Identification of *Pseudomonas aeruginosa* using functional magnetic nanoparticle-based affinity capture combined with MALDI MS analysis†

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PA-IL is a galactophilic lectin that is found on the outer membrane of *Pseudomonas aeruginosa*. Pigeon ovalbumin (POA), a phosphoprotein, contains high levels of terminal Galα(1→4)Gal units. Thus, magnetic nanoparticles with immobilized POA can be used as affinity probes for *P. aeruginosa*, functioning *via* the recognition of galactophilic PA-IL. We fabricated POA-bound nanoparticles (NPs) by immobilizing POA onto the surface of core/shell magnetic iron oxide/alumina NPs *via* metal–phosphate chelation. We then used the generated NPs to probe target bacteria from complex samples. The trapped bacterial cells were characterized based on their mass peak profiles obtained from MALDI MS analyses. In addition, we confirmed the determination of *P. aeruginosa* using a proteomic strategy: combining the resultant MALDI MS/MS spectra of its tryptic digest with protein database searching. The feasibility of using this approach to rapidly characterize *P. aeruginosa* from clinical samples without the need to perform culturing steps was also demonstrated.

Introduction

Pseudomonas aeruginosa is an opportunistic bacterial species that is notorious for causing human infections; it is commonly found in urinary tract infections.[1] Because conventional microbiological-examination is quite time-consuming and labor-intensive, we became interested in developing a method that is capable of rapidly identifying this bacterium. Previous approaches toward the rapid detection and identification of *P. aeruginosa* have included the use of the polymerase chain reaction,[2–5] capillary electrophoresis,[6] immunoassays,[7] and Fourier transform infrared spectroscopy.[8] When using these methods, sample pretreatment prior to detection is generally required to reduce the degree of interference during analysis. Affinity-based approaches that target bacteria are often employed for bacterial sample pretreatment.[9–18] Lectins,[9–11] immunoglobulin G (IgG),[12,13] and vancomycin[14–18] have all been used to functionalize the surfaces of affinity substrates because of their specific interactions with their target bacteria.

Several metal oxides, including titanium oxide,[19–24] aluminium oxide,[20,25,26] and zirconium oxide,[27,28] are capable of binding phosphorylated proteins and peptides through monodentate, bidentate, and tridentate interactions between their metal ion centers and the phosphate units.[29] Thus, the linking of phosphorylated molecules onto the surfaces of these metal oxides can be readily achieved through self-assembly. The feasibility of immobilizing biomolecules containing phosphate groups onto alumina-containing substrates, through alumina–phosphate chelation, has been demonstrated,[30–32] with such complexes being stable over a wide pH range.[30]

Pigeon ovalbumin (POA), a phosphoprotein, contains high levels of terminal Galα(1→4)Gal units.[33,34] The fabrication of POA-presenting alumina-coated magnetic iron oxide nanoparticles (POA-Fe_3O_4@Al_2O_3 NPs) is simply achieved by mixing POA with Fe_3O_4@Al_2O_3 NPs; the POA molecules are readily appended onto the surfaces of Fe_3O_4@Al_2O_3 NPs *via* phosphate–aluminium chelation.[32] The conjugation can be accomplished rapidly – within 30 s – under microwave irradiation.[32] Such POA-Fe_3O_4@Al_2O_3 NPs have been used previously as affinity probes to selectively trap P-fimbriated *Escherichia coli* from complex samples, because the P fimbriae of *E. coli* have specificity toward Galα(1→4)Gal units.[32] *P. aeruginosa* produces several lectins, including the galactophilic PA-IL and the mannose- and fucose-binding PA-IIL,[35,36] so we were interested in whether we could extend the applicability of the POA-Fe_3O_4@Al_2O_3 NPs as affinity probes to target *P. aeruginosa* in complex samples. We expected that the strains of bacteria trapped by the NPs could be rapidly characterized based on their mass peak profiles obtained from MALDI MS analyses or by employing proteomics-based strategies,[37,38] *i.e.* by matching the biomarker ions obtained from the tryptic digests of the bacteria and their resultant MS/MS data to their parent proteins *via* protein database searching. Previously, we demonstrated the effectiveness of performing the tryptic digestion of bacteria in the presence of iron oxide NPs under microwave irradiation.[38] To shorten the analysis time, we also employed microwave-assisted bacterial tryptic digestion in this study.

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Experimental

Reagents and materials

Iron(II) chloride tetrahydrate, non-ionic detergent (Nonidet P-40), and aqueous ammonium hydroxide were obtained from Fluka (Germany); hydrochloric acid (HCl) and sodium silicate were obtained from J. T. Baker (Phillipsburg, NJ). Trypsin, sinapinic acid (SA), tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), PNGase F, and iodoacetic acid (IAA) were obtained from Sigma (St. Louis, MO). Ammonium hydrogen carbonate, trifluoroacetic acid (TFA), iron(III) chloride hexahydrate, and urea were purchased from Riedel-de Haën (Germany). Aluminium isopropoxide and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Aldrich (Germany); tryptic soy broth (TSB), Luria Bertani (LB) broth, and granulated agar were obtained from Becton Dickinson (USA). POA was purified from pigeon egg white, which was kindly provided by Dr James Johnson (Minneapolis Veterans Affairs Medical Center and University of Minnesota, Department of Medicine), who also provided a sample of *E. coli* strain V21, which does not express P fimbriae.[39] Clinical bacterial samples were obtained from Mackay Memorial Hospital (Hsinchu, Taiwan). The utilization of the clinical samples complied with guidelines of the Ethics Committee of the Hospital.

