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Lysozyme-encapsulated gold nanocluster-based affinity mass spectrometry for pathogenic bacteria

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RATIONALE: Bacterial infections can be difficult to treat and can lead to irreversible damage to patients if proper treatment is not provided in time. Additionally, the emerging threat from antibiotic-resistant bacterial strains makes medical treatment even more difficult. Thus, rapid identification of infected bacterial strains is essential to assist diagnostics and medical treatment.

METHODS: Lysozymes are glycoside hydrolases that can bind with peptidoglycans on bacterial cell walls. In this work, we demonstrated that lysozyme-encapsulated gold nanoclusters (lysozyme-AuNCs) with red photoluminescence can be used as affinity probes to concentrate pathogenic bacteria. After bacteria had been probed by the lysozyme-AuNCs in a sample solution, the lysozyme-AuNC-bacteria conjugates were readily spun down at a low centrifugation speed. The red emission from the AuNCs on the conjugates could be visualized with the naked eye under illumination of ultraviolet light. The bacteria in the conjugates can be identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) combined with principal component analysis (PCA).

RESULTS: We demonstrated that pathogenic bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, pandrug-resistant *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, and vancomycin-resistant *Enterococcus faecalis* (VRE) can be readily concentrated by the lysozyme-AuNCs and distinguished by the results combining MALDI-MS and PCA. Additionally, the possibility of using the current approach to differentiate *E. faecalis* from VRE was also demonstrated. The lowest detection concentration for *E. coli* using the current approach is ~10^6 cells/mL.

CONCLUSIONS: The results indicated that the lysozyme-AuNCs are effective affinity probes for Gram-positive and Gram-negative bacteria. By combining the results from MALDI-MS and PCA, different bacteria can be easily distinguished. The current approach can be potentially used to assist the identification of bacteria from biological fluids.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a useful tool for the rapid characterization of microorganisms[1–11] such as bacteria[1–8] and fungi,[9–11] which can cause infections or food poisoning. However, bacterial samples obtained from infected biological fluids or food poisoning samples are difficult to be characterized directly by MALDI-MS because the ions generated from complex sample matrices may seriously suppress the ions generated from bacterial cells. Thus, cell culturing or sample pretreatment is generally required to obtain sufficient amounts of bacteria and minimize the number of undesirable species prior to MALDI-MS analysis. However, cell culturing is time consuming. Sample pretreatment through selective concentration of bacteria from complex samples using affinity approaches can shorten analysis time. Functional magnetic nanoparticles that can recognize bacteria have been used as probes in pretreating bacterial samples.[4–8] Non-target species can be subsequently eliminated by magnetic separation, and the isolated bacteria trapped by the functional probes can be characterized by MALDI-MS.

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Gold nanoclusters (Au NCs) with high water solubility, good stability, and observable photoluminescence have been extensively used in chemical and biochemical sensing.[12–23] Protein-directed synthesis of AuNCs (protein-AuNCs) has recently drawn considerable attention because of the straightforward generation of protein-encapsulated AuNCs.[12–15,24–28] In addition, the proteins on the generated AuNCs may retain their bioactivity.[13,25] Xie *et al.*[12] used bovine serum albumin (BSA) as the reagent to sequester and reduce Au precursors for the generation of AuNCs in aqueous tetrachloroauric acid solution. Although the protein structure on the BSA-AuNCs cannot be preserved,[12] further exploration using other types of proteins showed the feasibility of retaining protein bioactivity.[13,25] For instance, lysozymes are a family of enzymes that can catalyze the hydrolysis of peptidoglycans on bacterial cell walls.[29] Lysozyme-encapsulated AuNCs can be used to effectively inhibit the cell growth of bacteria, including antibiotic-resistant bacteria, indicating that the activity of lysozyme on lysozyme-AuNCs is retained.[13] To accelerate the generation of the lysozyme-AuNCs, microwave-heating has been used to accelerate the generation of protein-encapsulated AuNCs.[13] The cell growth of antibiotic-resistant bacteria can be effectively inhibited in the presence of lysozyme-AuNCs

generated under microwave-heating. Lysozyme-AuNCs can also be used as effective probes to trap bacteria from sample solutions because lysozyme can bind with *N*-acetylglucosamine on the peptidoglycans on the bacterial cell walls.[30] In this work, we used the lysozyme-AuNCs as the sensing/concentrating probes for pathogenic bacteria. The presence of bacteria in the samples could be observed by the naked eye under illumination of ultraviolet (UV) light. Bacterial characterization was performed by MALDI-MS and principal component analysis (PCA).

