

Unveiling Molecular Fingerprints of Bacteria on Nanoplasmonic Sensors

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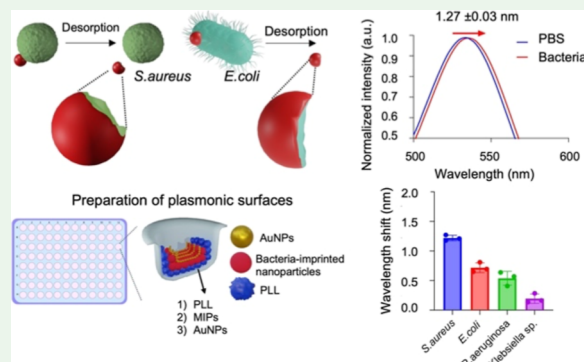
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ABSTRACT: The recent pandemic has underscored the severe global threat posed by infectious diseases. Disease diagnosis and surveillance need accurate and efficient detection systems. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are the main causes of infections in humans. We present a nanoplasmonic detection platform designed to simultaneously identify *E. coli* and *S. aureus* in a single system. This method involves synthesizing molecular fingerprints, i.e., molecularly imprinted polymers (MIPs), of bacteria for each target bacterium and integrating gold nanoparticles (AuNPs) to create a nanoplasmonic system for precise bacterial identification. Then, AuNP-decorated bacteria-MIPs were strategically placed in 96-well plates. This arrangement permitted the simultaneous detection of numerous bacteria on the same platform, with a detection range of 1×10^5 to 1×10^9 cfu/mL and a limit of detection of 4.8×10^4 cfu/mL for *E. coli* and 4.2×10^4 cfu/mL for *S. aureus*. Bacteria-MIPs were tested against Gram-specific bacteria and shown to be selective. Artificial urine and serum spiked samples were used to test the platform effectiveness in simulated infection scenarios. The target microorganisms were detected in complicated matrices with great sensitivity. Consequently, bacteria-MIPs' features make them an attractive choice for diagnostic system integration, allowing simultaneous detection of different bacteria on a plate and varied clinical and environmental applications.

KEYWORDS: molecular imprinting, bacteria detection, nanoplasmonic sensor, nanoparticles, multitarget detection



INTRODUCTION

The global prevalence of diseases has seen significant transformations throughout the past century. Unlike in the past, the implementation of proper sanitation and antibiotics has led to an epidemiological transition. Therefore, the types of diseases that were primarily spread through communication have now shifted to mostly nontransmissible diseases. However, infectious diseases remain a significant global health problem as deliberately faced during the latest pandemic.¹ *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are primary etiological agents responsible for healthcare-associated infections in humans. These bacteria are commonly found as commensals and can become highly infectious when penetrating sterile areas. They are responsible for about 50% of bacteremia cases that occur in the community or are acquired in hospitals.² Urinary tract infection (UTI), for instance, is recognized as the second most prevalent cause of infectious illnesses. UTIs are prevalent bacterial illnesses, occurring in around 50–60% of individuals during their lifetime, particularly among adult women.³ UTI arises when a pathogen successfully infiltrates the urinary tract system and reaches a concentration of more than 10^5 cfu/mL in the urine and constitutes over 40% of all infections acquired in hospitals and 50% of cases of bacteremia, which can lead to extended

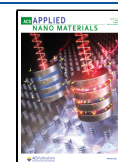
hospital stays and higher rates of illness and death among patients.⁴ Uropathogenic *E. coli* strains are the main reason behind UTIs. *E. coli* is mostly a facultative anaerobic Gram-negative bacterium. It establishes in the digestive tract of human neonates shortly after birth and contributes to the maintenance of appropriate intestinal balance.⁵ On the other hand, *S. aureus* is another highly contagious and prevalent bacterial pathogen, responsible for several skin infections. It is a primary cause of pneumonia and other infections in the respiratory tract, surgical sites, prosthetic joints, and cardiovascular system.⁶ Nevertheless, under particular circumstances, they have the ability to trigger the infectious and inflammatory process, resulting in developing UTIs.⁷ Furthermore, *S. aureus*-related bloodstream infection is a highly prevalent and severe bacterial infection. When the concentration of pathogens in the bloodstream is low (<10 cfu/mL), their growth is weak enough to have a negative result.⁸

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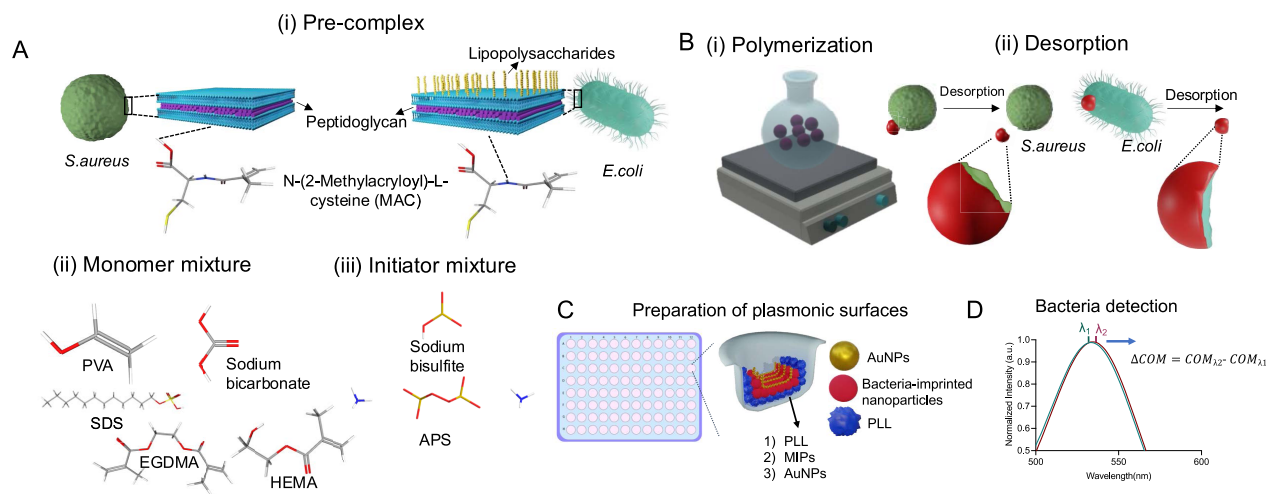
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Scheme 1. Depicting the Synthesis of Bacteria-Imprinted Polymers (MIPs) and Their Application in a Nanoplasmonic Sensor for Bacteria Detection. (A,B) Synthesis of *E. coli*-MIPs and *S. aureus*-MIPs. (A) (i) Preparation of a Pre-complex, (ii) Monomer, and (iii) Initiator Mixture for the Polymerization Process. (B) (i) Polymerization and (ii) Desorption Stages. (C) Preparation of Plasmonic Surfaces Using AuNPs and Bacteria-MIPs. (D) Utilization of Nanoplasmonic Surfaces for the Detection of Bacteria