Fabrication of alumina-coated magnetic iron oxide (Fe_3O_4@Al_2O_3) NPs

Magnetic iron oxide NPs were prepared *via* co-precipitation; the procedure has been described previously.[32] $FeCl_3$ (5.4 g) and $FeCl_2$ (2.0 g) were dissolved in 2 M HCl (25 mL) at room temperature under sonication. The mixture was degassed using a vacuum pump and then the flask was filled with N_2 gas after the salts had dissolved completely in the solution. Aqueous NH_3 (28%, 40 mL) was slowly injected into the mixture under nitrogen while stirring for 1 h at room temperature, followed by rinsing with deionized water three times and re-suspending in deionized water (40 mL). The iron oxide NPs (0.2 g) in deionized water (40 mL) were suspended through sonication under N_2 gas. To coat a thin film of SiO_2 onto the surfaces of the magnetic particles, aqueous sodium silicate solution (0.6%, pH 9, 40 mL) was added and then the mixture was stirred for 24 h at 37 °C, followed by rinsing with deionized water (3 × 40 mL) and then re-suspending in deionized water (40 mL). Alumina was then coated on the surface of the NPs by adding aluminium isopropoxide (20 mg). The mixture was sonicated for 30 min at room temperature and then it was placed in a water bath maintained at 80 °C under vigorous stirring for 1 h. The cap of the vial was then opened to release the generated gas (2-propanol) and the mixture was heated at 90 °C in a water bath for 30 min. The reaction vial was then loosely capped and the mixture heated at 90 °C for 2.5 h. After the mixture had cooled to room temperature, the Fe_3O_4@Al_2O_3 NPs were isolated through magnetic collection, rinsing with water (3 × 40 mL), and then re-suspension in water (40 mL). The suspension was stored in a refrigerator prior to use.

Fabrication of POA-bound Fe_3O_4@Al_2O_3 NPs

Using the Langmuir adsorption model (results not shown), the dissociation constant (k_d) for the interaction between POA and the Fe_3O_4@Al_2O_3 NPs was estimated to be *ca.* 1.1×10^{-5} M at pH 6.4. POA molecules were bound to Fe_3O_4@Al_2O_3 NPs immediately prior to performing the trapping experiment. Denatured POA (10^{-4} M) was diluted five-fold with 0.15% aqueous TFA solution. The Fe_3O_4@Al_2O_3 NPs (0.4 mg) were incubated with the solution of denatured POA (2×10^{-5} M, 200 μL) in 0.15% aqueous TFA under microwave irradiation (power: 900 W) for 30 s. The POA-Fe_3O_4@Al_2O_3 NPs were rinsed with deionized water (2 × 200 μL) and then re-suspended in deionized water (100 μL) prior to use.

Elimination of glycans on the POA-Fe_3O_4@Al_2O_3 NPs

Glycans on the NPs were eliminated based on the method presented in a previous report.[34] Briefly, the POA-Fe_3O_4@Al_2O_3 NPs (40 μg) was added with Nonidet P-40 (0.44 μL) in Tris buffer (44 μL, 12.5 mM, pH 7.4) and PNGase F (15 μL, 0.5 unit/μL). The mixture was placed in a water bath maintained at 37 °C for 24 h and then at 100 °C for 5 min. The NPs were isolated magnetically and rinsed by deionized water several times. The generated NPs were then employed as the affinity probes for target bacteria.

Preparation of bacterial samples

Bacterial samples should be prepared in a Biosafety Level 2 (BSL-2) laboratory. *E. coli* strain V21, *Staphylococcus saprophyticus*, and *Staphylococcus aureus* (obtained from the General Tzu-Chi Hospital, Hualien, Taiwan) were cultured in LB agar plates. After overnight incubation at 37 °C, the bacterial cells were transferred to Tris buffer (12.5 mM, pH 7.4, 1 mL). *P. aeruginosa*, *Enterococcus faecium*, and *Enterococcus faecalis* (obtained from the General Tzu-Chi Hospital, Hualien, Taiwan) were cultured in tryptic soy broth with yeast (TSBY) agar plates. 20 TSBY agar plates were prepared by dissolving TSB powder (12 g), yeast extract (2 g), and granulated agar (7.4 g) in deionized water (400 mL). The bacterial concentration was adjusted to the desired level by measuring the optical density (OD) at 600 nm; it was checked by plating serial dilutions of the bacterial samples on LB agar plates and counting the colony forming units (cfu) after overnight incubation at 37 °C by serial dilution quantitative culture. Freshly harvested bacteria after overnight-growth were used directly in all of the experiments described in this study.

Using POA-Fe_3O_4@Al_2O_3 NPs to selectively trap target bacteria

The POA-Fe_3O_4@Al_2O_3 NPs (40 μg) were vortex-mixed with a bacterial sample (1 mL or 0.8 mL) at given OD various concentrations for 30 min. The NP–bacterium conjugates were isolated through magnetic separation and then rinsed with Tris buffer (12.5 mM, pH 7.4, 3 × 1 mL). The isolated conjugates were mixed with SA (15 mg/mL, 2 μL) or CHCA (15 mg/mL, 2 μL). After standing for 3 min, the supernatant (1 μL) was deposited on a MALDI sample plate for MALDI MS analysis.

Melittin and ubiquitin were used as internal standards (i.s.) for mass calibration.