EXPERIMENTAL

Reagents and materials

Lysozyme (E.C. number: 235-747-3) from chicken egg white, BSA, and sinapinic acid were purchased from Sigma (St. Louis, MO, USA). Hydrogen tetrachloroaurate(III) tetrahydrate was obtained from Showa (Tokyo, Japan). Trifluoroacetic acid was acquired from Alfa Aesar (Ward Hill, MA, USA). Fetal bovine serum was obtained from Bi biological (Kibbutz Beit Haemek, Israel). Luria-Bertani broth (LB broth) and tryptic soy broth (TSB) were obtained from Becton Dickinson (Franklin Lakes, NJ, USA), and yeast extract was purchased from Alpha Bioscience (Baltimore, MD, USA). Amicon Ultra-4 centrifugal filters were purchased from Millipore (Billerica, MA, USA). Sodium hydroxide was obtained from Riedel-de Haën (Seelze, Germany).

Generation of the lysozyme-AuNCs

The synthesis of the lysozyme-AuNCs was performed in accordance with a previous report.[13] Tetrachloroaurate(III) tetrahydrate (100 mM, 10 μL) and lysozyme (10 mM, 20 μL) were added to deionized water (0.96 mL), and the resultant solution was vigorously stirred for 5 min. The solution pH was adjusted to ~12 by adding aqueous sodium hydroxide (2 M, 10 μL) and stirring the mixture for another 5 min at room temperature. The mixture was then placed in a domestic microwave oven (power: 90 W) for 7 heating cycles (5 min/cycle). The mixture was cooled to room temperature between each heating cycle. During heating, the sample vial cap was kept loose. The resultant lysozyme-AuNCs were isolated from the solution by centrifugation for 7 min in Amicon ultra-4 centrifugal filters (cutoff mass: 30 kDa) at 6000 rpm (rotor radius: 8.5 cm) to remove excess proteins. The isolated NCs were rinsed using deionized water (1 mL × 3), and the centrifugation and rinse steps were then repeated several times. The AuNCs were subsequently lyophilized for storage. A given concentration of lysozyme-AuNCs was dissolved in a given volume of deionized water prior to the experiments.

Preparation of bacteria

Klebsiella pneumoniae, *Pseudomonas aeruginosa*, pandrug-resistant *Acinetobacter baumannii* (PDRAB), *Staphylococcus aureus*, *Enterococcus faecalis*, and vancomycin-resistant *Enterococcus faecalis* (VRE) were originally collected from Tzu-Chi Hospital (Hualien, Taiwan) and then provided by Prof. Pei-Jane Tsai (National Cheng-Kung University, Taiwan). *Escherichia coli* J96 was provided by Prof. Hwei-Ling Peng of the National Chiao Tung University (Taiwan). All the bacteria were culture based on the standard method described previously[6] and the details of the preparation of the medium have been described elsewhere.[6] *K. pneumonia*, *P. aeruginosa*, *E. faecalis*, PDRAB, and VRE were cultured in LB broth, whereas *S. aureus* was cultured in a medium consisting of TSB and yeast extract.