Therefore, the rapid and precise identification of *S. aureus* is essential and plays a pivotal role in medicine.⁹

Conventional techniques for identifying and studying microorganisms usually involve genetic analysis using polymerase chain reaction (PCR),¹⁰ antibody-based tests,¹¹ culture methods, and microscopy.¹² However, culture-based methods can be time-consuming and usually take between 2 and 7 days,^{13–15} depending on the growth period of bacteria to be detected, and typically, biochemical tests (such as metabolic fingerprinting), molecular tests (usually PCR), or mass spectrometry are required to confirm that the isolate is the specific pathogen of interest.¹⁶ In addition, bacterial identification can be performed within a few hours with a portable, small-sized digital PCR (dPCR) system.¹⁷ However, these molecular diagnosis techniques hold some limitations: (i) high risk of contamination leading to false positives;¹⁸ (ii) lengthy detection times due to DNA preparation steps;¹⁹ and (iii) the need for costly reagents and equipment.²⁰ Moreover, although dPCR is a highly precise method, it also faces several challenges that limit its widespread application. Compared to standard quantitative PCR (qPCR), dPCR requires more expensive instrumentation and reagents,²¹ more complex sample preparation and handling,²² and generally has lower throughput compared to qPCR. The interpretation of data obtained from dPCR can be more complex, necessitating specialized software and expertise.²³ Additionally, multiplexing is more challenging in dPCR compared to the qPCR.²⁴ Overall, precise and rapid detection of microorganisms using various technologies, such as sensors, plays a crucial role in diagnostics.

On the other hand, extensive research over the past few decades has been explored plasmonic sensors for applications in environmental science²⁵ and clinical diagnostics.²⁶ Plasmonic sensors are valued for their high sensitivity, rapid response, label-free detection, and real-time investigation of biological interactions.²⁷ Nanostructured noble metals, such as gold nanoparticles (AuNPs), are particularly effective, leveraging localized surface plasmon resonance (LSPR) to amplify the absorption and scattering of incoming light. LSPR

sensors provide real-time, molecular-level responses with a tiny sample volume due to their sensitivity to changes in the dielectric constant of the surrounding medium.²⁸ The utilization of metal nanoparticles in drug delivery²⁹ and diagnostic applications³⁰ has emerged as a new development. The nanoplasmonic features of these nanoparticles offer a distinct advantage in monitoring alterations in light coupling on a sensor surface. The binding or separation of biological molecules onto metal nanoparticles results in a shift of the peak at the wavelength of resonance, allowing for a wide range of spectral observations with a strong signal-to-noise ratio.³¹ AuNPs, in particular, possess distinctive optical characteristics, low-toxicity, resistance to oxidation, and versatility in terms of surface chemistry, making them widely used in several sensor contexts.³²

Molecular recognition is crucial for the routine functioning of biological systems and commonly occurs between receptor–ligand,³³ antigen–antibody,³⁴ enzyme–inhibitor,³⁵ RNA–ribosome,³⁶ DNA–protein,³⁷ sugar–lectin,³⁸ and other pairs. Molecularly imprinted polymers (MIPs), designed to emulate receptors, present a cost-effective alternative to natural counterparts.³⁹ MIPs are recognition materials created by the polymerization of monomers, cross-linkers, and other essential components (such as initiators and porogens) in the presence of the target analyte, known as the “template”. Following removal of the template, distinct cavities emerge, meticulously preserving the dimensions, configuration, and chemical characteristics of the analyte. This precision allows for the accurate identification of the desired target analyte.⁴⁰ Bacteria-imprinted polymers are prepared by polymerizing functional monomers around a bacterial template, resulting in complementary binding sites once the template is removed. The contact mechanisms between MIPs and bacteria are mostly driven by the surface properties of the bacterial species undergoing imprinting. Interactions with *E. coli* primarily involve the surface lipopolysaccharides (LPS) located in the outer membrane. Functional monomers, such as methacrylic acid, establish hydrogen bonds with the hydroxyl and carboxyl groups of LPS during the imprinting process.⁴¹ The interaction

of *S. aureus* predominantly involves teichoic acids with the peptidoglycan layers within the bacterial cell wall. Functional monomers, including methacrylic acid, engage with the hydroxyl and phosphate groups in teichoic acids via hydrogen bonding and possible electrostatic interactions with the negatively charged phosphate groups.⁴² MIPs technology confers numerous advantages, notably encompassing convenient long-term storage, exceptional stability, enduring durability, cost-effectiveness, and remarkable specificity.⁴³

The combination of AuNPs with MIPs can potentially increase the sensitivity and selectivity of plasmonic sensors in different ways. The most important property of AuNPs is the excitement of localized surface plasmons and the enhancement of the local fields in the vicinity of AuNPs. As a result of this feature, molecular recognition-induced optical signals are amplified, and the limit of detection (LOD) is improved.⁴⁴ Moreover, the larger surface area of AuNPs improves the accessibility of targets to the bound MIPs, which synergistically increases both sensitivity and selectivity of the platform.⁴⁵ In addition to the sensitivity and selectivity parameters, the robustness and stability of AuNP-integrated MIP platforms are beneficial for both long-term and repeatable measurements.

Herein, we have expounded upon an accurate nanoplasmonic detection platform capable of simultaneously discerning two bacteria (*E. coli* and *S. aureus*) within a unified system. In this system, we synthesized *E. coli* and *S. aureus*-MIPs (molecular fingerprints of bacteria), separately, and these nanoparticles were meticulously adorned with AuNPs, thereby culminating in the establishment of a nanoplasmonic sensor expressly crafted for the determination of the bacterial presence (Scheme 1). The synthesis of bacteria-MIPs was executed via the mini-emulsion method, employing an amino-acid-based functional monomer, specifically *N*-methacryloyl-L-cysteine (MAC). Subsequently, bacteria-MIPs decorated with AuNPs which were synthesized by the growth seed method and were attached to the wells of 96-well plates, enabling the determination of multiple bacteria on the same plate with 1×10^5 – 1×10^9 cfu/mL detection range with the calculated LOD value 4.8×10^4 cfu/mL for *E. coli* and 4.2×10^4 cfu/mL for *S. aureus*. Furthermore, the selectivity of the prepared bacteria-MIPs was tested with bacteria with Gram-specific differences. Moreover, artificial urine and serum spiked samples were used to simulate infection environments, and it was observed that the bacteria used in the study could be detected with high sensitivity.