When tryptic digestion of the bacteria trapped by the NPs was performed, the bacteria were eluted prior to digestion by elution containing the globotriose derivative Galα(1→4)Galβ(1,4)-Gluβ(O→$(CH_2)_6$Cl) (Gb_3 derivative). Preparation of the Gb_3 derivative was based on published procedure.[40] The Gb_3 derivative solution (6 mg/mL, 1 μL) was mixed with the NP–target bacteria conjugates to release the bacteria from the NPs under microwave-heating (power: 900 W) for 90 s. The elution solution was then mixed with a solution of the Fe_3O_4@Al_2O_3 NPs (25 μg) and incubated in a microwave oven (power: 900 W) for 90 s. The NP–bacteria conjugates were isolated by magnetic separation. After rinsing, the conjugates were added with trypsin (18.75 μg, 1 μL) prepared in ammonium hydrogencarbonate (50 mM). The mixture was incubated in a microwave oven (power: 900 W) for 1 min. The cap of the sample vial was kept open during microwave-heating. The resulting solution was mixed with CHCA (25 mg/mL, 1 μL) containing 1% TFA for MALDI MS (or MS/MS) analysis. Mass spectra were calibrated using Bruker Daltonics peptide calibration reagents (M_w = 700–3200 Da) as internal standards.

Database searches

Fragment peaks (S/N > 3) resulting from precursor ions (S/N > 3) were submitted *via* Biotools (v. 3.0) to MASCOT (www.matrixscience.com) using the following search parameters: the database searched was NCBInr 20090109; taxonomy was limited to eubacteria; the enzyme was trypsin; MS and MS/MS tolerances were set at ±0.7 and ±1.0 Da, respectively; the number of missed cleavages was set to 1; and the significance threshold was set at $p < 0.05$ because the score was over 50. Only one MS/MS spectrum resulting from a precursor ion was searched in the protein database for each iteration of the procedure.

Instrumentation

All mass spectra were obtained using an Bruker Daltonics Autoflex III MALDI TOF MS/MS spectrometer (Germany) equipped with a 355 nm Nd:YAG laser. The laser intensity was set in the range of *ca.* 40–50% to obtain the optimized mass spectra. The absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Melbourne, Australia).

Results and discussion

Mass peak profiles of the bacteria trapped by the POA-Fe_3O_4@Al_2O_3 NPs obtained from MALDI MS analysis were used to confirm their identities. Thus, we first examined the mass peak profiles of the model bacteria, including *P. aeruginosa*, *S. aureus*, *S. saprophyticus*, and *E. coli* V21, through direct MALDI MS analysis. Panels (a)–(d) in Fig. 1 display the direct MALDI mass spectra of the intact cells of *P. aeruginosa*, *S. aureus*, *S. saprophyticus*, and *E. coli* V21, respectively. Potential biomarker peaks representing these bacteria appear in the region from *m/z* 4000 to 10 000. The peak at *m/z* 8565, marked 'i.s.', was derived from ubiquitin, which we added as an internal standard for mass calibration. The mass peak profiles for these four bacterial strains are all very different.

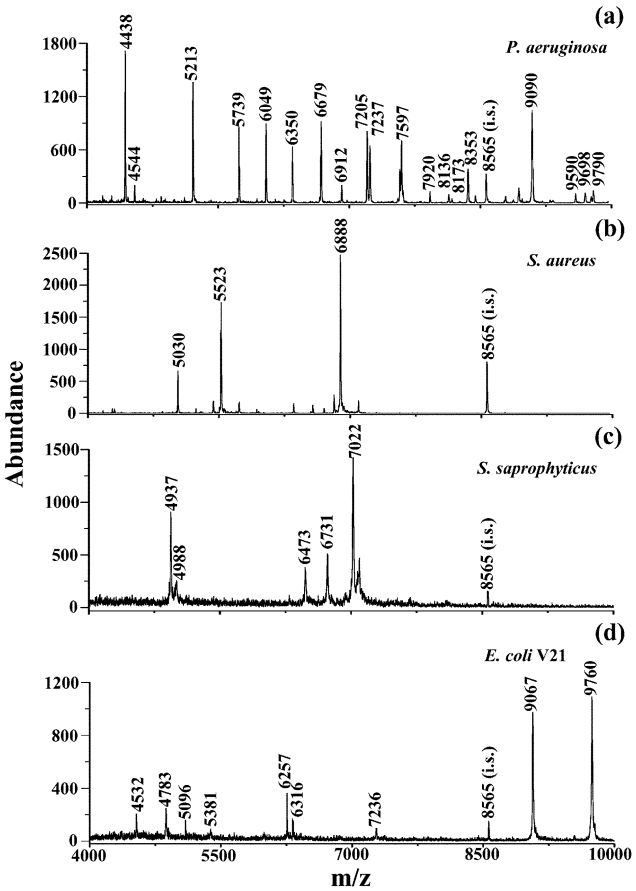

Fig. 1 Direct MALDI mass spectra of (a) *P. aeruginosa* (7.6×10^8 cells/mL, 1 μL), (b) *S. aureus* (8.9×10^8 cells/mL, 1 μL), (c) *S. saprophyticus* (5.0×10^8 cells/mL, 1 μL), and (c) *E. coli* V21 (5.0×10^8 cells/mL, 1 μL).