Lysozyme-AuNC-based affinity mass spectrometry for bacteria

After overnight culturing, freshly harvested bacteria were rinsed with a phosphate-buffered saline (PBS, pH 7.4) solution twice by centrifugation for 10 min at 4500 rpm (or 6000 rpm) in a Thermo centrifuge equipped with a rotor (rotor radius: 8.0 cm). The PBS solution (pH 7.4) was prepared by mixing aqueous disodium hydrogen phosphate (20 mM) and aqueous sodium dihydrogen phosphate (10 mM). The bacterial cells were resuspended in the PBS solution to prepare a given concentration of suspension solution. The stock bacterial solution was prepared at optical density (OD) at a wavelength of 600 nm (OD_{600}) equal to 1. The bacterial samples were then serially diluted with the PBS solution. Aqueous lysozyme-AuNCs (1 mg/mL) was freshly prepared in deionized water before the experiment. During the bacterium trapping/sensing experiments, the bacterial solution (900 μL) was vortex-mixed with the lysozyme-AuNCs solution (1 mg/mL, 100 μL) and BSA (1 mM, 10 μL) for 1 h, and then centrifuged at 3500 rpm for 5 min. BSA (1 mM, 10 μL) was added to the sample to stabilize the lysozyme-AuNC suspension. The sample tubes were then observed under UV light illumination, and photographs were taken. The precipitate was then analyzed by MALDI-MS using an Autoflex III time-of-flight mass spectrometer (Bruker Daltonics) equipped with a 355 nm laser. The precipitate was redissolved in the PBS solution. The suspension (1 μL) was mixed with the MALDI matrix, which consisted of sinapinic acid (1 μL, 25 mg/mL) prepared in an acetonitrile/deionized water (1:1, v/v) solution containing 1% trifluoroacetic acid. To determine the limit of detection of this approach, the supernatant (980 μL) was removed, and 1 μL of the remaining precipitate was mixed with the same MALDI matrix (1 μL, 25 mg/mL). After the solvent had evaporated, the sample was analyzed by MALDI-MS in linear mode.

RESULTS AND DISCUSSION

The absorption and photoluminescence properties of the lysozyme-AuNCs are similar to those previously reported.[13] The color of the AuNC solution is pale yellow, and the maximum photoluminescence of the lysozyme-AuNCs appears at ~640 nm when the excitation wavelength was set at 395 nm. Five nosocomial pathogens, namely, *E. coli* J96, *K. pneumoniae*, *P. aeruginosa*, PDRAB, and *S. aureus*, were used as the model bacteria. After the aforementioned bacterial incubation with the lysozyme-AuNCs and the subsequent centrifugation, tube *a*, which contained bacteria with the lysozyme-AuNCs, showed precipitation with bright red emission under illumination of UV light (λ_{max}= 365 nm) (Fig. 1). Tubes *b* and *c* on each panel in Fig. 1, which contained the bacterial sample and the lysozyme-AuNCs, respectively, served as controls and were prepared using the steps used for tube *a*. After centrifugation at 3500 rpm, the bacteria in tube *b* spun down to the bottom of

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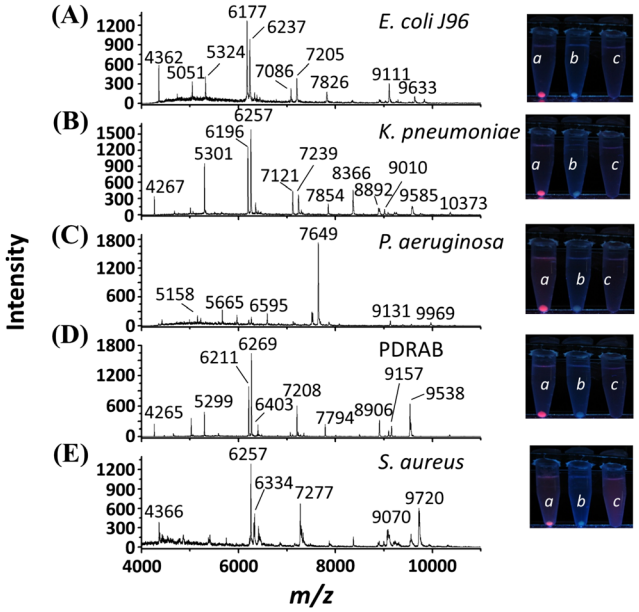

Figure 1. MALDI mass spectra of the samples obtained after using the lysozyme-AuNCs as affinity probes to probe bacteria from samples containing (A) *E. coli* J96, (B) *K. pneumoniae*, (C) *P. aeruginosa*, (D) pandrug-resistant *A. baumannii* (PDRAB), and (E) *S. aureus*. The samples were prepared in PBS solution (pH 7.4) containing BSA (~10 μM). The photographs on the right-hand side in each panel taken under UV light illumination (λ_{max} = 365 nm) correspond to the results after incubating the lysozyme-AuNCs with bacteria (tube *a*), while tubes *b* and *c*, which served as controls, contained bacteria and lysozyme-AuNCs, respectively.