Our study presents an alternative strategy that combines MIPs with AuNPs, achieving bacterial detection simultaneously on a single assay. Briefly, by utilizing a 96-well plate format, this scalable and efficient system offers high-throughput screening capabilities, making it suitable for large-scale processes in clinical applications. MIPs exhibit a high surface-to-volume ratio, providing multiple binding points and antigen-recognition sites with high sensitivity, while the plasmonic properties of AuNPs enable rapid, label-free, and real-time detection. Our approach, which integrates MIPs with plasmonics, represents a notable advancement over traditional techniques, offering a dual functionality and enabling both considerable specificity and sensitivity without the need for additional labeling steps. In summary, our nanoplasmonic technique would potentially emerge as a viable opportunity for seamless integration into diagnostic systems in the near future. This innovative platform not only enables the simultaneous detection of multiple bacterial strains on a single platform but

also demonstrates its applicability in diverse clinical and environmental settings.

EXPERIMENTAL SECTION

Chemicals and Bacteria. *E. coli* (American Type Culture Collection (ATCC) 25922) and *S. aureus* (ATCC 29213) were obtained from ATCC, USA. Luria–Bertani (LB) broth, sodium chloride (NaCl), sodium citrate dihydrate ($C_6H_9Na_3O_9$), tetrachloroauric (III) acid trihydrate ($HAuCl_4 \cdot 3H_2O$), and poly-L-lysine (PLL) was purchased from Sigma-Aldrich (MO, USA). Phosphate-buffered saline (PBS) was purchased from Gibco.

Synthesis of Bacteria-Imprinted and Nonimprinted Nanoparticles. Two different bacteria (*E. coli* and *S. aureus*) were selected as templates for MIPs production. Bacteria-MIPs were prepared separately by the following procedure as described in our previous study.⁴⁶ The preparation of bacteria solutions was summarized in the Supporting Information. Briefly, an amino-acid-based monomer, MAC, and 1×10^6 cfu/mL of bacteria solution were mixed to form a precomplex for an hour before the polymerization. The two phase-emulsion method was employed preparing bacteria-MIPs. These nanoparticles were then centrifuged using ethanol and water sequentially to remove unreacted monomer, surfactant, and initiator. In order to remove bacteria from polymeric nanoparticles, these MIPs were mixed with 0.5 M of NaCl solution at 20 rpm for an hour. The nonimprinted (NIPs) were also synthesized with the same procedure without the bacteria template. The prepared bacteria-MIPs and NIPs solutions were determined as 1X concentration and kept in a dark condition at +4 °C until it was ready to be used.

Synthesis of AuNPs. Spherical AuNPs were synthesized by following the seed growth synthesis method. Briefly, 90 mL of sodium citrate (60 mg) solution was prepared in deionized water and agitated at 700 rpm and 100 °C for 10 min. Subsequently, 6 mg of $HAuCl_4 \cdot 3H_2O$ was introduced into the solution, and the same procedure was sustained for 30 min. Once the temperature reduced to 90 °C, 25 mM of sodium citrate and 1 mM of $HAuCl_4 \cdot 3H_2O$ solution were introduced into the mixture. The resulting mixture was shaken for a further 30 min under the same conditions. Once measuring the average diameter of AuNPs, their concentration was calculated according to the formula which is specified in the Supporting Information. The prepared AuNPs solution was calculated as 2.7 nM concentration, and it was kept in a dark condition at +4 °C until it was ready to be used.³⁰

Characterization of Nanoparticles. The bacteria-MIPs and NIPs were initially examined using dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern, UK) and nanoparticle tracking analysis (NTA) (Nanosight NS300, Malvern, UK). Functional monomer (MAC), MIPs, and NIPs were characterized using a Fourier transform infrared (FTIR) spectrophotometer (Vertex 70 FTIR Spectroscopy, Bruker, Germany) to determine the functional groups. The samples were centrifuged at 14,500 rpm for 30 min and resuspended in ethanol and from 400 to 4000 cm^{-1} to determine surface chemical groups. X-ray photoelectron spectroscopy (XPS) (K-Alpha XPS, Thermo Fisher Scientific, USA) was also used to characterize MIPs and NIPs nanoparticles with an elemental map of the compositions. The surface morphology of MIPs and NIPs nanoparticles was examined by using scanning electron microscopy (SEM, FEI Quanta 200 FEG, USA) and transmission electron microscopy (TEM, FEI Tecnai G2 F30, USA). Likewise, AuNPs were characterized using DLS, zeta potential analysis, and TEM, with the particle size measured using ImageJ.

Determination of the Optimum Condition for Nanoplasmonic Sensor Preparation. Before the nanoplasmonic surfaces were prepared, optimum conditions were determined by decorating 96-wells with AuNPs. The attachment of AuNPs was achieved via PLL.^{31,47,48} In this regard, the optimum concentration of PLL was determined by keeping the AuNPs concentration constant (2X concentration). Various concentrations of PLL solutions (dissolved in 1X PBS) within the range of 0.01–0.1 mg/mL were prepared and incubated overnight at +4 °C. After the incubation, the wells were