To demonstrate the selectivity of the POA-Fe_3O_4@Al_2O_3 NPs toward *P. aeruginosa*, we examined a sample containing *S. aureus* and *P. aeruginosa*. Fig. 2a displays the direct MALDI mass spectrum of the mixture (1 μL) containing *S. aureus* (3.0×10^8 cells/mL) and *P. aeruginosa* (1.1×10^8 cells/mL). Because the cell concentration of *S. aureus* in the sample was higher than that of *P. aeruginosa*, the mass spectrum presents peaks at *m/z* 5030, 5523, and 6888 attributable to *S. aureus* (*cf.* Fig. 1b). Also, ion suppression between peaks may have been involved during the analysis. When using POA-Fe_3O_4@Al_2O_3 NPs to selectively trap the target bacteria from the same sample, the MALDI mass spectrum (Fig. 2b) displays peaks at *m/z* 4437, 5211, 6047, 6348, 6676, 7203, 7236, 7918, 8174, and 8356 that match those derived from *P. aeruginosa* (*cf.* Fig. 1a). The matched masses of these peaks may have mass differences of ±3 because of the limited mass resolution of high masses when operating the spectrometer in the linear mode. Thus, these spectra reveal that the POA-Fe_3O_4@Al_2O_3 NPs have specificity for *P. aeruginosa*. Because the binding between *P. aeruginosa* and POA is assumed arising between the galactose on POA of the POA-Fe_3O_4@Al_2O_3 NPs and galactophilic PA-IL, we also preformed a control experiment by eliminating the glycans from the POA[34] followed by employing the NPs to trap target bacteria from the same sample as used for obtaining Fig. 2b. MALDI MS analysis was then

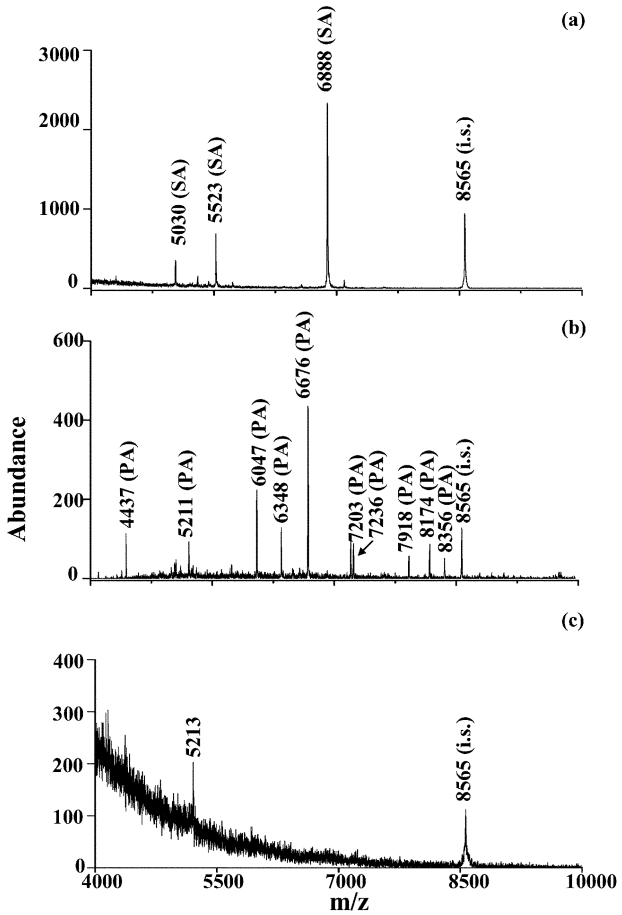

Fig. 2 (a) Direct MALDI mass spectrum of a mixture (1 μL) of *S. aureus* (3.0×10^8 cells/mL) and *P. aeruginosa* (1.1×10^8 cells/mL). (b) MALDI mass spectrum obtained after using the POA-Fe_3O_4@Al_2O_3 NPs (40 μg) as affinity probes to selectively enrich target bacterial cells from the mixture (0.5 mL) used to obtain panel (a). (c) MALDI mass spectrum obtained after using the PNGase F-treated POA-Fe_3O_4@Al_2O_3 NPs (40 μg) as affinity probes to selectively enrich target bacterial cells from the mixture (0.5 mL) used to obtain the result shown in panel (a). The peak marked 'i.s.' represents ubiquitin, used as a standard for mass calibration. The peaks marked 'SA' and 'PA' were derived from *S. aureus* and *P. aeruginosa*, respectively.