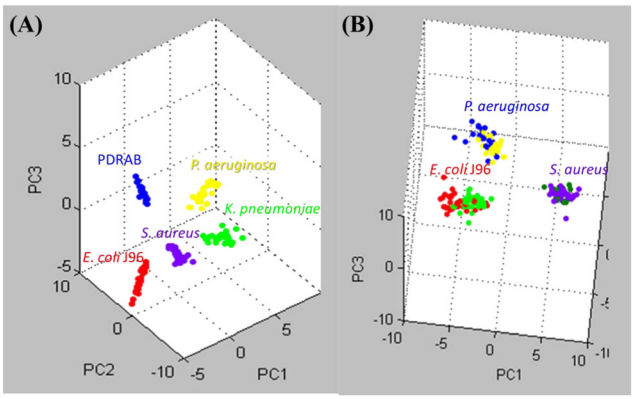

Figure 2. (A) PCA plot of the data obtained using the lysozyme-AuNCs as the affinity probes to trap samples containing *E. coli* J96 (red dots), *K. pneumoniae* (light green dots), *P. aeruginosa* (yellow dots), PDRAB (blue dots), and *S. aureus* (purple dots). The samples were prepared in a PBS solution (pH 7.4) containing BSA (~10 μM). The experiments on each bacterial sample were repeated 30 times. (B) PCA plot of the data obtained using the lysozyme-AuNCs as the affinity probes for *E. coli* J96 (red dots), *P. aeruginosa* (blue dots), and *S. aureus* (purple dots). The light green, yellow, and green dots represent the direct MALDI mass spectra of *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively. The results were obtained from 30 replicates.

the sample tube. Meanwhile, no precipitation was observed in tube *c* after centrifugation. These results indicate that the lysozyme-AuNCs can interact and settle down with these bacteria after low-speed centrifugation. MALDI-MS was further used to determine the identities of the bacteria obtained from the tube *a* in each panel in Fig. 1. Figures 1(A) to 1(E) show the MALDI mass spectra of *E. coli* J96, *K. pneumoniae*, *P. aeruginosa*, PRDAB, and *S. aureus*, respectively. The mass spectral profiles differ.

Principal component analysis (PCA) was then used to differentiate the bacteria by repeating the experiment 30 times for each bacterial sample and then conducting PCA data analysis. Figure 2(A) shows the resultant PCA plot. Different bacteria can be clearly allocated in different clusters on the plot, indicating the feasibility of using the current approach to distinguish these bacteria. Urinary tract infections are commonly caused by *E. coli* J96, P. *aeruginosa*, and *S. aureus*. However, the antibiotics used to inhibit the cell growth of these bacteria differ. Thus, using an analytical method capable of distinguishing these three bacteria will be very helpful for assisting diagnostics. Thus, we examined the possibility of using our approach to distinguish these three uropathogenic bacteria by combining MALDI-MS results and PCA analysis. Figure 2(B) shows the resultant PCA plot of these three bacterial samples. These three bacteria formed clusters distributed in three different positions on the PCA plot. These results indicate the feasibility of applying our approach to the differentiation of different uropathogenic bacteria. Our proposed method would be helpful in diagnoses and in prescribing suitable antibiotics for medical treatment. Furthermore, the direct MALDI mass spectra of intact bacterial cells and those obtained after the bacteria were trapped by the lysozyme-AuNCs were compared. The clusters marked with yellow, green, and pale green colors in Fig. 2(B) represent the direct MALDI mass spectra of the intact cells of *P. aeruginosa*, *S. aureus*, and *E. coli* J96, respectively. The clusters marked in blue, purple, and red dots in the same PCA plot represent the mass spectra obtained after trapping *P. aeruginosa*, *S. aureus*, and *E. coli* J96, from the sample solutions using the lysozyme-AuNCs as the probe, respectively. For the same bacterial species, the direct MALDI-MS data and those obtained after treatment with lysozyme-AuNCs are located in the same area on the PCA plot. These results indicate that the presence of the lysozyme-AuNCs in the MALDI sample does not affect the MALDI-MS results.