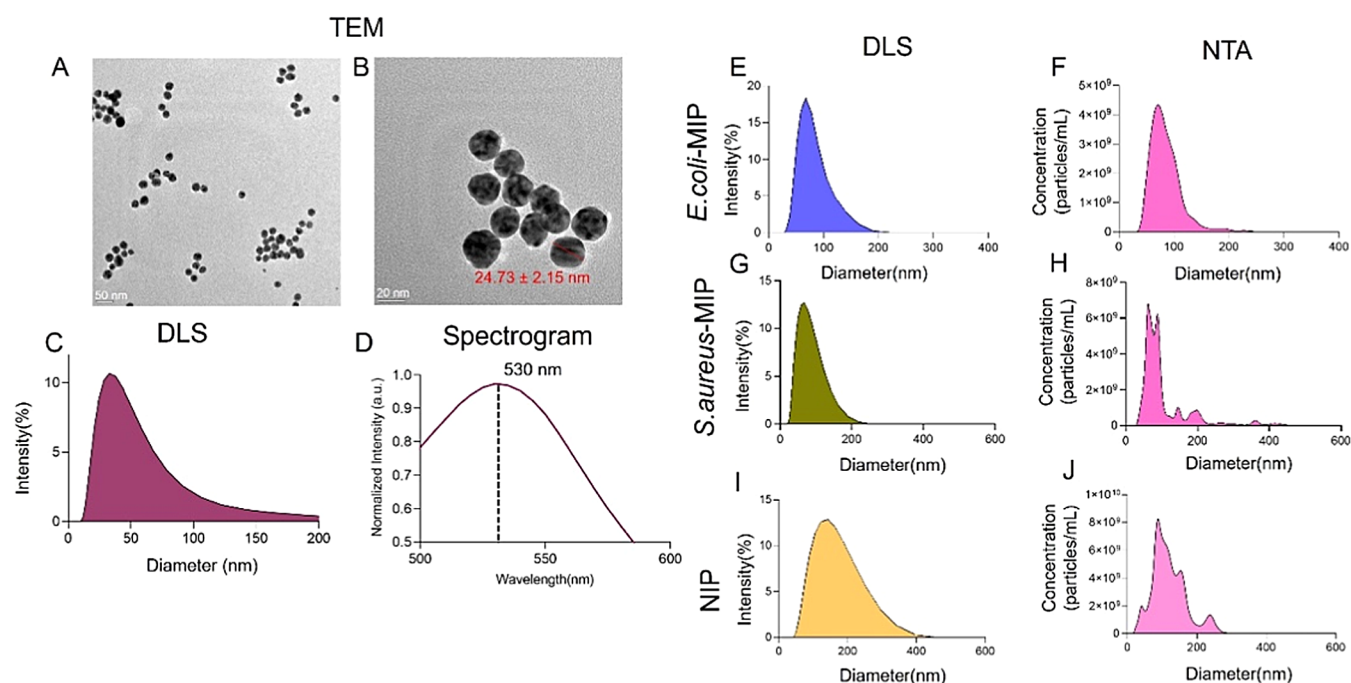


Figure 1. Characterization of AuNPs (A–D), bacteria-MIPs (E–H), and NIPs (I,J). (A,B) TEM images, (C) DLS measurement, and (D) the spectrogram of AuNPs. DLS measurement of (E) *E. coli*-MIPs, (G) *S. aureus*-MIPs, and (I) NIPs. NTA measurement of (F) *E. coli*-MIPs, (H) *S. aureus*-MIPs, and (J) NIPs.

washed three times with 1X PBS. After being centrifuged at 10,000 rpm for 30 min, the AuNPs at 2X concentration were added to the wells and incubated again at +4 °C overnight. After the experiments were continued at that concentration, the optimum PLL concentration was determined. In order to determine the optimum AuNPs concentration, AuNPs solutions were prepared at different concentrations (0.5X–10X) by keeping the PLL concentration constant (0.1 mg/mL). Likewise, after the optimum AuNPs concentration was determined, the experiments were continued accordingly. In order to determine the optimum bacteria-MIPs concentration, constant concentrations of PLL (0.1 mg/mL) and bacteria-MIPs (1:1 to 1:10) were incubated overnight at +4 °C. Afterward, the optimum concentration of AuNPs (1X concentration) were incubated again at +4 °C overnight. After the optimum bacteria-MIPs concentration was determined after incubation, the experiments were continued at that concentration. Absorbance measurements were taken at 1 nm intervals using a microplate reader (SpectraMax M5, San Jose, USA) within the range of 500–600 nm. All the conditions were tested with 3 replicates. The data were plotted using Graphpad Prism (v9), along with the statistical analyses one-way ANOVA (Friedman test) (the sample size was stated in each figure; $p < 0.05$).

Preparation and Characterization of AuNP-Modified MIP Surfaces. After determining the optimal parameters for PLL, AuNPs, and MIPs, we carried out our experiments under those circumstances. Briefly, in order to prepare bacteria-specific nanoplasmonic surfaces, the optimum PLL (0.1 mg/mL) and bacteria-MIPs solutions were mixed in a 1:1 ratio and added to the wells and incubated overnight at +4 °C. After the wells were washed with PBS, AuNPs solution (1X concentration) was added and incubated overnight again. For the characterization, contact angle measurement (Dataphysics, OCA 30, Filderstadt, Germany) and SEM were used for the characterization of the nanoplasmonic sensor surfaces. For contact angle measurement, 2 μ L of ultrapure water from the system was dropped on the surfaces, measurements were taken from 3 different regions, and the angles were calculated with the software of the device. Before SEM images were taken from the surfaces, they were coated with 10 nm Au/Pd.

***E. coli* and *S. aureus* Detection Assays.** Bacteria cultures were prepared as stated in Supporting Information. The absorbance values were adjusted to 0.08–0.13 au at 600 nm equivalent to the 0.5

McFarland turbidity standard (1×10^8 cfu/mL),⁴⁹ and the other concentrations were prepared by serial dilution. For studies on the detection of *E. coli* and *S. aureus* with the prepared sensor surfaces, primarily, 200 μ L of PBS solution was added to the wells, and spectrum scanning measurement was performed in the 500–600 nm range. Subsequently, 200 μ L of bacterial solutions in the concentration range of 1×10^5 – 1×10^9 cfu/mL were added to the wells and incubated for an hour at 37 °C using a microplate shaker, washed with PBS, and rescanned. Additionally, the binding of the two bacteria to NIPs was also analyzed. A concentration of 1×10^8 cfu/mL for both bacteria was incubated under the same conditions as previously mentioned, and spectrum scanning was performed and compared to the results with MIPs. In order to make a comparison with standard methods from a method-verification perspective, bacteria were added in LB medium at the concentrations used in sensor experiments and incubated at 37 °C overnight. Afterward, absorbance measurements were performed at 600 nm. To verify the accuracy of the plasmonic sensor for detecting bacterial concentration, we compared the sensor response to those obtained using the standard UV–vis spectra method. Based on the differences between bacterial cell wall of Gram-positive and -negative bacteria, *S. aureus*, a Gram-positive bacterium, was chosen to evaluate the selectivity performance of *E. coli*-MIPs, while *E. coli*, a Gram-negative bacterium, was chosen for *S. aureus*-MIPs. In order to conduct selectivity studies, the indicated bacteria were prepared at a concentration of 1×10^8 cfu/mL and incubated under the same conditions as previously indicated.

To evaluate the performance of the sensor, artificial urine and artificial serum samples were used to create conditions similar to clinical samples, and both bacteria were prepared at a concentration of 1×10^8 cfu/mL and as described, followed by spectrum scanning. The performance of the sensor was also tested under varying pH conditions (pH 4.0–9.0) using PBS solution spiked with 10^8 cfu/mL bacteria to simulate physiological or environmental diversity.