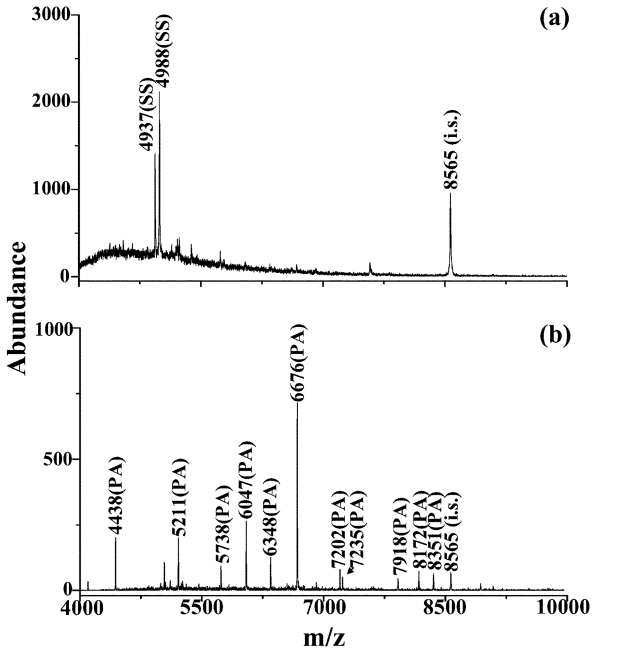

Fig. 3 (a) Direct MALDI mass spectrum of a mixture (1 μL) of *S. saprophyticus* (3.20×10^8 cells/mL) and *P. aeruginosa* (1.13×10^8 cells/mL). (b) MALDI mass spectrum obtained after using the POA-Fe_3O_4@Al_2O_3 NPs (40 μg) as affinity probes to selectively enrich target bacterial cells from the mixture (0.5 mL) used to obtain panel (a). The peak marked 'i.s.' represents ubiquitin, used as a standard for mass calibration. The peaks marked 'SS' and 'PA' were derived from *S. saprophyticus* and *P. aeruginosa*, respectively.

conducted. Fig. 2c presents the resultant MALDI mass spectrum. Apparently, the peaks derived from *P. aeruginosa* as shown in Fig. 2b do not appear in this mass spectrum, while only a peak at *m/z* 5213 with weak intensity, likely derived from *P. aeruginosa*, appears in the mass spectrum. The results indicate that the presence of the glycans on the NPs is essential for the binding between the bacteria and the POA-Fe_3O_4@Al_2O_3 NPs.

S. saprophyticus is a Gram-positive bacterial species that is found commonly in urinary tract infections.[41] We examined the selectivity of our probes for *P. aeruginosa* in the presence of *S. saprophyticus*. Fig. 3a displays the direct MALDI mass spectrum of a mixture (1 μL) containing *S. saprophyticus* (3.2×10^8 cells/mL) and *P. aeruginosa* (1.1×10^8 cells/mL). The peaks at *m/z* 4937 and 4988, which match those derived from *S. saprophyticus* (*cf.* Fig. 1c), dominate the mass spectrum, presumably because the cell concentration of *S. saprophyticus* was higher than that of *P. aeruginosa* in the sample. After selective enrichment using the POA-Fe_3O_4@Al_2O_3 NPs, peaks marked 'PA' appear in the mass spectrum (Fig. 3b); they matched those derived from *P. aeruginosa* (*cf.* Fig. 1a). We observed no ions matching those derived from *S. saprophyticus*. These results indicate that the POA-Fe_3O_4@Al_2O_3 NPs can concentrate *P. aeruginosa* specifically from bacterial mixtures, even at higher cell concentrations of *S. saprophyticus*.

E. coli V21 does not express P fimbriae.[39] We used a mixture of *P. aeruginosa* and *E. coli* V21 to examine the selectivity of the POA-Fe_3O_4@Al_2O_3 NPs toward *P. aeruginosa*. Fig. 4a displays the direct MALDI mass spectrum of this mixture. The peaks at *m/z* 5096, 5382, 6258, 6317, 7235, and 9066 that are marked 'V21' match those derived from *E. coli* V21 (*cf.* Fig. 1d). The remaining peaks, marked 'PA' were derived from *P. aeruginosa*. In contrast, after using the POA-Fe_3O_4@Al_2O_3 NPs to selectively trap the bacteria from the mixture, the peaks appearing in the resulting mass spectrum were all derived from *P. aeruginosa* (Fig. 4b). Again, these results demonstrate that our probe has great specificity for *P. aeruginosa*.

In addition to *S. aureus* and *S. saprophyticus*, *E. faecium* and *E. faecalis* are also commonly found in urinary tract bacterial infections. Thus, we investigated their binding affinity with the POA-Fe_3O_4@Al_2O_3 NPs by investigating the trapping capacity of the NPs toward these bacteria. Fig. 5 displays the trapping capacity (cells/mg) of the probes for four bacterial strains: *E. coli* V21, *E. faecium*, *E. faecalis*, and *P. aeruginosa*. The trapping capacity of the probes for *P. aeruginosa* (*ca.* 8.5×10^7 cells/mg)

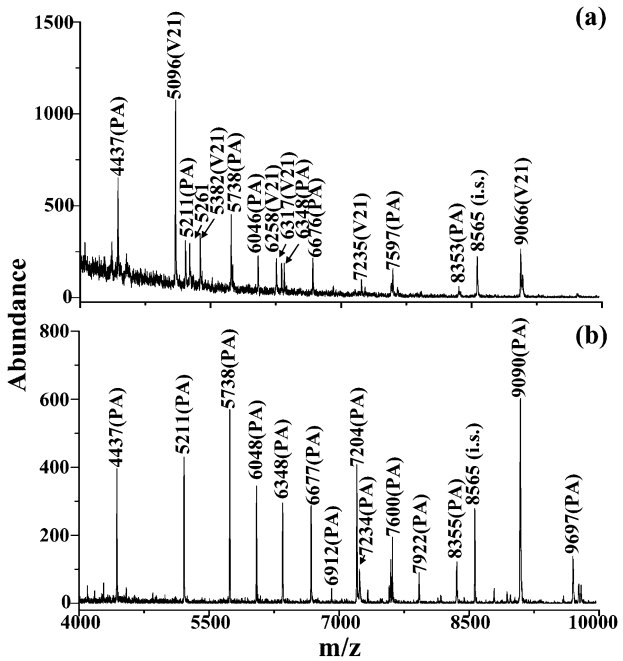

Fig. 4 (a) Direct MALDI mass spectrum of a mixture (1 μL) of *E. coli* V21 (2.26×10^8 cells/mL) and *P. aeruginosa* (1.28×10^8 cells/mL). (b) MALDI mass spectrum obtained after using the POA-Fe_3O_4@Al_2O_3 NPs (40 μg) as affinity probes to selectively enrich target bacterial cells from the mixture (0.5 mL) used to obtain panel (a). The peak marked 'i.s.' represents ubiquitin, used as a standard for mass calibration. The peaks marked 'V21' and 'PA' were derived from *E. coli* and *P. aeruginosa*, respectively.