Additionally, we also used a bacterial mixture containing *E. coli* J96, *K. pneumoniae*, and *S. aureus* as the samples to examine the feasibility of using the current approach in a mixture sample. Figure 3 shows the resultant PCA plot. The cluster of the mixture marked with blue color was located in the center of these three individual bacterial clusters including *E. coli* J96 (red), *S. aureus* (yellow), and *K. pneumonia* (pale green). Generally, only one type of bacterial strain would dominate a biological sample. If more than two types of bacteria are present in the sample, our PCA result shows the cluster of the bacterial mixture will be located among the clusters of individual bacteria.

The feasibility of using the current approach to distinguish antibiotic-resistant strains from their wild types was also determined. *E. faecalis* and VRE were used as the model samples. The insets in Figs. 4(A) and 4(B) show the photographs of the samples containing VRE and *E. faecalis*, respectively, obtained after vortex-mixing with the lysozyme-AuNCs for 1 h followed

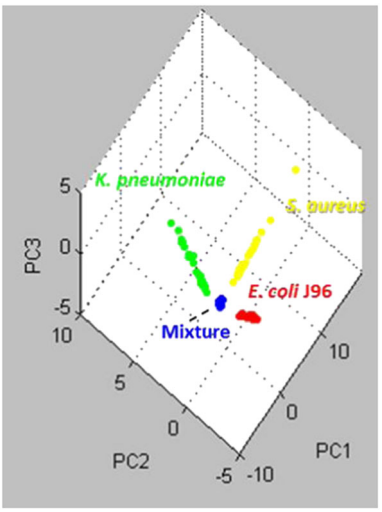

Figure 3. PCA plot acquired from the MALDI-MS results obtained using the lysozyme-AuNCs (1 mg/mL, 0.1 mL) as affinity probes to trap a bacterial mixture (0.9 mL) (blue cluster) containing *E. coli* J96 (OD_{600}= 1, 0.3 mL), *K. pneumoniae* (OD_{600}= 1, 0.3 mL), and *S. aureus* (OD_{600}= 1, 0.3 mL) and three other bacterial samples including *E. coli* J96 (red cluster), *K. pneumoniae* (pale green cluster), and *S. aureus* (yellow cluster). All the samples were prepared in a PBS solution (pH 7.4) containing BSA (~10 μM). 30 replicates were conducted.

by centrifugation at 3500 rpm for 5 min. Tube *a* in the insets in Figs. 4(A) and 4(B) show precipitation with a reddish emission, which corresponds to the lysozyme-AuNC-bacteria conjugates. Tubes *b* and *c* were control samples containing bacteria and lysozyme-AuNCs, respectively. MALDI-MS was then used to characterize the conjugates in tube *a*. Figures 4(A) and 4(B) show the corresponding spectra. A number of major peaks at *m/z* 4331, 6011, 6160, 6339, 6808, and 8963 appeared in both mass spectra; however, additional peaks at *m/z* 5277, 9564, 10593, and 11209 were found in Fig. 4(B) only. These results indicate that the mass spectra can be used to distinguish VRE from *E. faecalis*. Figure 4(C) shows the PCA plot drawn on the basis of the MALDI-MS results for the samples; the data were obtained by repeating the same experiment 30 times. The results for the different bacterial samples exhibit different distributions on the PCA plot, indicating that the current approach can be used to distinguish these two bacterial strains.

Additionally, we also examined if this approach is suitable to be used in characterization of bacteria present in serum samples. Thus, *S. aureus* spiked in 50-fold diluted fetal bovine serum (FBS) was used as the model sample. Figure 5 shows the resultant PCA plot. The cluster marked with blue color was acquired from the MALDI mass spectra obtained after using the lysozyme-AuNCs as affinity probes to trap bacteria from a sample solution containing *S. aureus* prepared in PBS (pH 7.4), while the cluster marked with yellow color was acquired from the MALDI mass spectra obtained after using the same AuNCs as affinity probes to trap bacteria from the FBS sample containing *S. aureus*. These two clusters were almost overlapped, indicating this approach can be used to identify bacteria even from complex serum samples. Additionally, the clusters marked in red and pale green colors correspond to the results obtained from the samples containing *E. coli* J96 and *K. pneumonia*, respectively. These clusters from different bacteria were well separated from