In order to determine the long-term stability and reproducibility, bacteria-MIPs synthesized 24 months ago and stored at +4 °C were used to perform detection experiments under the same conditions as the original study. Five independent experiments were also performed to evaluate the reproducibility. Each experiment was performed with

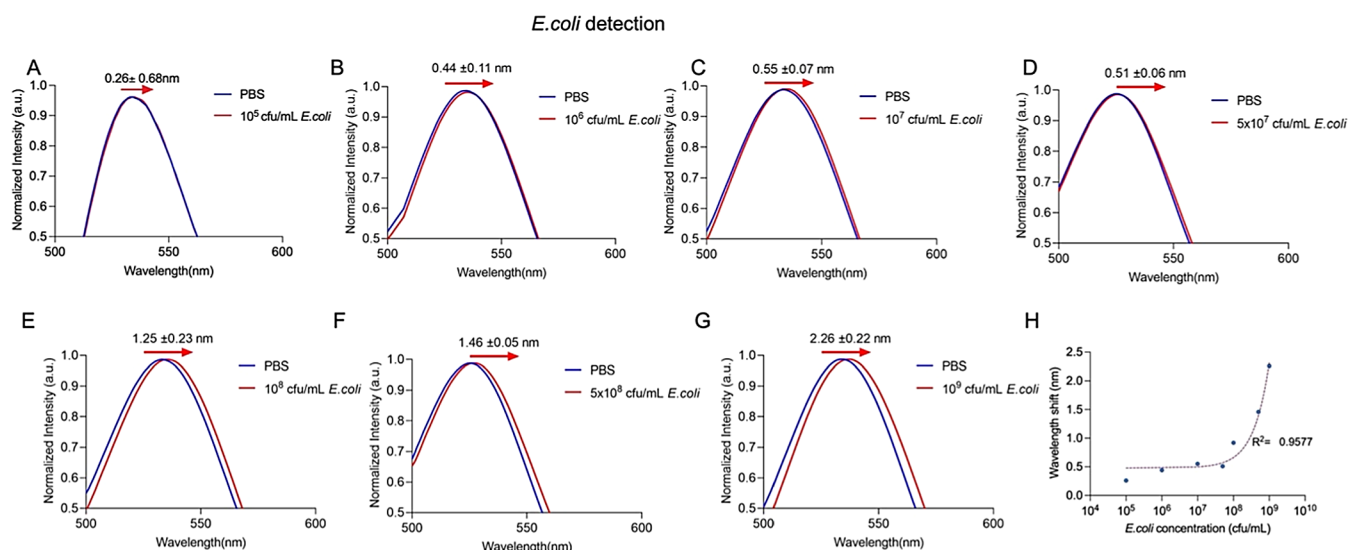


Figure 2. *E. coli* detection on nanoplasmonic surfaces. *E. coli* detection results with different bacteria concentrations, including (A) 1×10^5 , (B) 1×10^6 , (C) 1×10^7 , (D) 5×10^7 , (E) 1×10^8 , (F) 5×10^8 , (G) 1×10^9 cfu/mL, and (H) analysis of wavelength shift and logarithmic concentrations of *E. coli*.

the same protocol, including sample preparation, particle functionalization, and measurement. Detection performances were evaluated by comparing the wavelength shifts.

Spectral Measurements and Data Analysis. Each binding study was characterized with a detected shift of the maximum extinction point of AuNPs with the results obtained from the spectrum scan. In order to find the center of mass (CoM) of each recorded spectrum, a MATLAB code was written. Detailed information about the data analysis can be found in the [Supporting Information](#).

FDTD Simulation. The electromagnetic simulation was performed on commercial FDTD software Ansys Lumerical (Pennsylvania, USA). A simulation mesh size of 2 nm was employed for calculating the electric field intensity distribution around AuNPs and determining the absorption cross section of the AuNPs-modified MIPs. The RI of the PLL layer was set to 1.37, and the RI of the PS substrate was set to 1.59. The RI value of the MIP was set to 1.451 for modeling optical properties of 2-hydroxyethyl methacrylate. A total-field scattered-field (TFSF) source, covering the spectral range from 300 to 600 nm, was used to irradiate the AuNPs.

RESULTS AND DISCUSSION

Characterization of MIPs, NIPs, and AuNPs. The diameters of synthesized AuNPs were determined by using TEM analysis and DLS measurements. As per the result, the particle diameter was measured as 24.73 ± 2.15 nm and 38.6 ± 4.30 nm (Figure 1A,B). Since the DLS technique also measures a hydration layer around the nanoparticles, approximately 10 nm higher particle diameter was observed in this method (Figure 1C). On the other hand, surface charge is typically measured by zeta potential, and it presents information about the charge of particles in a liquid medium.⁵⁰ The zeta potential value of the AuNPs was measured as -35.8 mV. The maximum wavelength of the AuNPs was observed at 520 nm at the absorbance measurement (Figure 1D).

DLS, NTA, FTIR spectrophotometer, and XPS measurements were conducted in characterizing *E. coli*- and *S. aureus* MIP and NIP nanoparticles. The DLS and NTA results for *E. coli*-MIPs, *S. aureus*-MIPs, and NIPs were given in the [Table S1](#) and [Figures 1E–J](#) and [S1](#). Considering all these results, the average size values in the NTA and DLS results were comparable to each other.

The XPS analysis confirmed the chemical composition of bacteria-MIPs and NIPs nanoparticles, showing different types of oxygen, carbon, and sulfur atoms, with a notable sulfur peak present only in bacteria-MIPs (Figure S2A–F), and FTIR analysis demonstrated shifts and intensity changes in the characteristic bands of the functional monomer and bacteria-MIPs, indicating successful polymerization (Figure S2G). Detailed results of XPS and FTIR are provided in the SI. According to the TEM and SEM analyses, the *E. coli*-MIPs, *S. aureus*-MIPs and NIPs had spherical shapes and rough surfaces, as shown in [Supporting Information](#).

Preparation of AuNP-Modified MIP Sensor Surfaces.

The optimum conditions of nanoplasmonic sensor surfaces were determined by assessing the wavelength shifts in proportion to various concentrations of PLL, AuNPs, and bacteria-MIPs solutions. Briefly, PLL—a cationic polypeptide, interacts electrostatically with negatively charged polymeric nanoparticles and AuNPs.⁵¹ Four different concentrations of PLL (0.01 to 0.1 mg/mL) were tested, followed by AuNPs at a fixed 2.70 nM concentration. To optimize the AuNP dilution, concentrations from 1.35 to 27 nM were examined with a constant 0.1 mg/mL PLL concentration. For bacteria-MIPs, concentrations were varied from 0.1X to 1X, maintaining PLL at 0.1 mg/mL and AuNPs at 2.7 nM. The spectrometric analysis was performed in the 500–600 nm range, with optimal conditions detailed in the [Supporting Information](#). During the process of fabricating nanoplasmonic sensor surfaces, bacteria-MIPs were attached to the surfaces. For the purpose of characterizing bacteria-MIPs sensor surfaces modified with AuNPs, contact angle measurements were conducted initially. The measurement results for the different surfaces were detailed in [Figures S4–S6](#). The reduction in the contact angle value following surface changes indicates the effective modification of PLL and MIPs to the surfaces (Figure S7).

