5, and 7.4×10^4 cfu/mL, respectively. For the sake of comparison, Figure 11d displays the MALDI mass spectrum obtained after using the vancomycin-modified probe to trap target analytes from the urine blank. The ions at m/z 3892, 4369, 4611, 4756, 5034, 6094, and 8550 (marked with the letter "U") were all generated from the complex urine matrix. In addition of the interfering "U" ions in

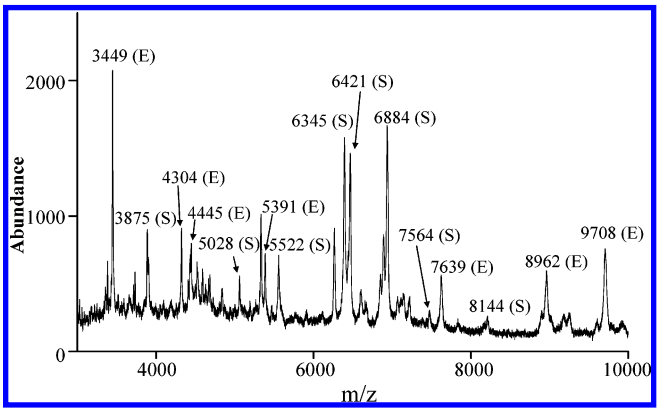

Figure 10. MALDI mass spectrum obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria in a sample solution (3 mL) containing *S. aureus* (3.4×10^6 cfu/mL) and *E. faecalis* (1.3×10^6 cfu/mL). The peaks marked with the letter "S" were generated from *S. aureus*, while the peaks marked with the letter "E" were generated from *E. faecalis*.

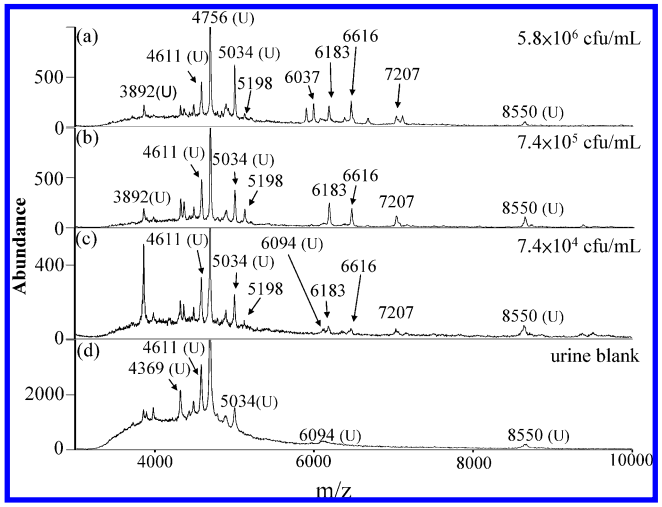

Figure 11. MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria in samples of urine solution (3 mL) containing *S. saprophyticus* at concentrations of (a) 5.8×10^6, (b) 7.4×10^5, and (c) 7.4×10^4 cfu/mL. (d) MALDI mass spectrum obtained directly from the urine solution. The peaks marked with the letter "U" represent ions concentrated from urine.

the lower-mass region, ions at m/z 5198, 6037, 6183, 6616, and 7207, representing *S. saprophyticus*, appear in the higher-mass region (cf. the appearance of the mass spectra presented in Figure 3). The lowest detection limit of *S. saprophyticus* was $\sim 7.4 \times 10^4$ cfu/mL from a 3-mL sample of urine solution; this limit is comparable to the value obtained from the aqueous solutions (cf. Figure 3c). These results confirm that the vancomycin-modified nanoscale probes have a high specificity toward *S. saprophyticus*.

Panels a−c in Figure 12 present the MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to selectively concentrate *S. aureus* bacteria, at concentrations of 4.8×10^6, 7.8×10^5, and 7.8×10^4 cfu/mL, respectively, from urine samples. By comparison with the ions generated from *S. aureus* in the mass spectra presented in Figure 4, we assign the ions at m/z 3875, 5522, 6345, 6421, 6884, 7474, and 8883 as being generated from *S. aureus*. The remaining ions, at m/z 4369, 4756, 5034, and 6094 marked with the letter "U", derive from the complex urine matrix (cf. Figure 11d). The lowest detection limit of *S. aureus* was $\sim 7.8 \times 10^4$ cfu/mL from a 3-mL sample of urine solution. Although interference from the complex matrix in urine could not be removed totally from the spectra in either Figure 11 or 12, it is still possible to identify the microorganism species based on the limited number of ion peaks. It is possible that endogenous Gram-positive bacteria may be present in urine samples, but usually they have concentrations of $<10^4$ cfu/mL; i.e., they are present at concentrations lower than the current approach can detect.

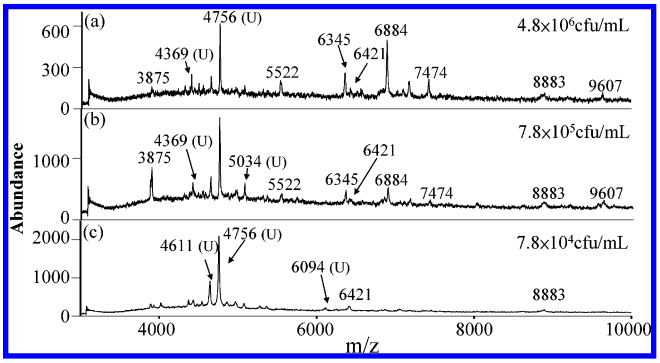

Figure 12. MALDI mass spectra obtained after using the vancomycin-immobilized magnetic nanoparticles to probe target bacteria in samples of urine solution (3 mL) containing *S. aureus* at concentrations of (a) 4.8×10^6, (b) 7.8×10^5, and (c) 7.8×10^4 cfu/mL. The peaks marked with the letter "U" represent ions concentrated from urine.

CONCLUSIONS

Gram-positive bacteria, such as *S. saprophyticus*, *S. aureus*, and *E. faecalis*, can be concentrated effectively from aqueous solution by the use of vancomycin-immobilized magnetic nanoparticles. Furthermore, these nanoscale probes are capable of selectively binding a Gram-positive bacterium, *S. aureus,* in the presence of a high concentration of Gram-negative *E. coli* JM 109 cells in a sample solution. Additionally, we have demonstrated that the nanoparticles can be used to trap *S. saprophyticus* and *S. aureus* selectively from urine samples. Although impurities from the complex urine matrix were not eliminated totally from the MALDI mass spectrum when this trapping treatment process was used, the limited number of ions generated from the bacteria suggests that this method is suitable for the rapid identification of a microorganism's species. Combining the concentration of Gram-positive pathogens selectively on vancomycin-immobilized magnetic nanoparticles with the sensitivity of MALDI-MS analysis makes it possible to rapidly identify trace pathogens in urine samples. Nevertheless, further improvements in this approach, e.g., by discovering appropriate methods to effectively reduce the influence of complex biological fluids, may allow us to lower the detection limit.

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