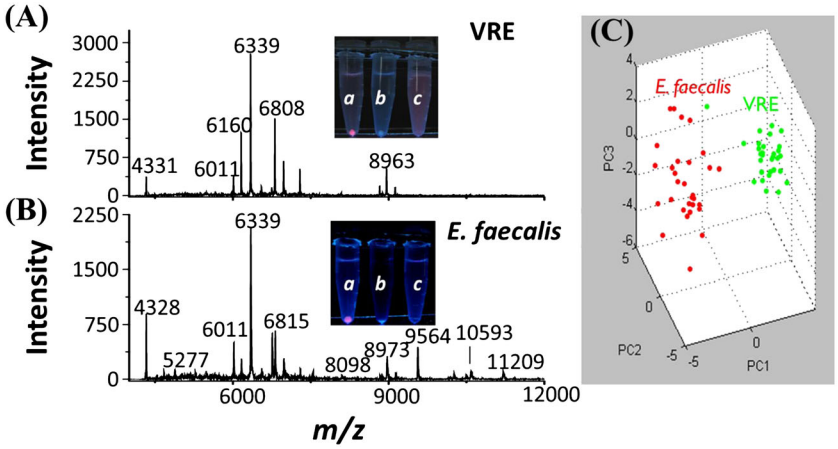

Figure 4. MALDI mass spectra obtained after using the lysozyme-AuNCs as the affinity probes to trap bacteria from samples containing (A) VRE and (B) *E. faecalis*. The inset photographs are the sensing results for panels (A) and (B), which were obtained by vortex-mixing the lysozyme-AuNCs with VRE and *E. faecalis* for 1 h, respectively, followed by centrifugation at 3500 rpm for 5 min. The photographs were taken under UV light illumination (λ_{max} = 365 nm). The tubes in the insets from left to right contained bacteria/lysozyme-AuNCs (tube *a*), bacteria (tube *b*), and lysozyme-AuNCs (tube *c*). The MALDI mass analysis for the samples VRE and *E. faecalis* was repeated for 30 times. (C) PCA plot obtained from 30 replicates of the same experiments for obtaining panels (A) and (B). The red dots represent the results obtained for the *E. faecalis* samples, and the pale green dots represent the results for the VRE samples. All samples were prepared in a PBS solution (pH 7.4) containing BSA (~10 μM).

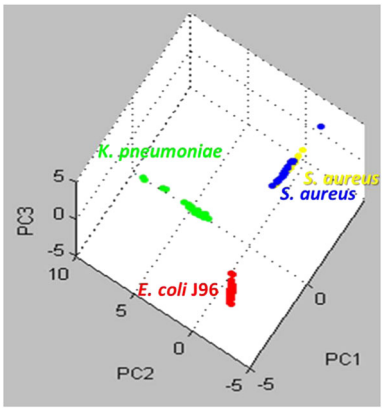

Figure 5. PCA plot acquired from the MALDI mass spectral results obtained using the lysozyme-AuNCs (1 mg/mL, 0.1 mL) as the affinity probes to probe bacterial samples containing different bacteria (OD_{600} = 1, 0.9 mL) including *E. coli* J96 (red cluster) prepared in PBS buffer (pH 7.4), *K. pneumonia* (pale green cluster) prepared in PBS buffer (pH 7.4), *S. aureus* prepared in PBS buffer (blue cluster), and *S. aureus* (yellow cluster) prepared in 50-fold diluted fetal bovine serum. All the samples contained BSA (~10 μM). 30 replicates were conducted.