***E. coli* and *S. aureus* Detection Assays.** Regarding the detection of *E. coli* on the nanoplasmonic sensor surfaces, seven different concentrations in the range of 1×10^5 – 1×10^9 cfu/mL were evaluated. As a result of plasmonic measurements, the CoM values of the spectra and corresponding wavelength shifts were calculated. Considering the results

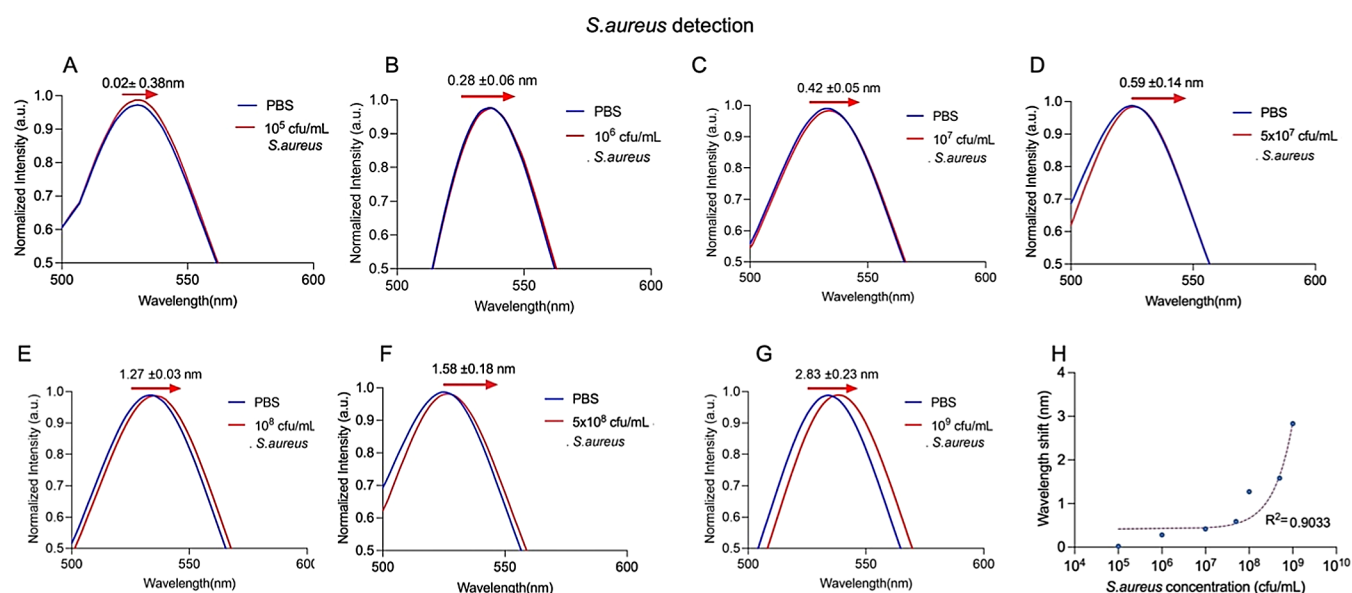


Figure 3. *S. aureus* detection on nanoplasmonic surfaces. *S. aureus* detection results with different bacteria concentrations, including (A) 1×10^5 , (B) 1×10^6 , (C) 1×10^7 , (D) 5×10^7 , (E) 1×10^8 , (F) 5×10^8 , (G) 1×10^9 cfu/mL. (H) Analysis shifts and logarithmic concentrations of *S. aureus*.

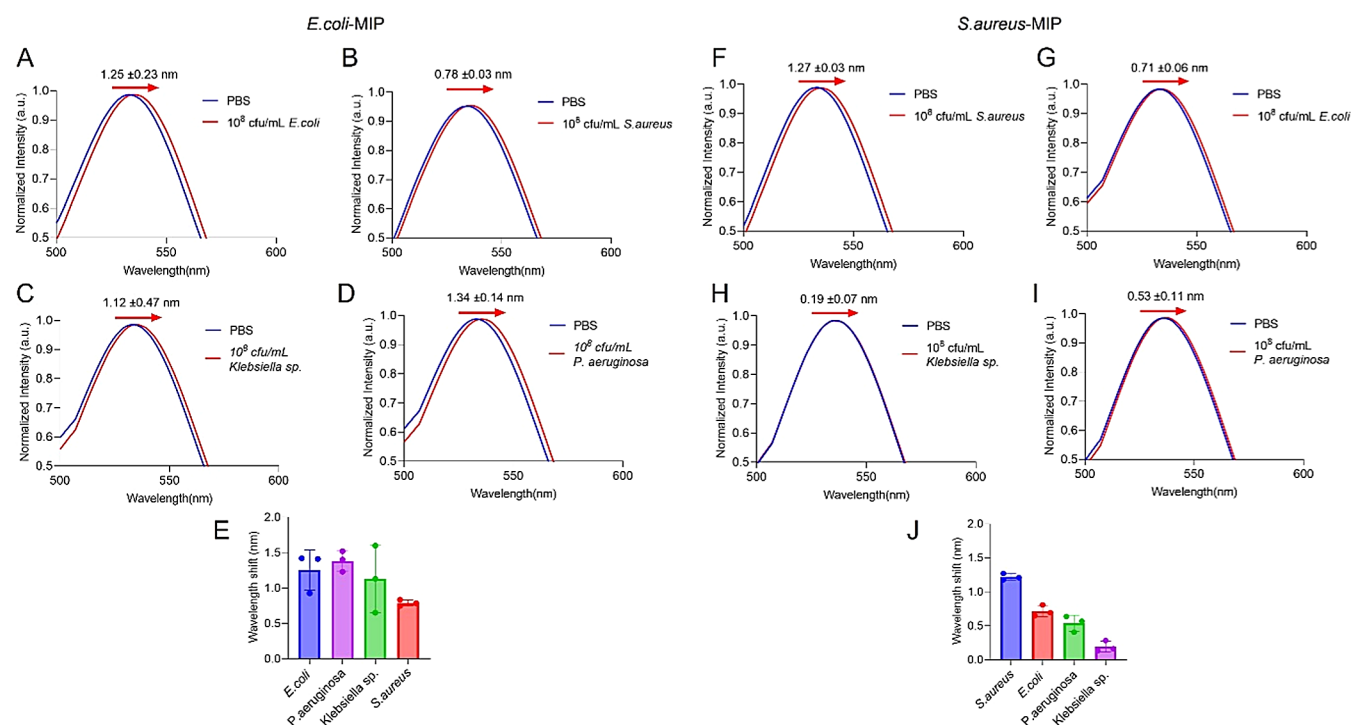


Figure 4. Selectivity results for (A–E) *E. coli*-MIPs and (F–J) *S. aureus*-MIPs.