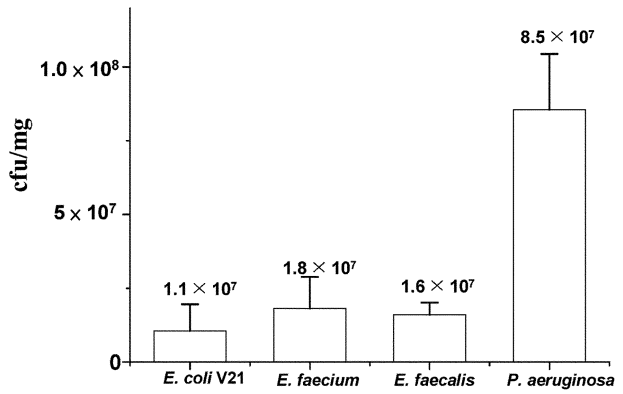

Fig. 5 Bar graph presenting the trapping capacity of the POA-Fe_3O_4@Al_2O_3 NPs toward their target bacteria.

was much higher than those for the other three types of bacteria. Thus, we conclude that the binding affinity of the NPs toward *P. aeruginosa* is much higher than it is toward other bacterial strains.

To confirm our results, we employed an NP-based proteomics strategy[39] to characterize the bacteria targeted by the POA-Fe_3O_4@Al_2O_3 NPs from a bacterial sample containing *P. aeruginosa*. After the bacteria had been trapped and isolated using the POA-Fe_3O_4@Al_2O_3 NPs, they were eluted from the Fe_3O_4@Al_2O_3 NPs using a Galα(1–4)Gal terminated trisaccharide (Gb_3), subjected to tryptic digestion under microwave irradiation (1 min), and then submitted to MALDI MS analysis. Fig. 6a presents the MALDI mass spectrum of the tryptic digest of the bacteria trapped by the POA-Fe_3O_4@Al_2O_3 NPs. The peaks at *m/z* 1234.2, 1406.8, 1464.2, 1630.6, and 1728.9, marked 'PA', are derived from *P. aeruginosa*. To confirm that the digestion was complete, the inset displays the mass spectrum in the region from *m/z* 4000 to 10 000. The mass peak profile representing *P. aeruginosa* (*cf.* Fig. 1a) was absent, suggesting that the proteins that should have provided the peaks in this region had been digested to smaller peptides after tryptic digestion. In addition, we suspect that the peaks at *m/z* 2203 and 2233 were derived from POA. Although we had specifically desorbed the

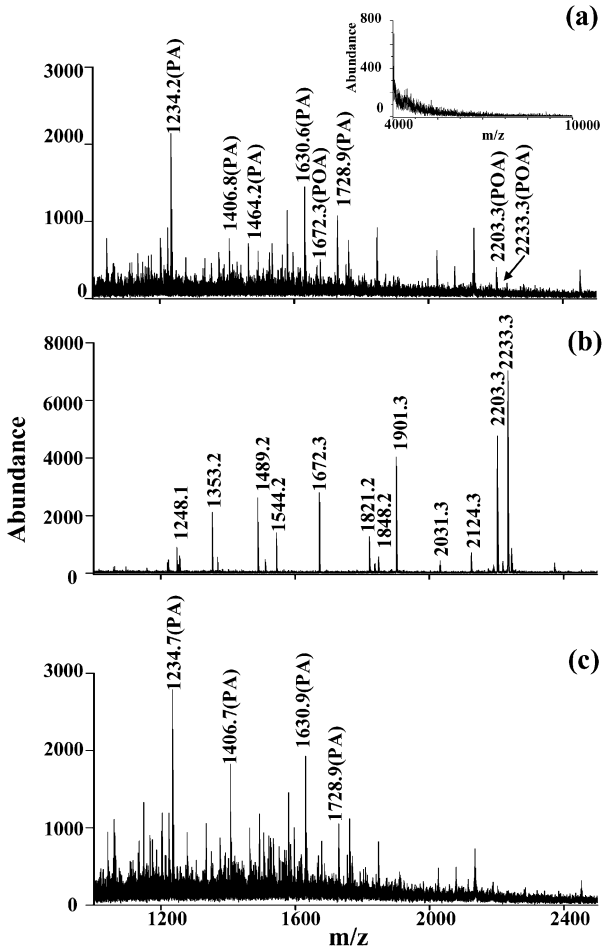

Fig. 6 (a) MALDI mass spectrum of the tryptic digest of the bacteria targeted by POA-Fe_3O_4@Al_2O_3 NPs (40 μg) from a solution (1 mL) containing *P. aeruginosa* (1.0×10^8 cells/mL, 1 mL). The bacteria were eluted from the NPs using Gb_3 prior to tryptic digestion. Bacterial tryptic digestion was performed in the presence of the Fe_3O_4@Al_2O_3 NPs under microwave-heating (power: 900 W) for 1 min in an Eppendorf tube. The inset shows the mass spectrum at the region of *m/z* 4000–10 000. (b) MALDI mass spectrum of the tryptic digest of the POA-Fe_3O_4@Al_2O_3 NPs (10 μg) under microwave-heating (power, 900 W) for 1 min. (c) MALDI mass spectrum of the tryptic digest of the bacteria targeted by POA-Fe_3O_4@Al_2O_3 NPs (40 μg) from a sample (0.8 mL) containing *S. saprophyticus* (2.25×10^8 cells/mL) and *P. aeruginosa* (2.75×10^7 cells/mL). The sample treatment was similar to that for obtaining the result shown in panel (a). CHCA (25 mg/mL, 1 μL) containing 1% TFA was used as the MALDI matrix.