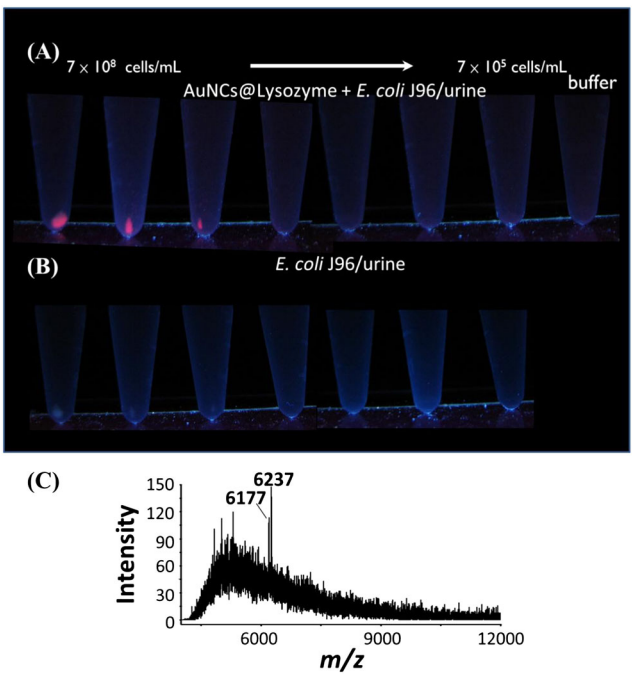

Figure 6. Photographs obtained by vortex-mixing (A) the lysozyme-AuNCs with *E. coli* J96 at different cell concentrations, and (B) *E. coli* J96 alone for 1 h at different cell concentrations, followed by centrifugation at 3500 rpm for 5 min. The samples were prepared in urine that was diluted 50-fold with PBS solution (pH 7.4) containing BSA (~10 μM). The photographs were taken under illumination of UV light (λ_{max} = 365 nm). (C) Examination of the limit of detection. MALDI mass spectrum obtained after using the lysozyme-AuNCs (1.36 mg/mL, 0.1 mL) as affinity probes to concentrate target species from a urine sample (0.90 mL) containing *E. coli* J96 (1.59×10^6 cells/mL) for 1 h. The urine sample was diluted 50-fold with PBS solution (pH 7.4) containing BSA (~10 μM) prior to bacterial spiking.

those clusters representing *S. aureus*. The results demonstrated again the capability of this approach in characterizing the identity of bacteria from complex samples.

The detection limit of the proposed approach was then determined using urine spiked with *E. coli* J96 as the sample. Figure 6(A) shows the photographs obtained after using the lysozyme-AuNCs as the probe for bacteria in urine samples containing different concentrations of *E. coli* J96. As the concentration of *E. coli* J96 in the samples decreases, the red precipitate corresponds to the lysozyme-AuNC-bacteria conjugates on the bottom of the sample tubes. However, compared with the control experimental sample tubes (i.e., without the lysozyme-AuNCs; Fig. 6(B)), the presence of the bacteria in the sample tubes in Fig. 6(A) was more easily observable. MALDI-MS was used to characterize the conjugates on the bottom of the samples in Fig. 6(A). Figure 6(C) shows the MALDI mass spectrum of the conjugates containing *E. coli* J96 (~1.59×10^6 cells/mL). The peaks at *m/z* 6177 and 6237 in the mass spectrum (cf. Fig. 1(A)) correspond to *E. coli* J96. The lowest detectable concentration using this approach for *E. coli* J96 was ~10^6 cells/mL. Generally, the concentration of uropathogens in the urine samples of patients with urinary tract infections is $\geqq 10^6$ cells/mL.[31] Thus, the current results indicate that our proposed approach may be potentially suitable for analyzing the samples obtained from patients with urinary tract infections.

CONCLUSIONS

This study demonstrated that bacterial identity can be readily determined by trapping bacteria using the lysozyme-AuNCs as affinity probes and analyzing the conjugates using MALDI-MS. The resultant data can then be analyzed by PCA. In the proposed approach, the time-consuming cell culturing is eliminated. Furthermore, the current method can be used to analyze uropathogenic bacteria from urine and serum samples. The possibility of using this approach to distinguish antibiotic-resistant strains from wild types was also demonstrated. The advantages of this approach include speed and simplicity. The results suggest that the lysozyme-AuNCs are universal affinity probes for pathogens including Gram-positive/negative and antibiotic-resistant bacteria. Thus, the current approach may be used in the medical diagnosis of bacterial infections.

Acknowledgements

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