which were shown in Figure 2A–H, wavelength shifts of 0.26 ± 0.68 nm for 1×10^5 cfu/mL, 0.44 ± 0.11 nm for 1×10^6 cfu/mL, 0.55 ± 0.07 nm for 1×10^7 cfu/mL, 0.51 ± 0.06 nm for 5×10^7 cfu/mL, 1.25 ± 0.23 nm for 1×10^8 cfu/mL, 1.46 ± 0.05 nm for 5×10^8 cfu/mL, and 2.26 ± 0.22 nm for 1×10^9 cfu/mL was observed. Consequently, nanoplasmonic sensor surfaces prepared with *E. coli*-MIPs have a measuring accuracy of 95.77% within the concentration range of 1×10^5 – 1×10^9 cfu/mL with a calculated LOD value (4.8×10^4 cfu/mL). The measured data were fitted by a linear regression model. For a linear calibration curve, the instrument response “y” is assumed

to have a linear relationship with standard concentration “x” within a certain concentration range. This relationship can be represented by the model $y = a + bx$. This model was utilized to determine the LOD. The LOD can be calculated using the formula $\text{LOD} = 3S/b$, where S is the standard deviation of the response, estimated by the standard deviation of the y-intercept of the regression lines.⁵²

Considering the LOD and potential applications in infections, bacteriuria is a mandatory finding in any case of UTI; typically with growth of 10^5 cfu/mL or more Gram-

negative bacteria⁵³ and the detection limit, we obtained matches this threshold/limit.

Likewise, seven different concentrations were assessed for the determination of *S. aureus*, and the wavelength shifts were calculated accordingly. Considering the results which were depicted in Figure 3A–H, the wavelength shifts of 0.02 ± 0.38 nm for 1×10^5 cfu/mL, 0.28 ± 0.06 nm for 1×10^6 cfu/mL, 0.42 ± 0.05 nm for 1×10^7 cfu/mL, 0.59 ± 0.14 nm for 5×10^7 cfu/mL, 1.27 ± 0.03 nm for 1×10^8 cfu/mL, 1.58 ± 0.18 nm for 5×10^8 cfu/mL, and 2.83 ± 0.23 nm for 1×10^9 cfu/mL was observed. Hence, nanoplasmonic sensor surfaces decorated with *S. aureus*-MIPs achieved an accuracy of 90.33% within the concentration range of 1×10^5 – 1×10^9 cfu/mL with a calculated LOD value (4.2×10^4 cfu/mL), as well. In practice, the concentration of bacteria in the blood for a bacteremia sample is in the range of 10 – 10^4 cfu/mL. In particular, considering pediatric cases, this value is higher than 10^3 cfu/mL.^{54,55} Our plasmonic sensor is capable of detecting bacterial concentrations starting from approximately 10^4 cfu/mL, making it suitable for use in the diagnosis of bloodstream infections. According to the comparison results with UV–Vis Spectra method, as shown in Figure S8, both methods demonstrate a similar trend of increasing response with rising bacterial concentrations, highlighting the consistency and reliability of the measurements.

Selectivity Assays. The major component of bacterial cell walls in both Gram-positive and Gram-negative bacteria is peptidoglycan. Gram-positive bacteria have thick cell walls consisting of a multilayered peptidoglycan region with a wall of teichoic acid and lipoteichoic acid attached to the peptidoglycan layer and the cell membrane, respectively. On the other hand, considering Gram-negative bacteria, there is a thin peptidoglycan layer between the cell and the outer membranes, located in the region called the periplasmic space. Instead of teichoic acids, Gram-negative bacteria synthesize LPS, which form different domains within the outer phospholipid bilayer.⁵⁶ Therefore, in order to assess the cross-selectivity performance of *E. coli*-MIPs, *S. aureus* (a Gram-positive bacterium), *Klebsiella* sp. and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Gram-negative bacteria) were used. Likewise, the selectivity of *S. aureus*-MIPs was evaluated using *E. coli*, *Klebsiella* sp., and *P. aeruginosa* (Gram-negative bacteria). The plasmonic measurements indicated that there was a wavelength shift of 1.25 ± 0.23 nm observed for *E. coli*; yet, for *S. aureus*, *Klebsiella* sp. and *P. aeruginosa* at the same bacteria concentration (1×10^8 cfu/mL), the value was calculated to be 0.78 ± 0.03 , 1.12 ± 0.47 , and 1.34 ± 0.14 nm, respectively, as shown in Figure 4A–D. Upon analyzing these findings, the *E. coli*-MIPs exhibited 1.60 times more selectivity in detecting *E. coli* compared to *S. aureus*. When we compare this value with those of other Gram-negative bacteria (*Klebsiella* sp. and *P. aeruginosa*) evaluated for selectivity, we observed that they yielded comparable results. (Figure 4E). This might be due to the similarity of the LPS structure in those bacteria's cell wall. LPS, also referred to as lipoglycans and endotoxins, constitutes the primary component of the outer membrane in Gram-negative bacteria.⁵⁷ During the imprinting process, bacteria cells are mixed with polymeric solutions, and the imprinting takes places in multiple locations along with a randomized process. Hence, with a high potential, MIPs are largely produced by imprinting the most abundant material (such as LPS for Gram-negative bacteria) on their surfaces. This limitation could potentially be addressed by selecting more specific proteins and imprinting

such proteins for recognition rather than focusing on the entire surface of bacteria.

In addition, another experiment was carried out with *S. aureus*-MIPs, with the similar concentrations of *E. coli*, *Klebsiella* sp., and *P. aeruginosa* (1×10^8 cfu/mL). The plasmonic measurements indicated a wavelength shift of 1.27 ± 0.03 nm for *S. aureus* at the same concentration; yet, for *E. coli*, *Klebsiella* sp., and *P. aeruginosa*, the shifts were 0.41 ± 0.14 , 0.53 ± 0.11 , and 0.19 ± 0.07 nm, respectively (Figure 4F–I). Upon the examination of the data, *S. aureus*-MIPs exhibited 1.78, 2.39, and 6.68 times more selectivity in detecting *S. aureus* compared to *E. coli*, *Klebsiella* sp., and *P. aeruginosa* (Figure 4J). The selectivity coefficient (k) was calculated according to eq 1 as follows:

$$k = \text{Shift}_{E.coli} / \text{Shift}_{S.aureus} \quad (1)$$

Considering the earlier reports,⁵⁸ the k value greater than 1 indicates high affinity between MIPs and the target molecules.⁴⁶ Bacteria detection assays were also conducted on NIP-modified surfaces. Figure S9A,B demonstrates that the nanoplasmonic sensor surfaces fabricated using NIPs had a reduced change in wavelength compared to the sensor prepared with bacteria-MIPs. According to the results, the measurement for *E. coli* was 0.43 ± 0.57 nm, whereas for *S. aureus*, it is 0.94 ± 0.33 nm.