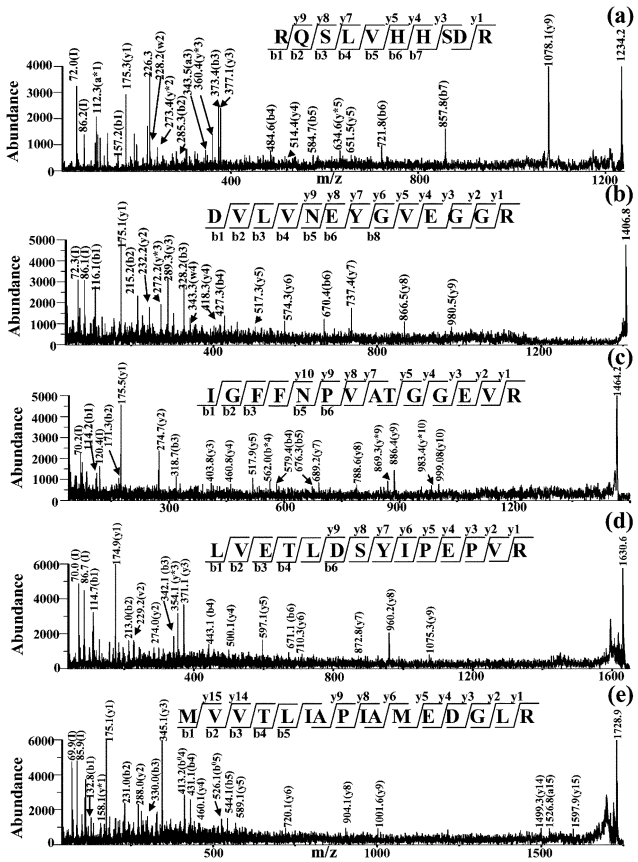

Fig. 7 MALDI MS/MS spectra of the sample used to obtain panel (a) in Fig. 6; the peaks at *m/z* (a) 1234.2, (b) 1406.8, and (c) 1464.2 (d) 1630.6 (e) 1728.9 were selected as precursor ions. CHCA (25 mg/mL, 1 μL) containing 1% TFA was used as the MALDI matrix.

bacteria from the POA-Fe_3O_4@Al_2O_3 NPs, traces of POA might have been present in the elution solution and, therefore, their digestion products appeared in the mass spectrum. Fig. 6b presents the MALDI mass spectrum obtained after performing the tryptic digestion of the POA-Fe_3O_4@Al_2O_3 NPs solely under microwave-heating for 1 min. Many peaks appear in the mass region from *m/z* 1200 to 2400, including two at *m/z* 2203 and 2233 that we confirm were derived from POA. We also employed the sample obtained by employing the POA-Fe_3O_4@Al_2O_3 NPs as affinity probes to trap target bacteria from a mixture containing *S. saprophyticus* and *P. aeruginosa* followed by rinsing and tryptic digestion under microwave-heating for 1 min. The tryptic digestion steps were similar to those for preparing the sample as shown in Fig. 6a. Fig. 6c displays the resultant MALDI mass spectrum of the tryptic digest product. Apparently, the mass spectrum looks similar to that shown in Fig. 6a. The major peaks at *m/z* 1234.7, 1406.7, 1630.9, and 1728.9, as revealed in Fig. 6a, appear in this mass spectrum. The results indicate that our probes were capable of interacting with target bacteria from a bacterial mixture. Furthermore, the identity of the bacteria can be further characterized by employing enzymatic digestion in a very short period of time under the assistance of microwave-heating.

To further confirm the identity of the peaks at *m/z* 1234.2, 1406.8, 1464.2, 1630.6, and 1728.9, we combined MALDI MS/MS with protein database searching. Panels (a)–(e) in Fig. 7 display the MALDI MS/MS mass spectra obtained after selecting the peaks at *m/z* 1234.2, 1406.8, 1464.2, 1630.6, and 1728.9 as precursor ions, respectively. Protein database searching allowed us to identify each peak; Table 1 lists their possible identities and corresponding peptide sequences. These peptides were derived from either the membrane proteins or ribosome proteins of *P. aeruginosa*. The results demonstrate the suitability of using this NP-based proteomic approach to rapidly confirm the presence of *P. aeruginosa* in bacterial samples.

Next, we examined the feasibility of using this approach to investigate the presence of *P. aeruginosa* in a clinical sample. Fig. 8a displays the MALDI mass spectrum of the bacteria obtained after using the POA-Fe_3O_4@Al_2O_3 NPs to selectively trap target species from a clinical sample collected from a patient with urinary tract infection caused by *P. aeruginosa*. The peaks at *m/z* 4437, 5211, 5739, 6049, 6678, and 7597 also appear in Fig. 1a, which suggests that the trapped species was *P. aeruginosa*. To further confirm the identity, the bacteria trapped by the NPs were further digested by trypsin under microwave irradiation for 1 min. Fig. 8b displays the MALDI mass spectrum of the tryptic digestion product. The potential biomarker ions at *m/z* 1234, 1464, and 1630 that we observed in Fig. 6a are also present in this mass spectrum. We confirmed the identity of these three peaks through a combination of MS/MS (see ESI†) and protein database searches. Similar to the results listed in Table 1, these ions also appear to be derived from the proteins of *P. aeruginosa*. Therefore, these results demonstrate that our NP-based proteomic approach is suitable for the direct characterization of

Table 1 Proteins identified in Fig. 7

	AC[a]	Protein	Peptide sequence	MH_{obs}^+	Size (aa)	Score
P. aeruginosa	gi|152989588	Hypothetical protein	RQSLVHHSDR	1234.2	263	65
	gi|152987689	Hypothetical protein			116	
	gi|84321510	Outer membrane protein and related peptidoglycan-associated (lipo)proteins	DVLVNEYGVEGGR	1406.8	331	110
	gi|4186419	Major outer membrane protein OprF precursor			350	
	gi|14195196	30S ribosomal protein S16	IGFFNPVATGGEVR	1464.2	83	98
	gi|15599461	Elongation factor Tu	LVETLDSYIPEPVR	1630.3	397	106
	gi|15599461	Elongation factor Tu	MVVTLIAPIAMEDGLR	1728.9	397	94

[a] AC: accession number.

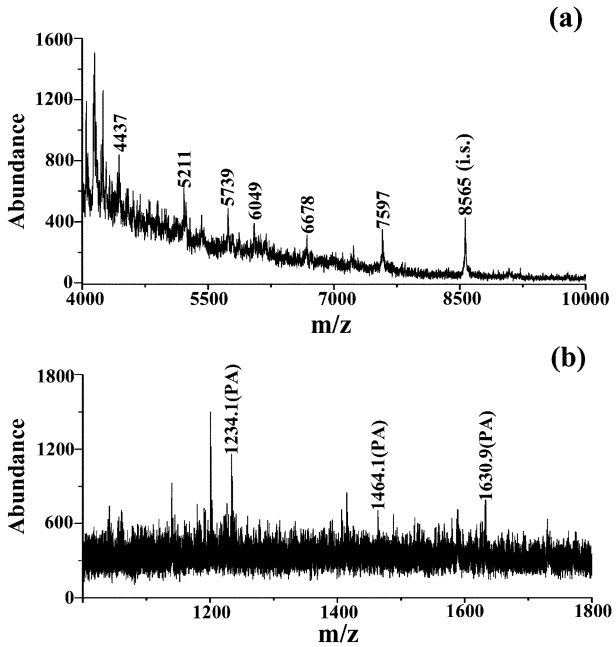

Fig. 8 (a) MALDI mass spectrum obtained after using the POA-Fe_3O_4@Al_2O_3 NPs (40 μg) as affinity probes to selectively enrich target bacterial cells from clinical urine sample (1 mL) diluted with deionized water (0.5 mL) containing *P. aeruginosa*. (b) MALDI mass spectrum of the tryptic digest of the bacteria targeted by the POA-Fe_3O_4@Al_2O_3 NPs from the same sample (1.5 mL) as that used to obtain panel (a). Gb_3 (6 mg/mL, 10 μL) was used to elute the bacteria from the NPs prior to tryptic digestion. Bacterial tryptic digestion was performed in the presence of the Fe_3O_4@Al_2O_3 NPs under microwave-heating (power: 900 W) for 1 min. The peaks marked 'PA' and 'POA' were derived from *P. aeruginosa* and pigeon ovalbumin, respectively.

P. aeruginosa from very complex samples. Note that bacterial cell culturing is not required when using this approach for the analysis of the clinical samples.

Additionally, it has been demonstrated that the POA-Fe_3O_4@Al_2O_3 NPs are also capable of interacting with uropathogenic *E. coli* that generally can cause urinary tract infections. It is curious to know how the resultant MALDI mass spectra look when using the NPs as affinity probes to trap target bacteria from a urine sample containing *E. coli* and *P. aeruginosa*. We spiked these two bacteria in a urine sample and conducted the NP-based trapping experiment. The results demonstrate that the MALDI mass spectra of the samples obtained prior to and after NP-enrichment resemble each other (see Fig. S3 in the ESI†). The peaks derived from *E. coli* J96 and *P. aeruginosa* appear simultaneously in the same mass spectra. The results demonstrate that the POA-Fe_3O_4@Al_2O_3 NPs are capable of enriching both bacteria simultaneously when both bacteria present in the same sample.

Conclusions

We have demonstrated the feasibility of using a functional NP-based affinity approach to selectively concentrate *P. aeruginosa* from complex samples. The targeted *P. aeruginosa* can be identified in two ways, based on (1) its mass peak profile and (2) the presence of its biomarker ions in the tryptic digest obtained after enriching the sample using the affinity probes and then performing MALDI MS analysis. Although most clinical samples are complex, the POA-Fe_3O_4@Al_2O_3 NPs can readily target *P. aeruginosa*, thereby greatly reducing the degree of matrix interference. Furthermore, the need for a time-consuming bacterial culturing step is eliminated; in addition, the bacterial tryptic digestions require only 1 min under microwave irradiation. The short analysis time is the major advantage of this approach. Our results suggest the potential application of this approach for the rapid identification of *P. aeruginosa* in clinical samples.

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