Furthermore, the imprinting factor, also known as the relative selectivity coefficient (k'), quantifies the impacts of molecular grooves on the binding of target molecules. It allows for a comparison of the binding effectiveness between MIPs and NIPs. The k' value was calculated using the eq 2 as 2.93 for *E. coli*-MIPs and 1.35 for *S. aureus*-MIPs.

$$k' = k_{MIP} / k_{NIP} \quad (2)$$

E. coli and *S. aureus* Detection in Artificial Urine, Artificial Serum Samples, and Various pH Values.

UTIs—generally caused by *E. coli* affecting 50–60% of individuals, particularly adult women, can lead to complications like kidney dysfunction and urosepsis, while bloodstream infections caused by *E. coli* and *S. aureus* are a leading cause of mortality in infectious diseases.^{3,59} This is crucial for monitoring infections related to healthcare and understanding the development of antibiotic resistance in the context of global health.⁵⁹ Nevertheless, it is crucial to identify certain diseases or observe specific interactions of cells or macromolecules, particularly in complex environments such as blood and urine. During analyses conducted in complex environments, several drawbacks may develop, such as nonspecific adsorption and the potential increased background development.⁶⁰ Detecting the target molecule with high selectivity in a densely complicated background might pose challenges in practical clinical applications.⁶¹

In order to evaluate the performance of the sensor under conditions similar to the clinical samples, this study employed artificial urine and serum samples to mimic real-sample conditions. The synthetic urine appears to be the model matrix, as it possesses different salt contents including sodium, potassium, and chloride, as well as urea and organic compounds, i.e., creatinine and ammonia. These elements are commonly found to be interfering factors in normal urine samples.⁶² Artificial serum was used to imitate a biological fluid being rich in proteins, which would include albumin, about 50 percent of which is serum albumin, globulins, and other macromolecules that could potentially influence sensor

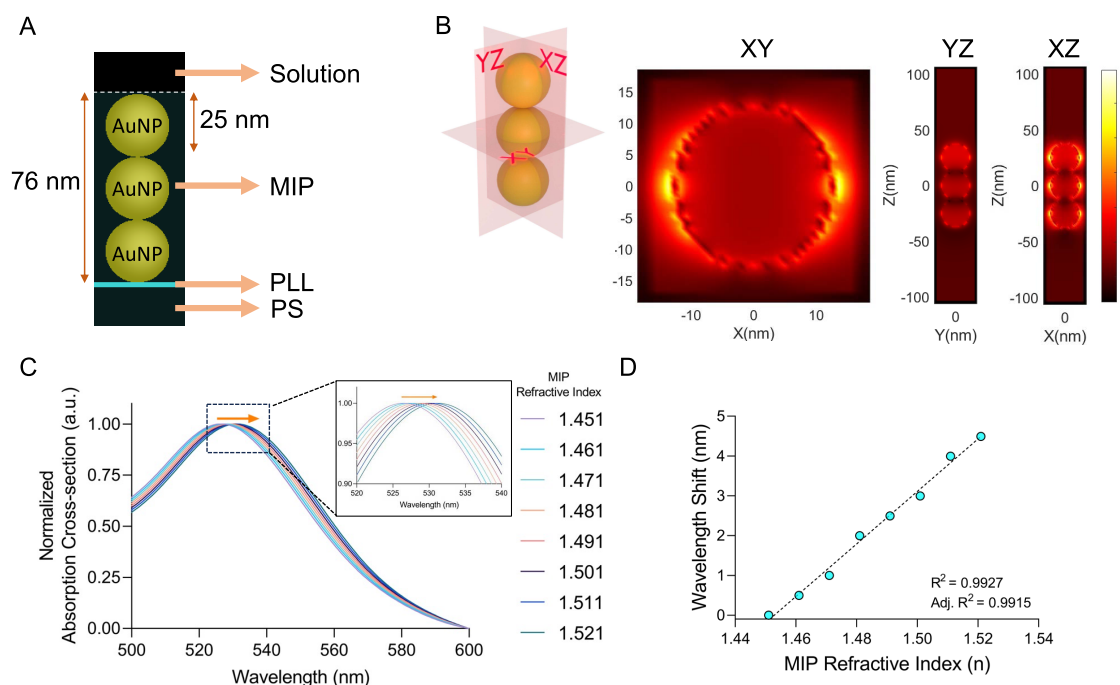


Figure 5. Electromagnetic simulation of AuNPs-modified MIPs. (A) The simulation design demonstrates the integration of MIPs and AuNPs with the characterized dimensions. (B) Localized electric field distribution around AuNPs at 527 nm and a surrounding RI of 1.33 is depicted from XY, YZ, and XZ cross sections. (C) Normalized absorption cross-section spectrum illustrates the spectral behavior of AuNP-modified MIPs in response to changes in RI, simulating bacterial surface adhesion. (D) The wavelength shift of the resonance peak demonstrates an approximate redshift of 5 nm due to the change in RI values of the MIP-target complex.

performance.⁶³ These matrices allowed the establishment of the ability of the sensor to selectively detect bacteria in the presence of such potential interfering agents and ensured the platform's robustness and applicability for practical diagnostic scenarios. In order to streamline the study process using these samples, two bacteria with a concentration of 1×10^8 cfu/mL were used. The findings from experiments using synthetic urine indicated that the measurement for *E. coli* was 0.74 ± 0.07 nm, whereas it was 1.41 ± 0.13 nm for *S. aureus*, as shown in Figure S9C,D. Similarly, the findings from experiments using artificial serum indicated measurements of 2.88 ± 0.32 and 2.82 ± 0.18 nm for *E. coli* and *S. aureus*, respectively (Figure S9E,F).

The performance of the sensor was also tested under varying pH conditions (pH 4–9) to simulate physiological or environmental diversity. Considering the results which was depicted in Figure S10, wavelength shifts of 1.65 ± 0.03 nm for pH 4.0, 1.56 ± 0.26 nm for pH 5.0, 1.44 ± 0.24 nm for pH 6.0, 1.56 ± 0.24 nm for pH 8.0, and 1.07 ± 0.52 nm for pH 9.0 were observed for *E. coli*. For *S. aureus*, these results are as follows: 1.51 ± 0.48 nm for pH 4.0, 1.92 ± 0.15 nm for pH 5.0, 1.83 ± 0.18 nm for pH 6.0, 1.71 ± 0.56 nm for pH 8.0, and 1.63 ± 0.42 nm for pH 9.0 (Figure S11). These findings indicate that the performance of the sensor was not significantly affected by the pH variations within the tested range.

Long-term Stability and Reproducibility Assays. To determine long-term stability and reproducibility, bacteria-MIPs synthesized 24 months ago and stored at +4 °C were used to perform detection experiments under the same conditions. Herein, five independent experiments were performed to assess the reproducibility. According to the results, the decrease in the wavelength shifts for *E. coli*-MIPs

was greater than the decrease for *S. aureus*-MIPs (Figure S12A,B). The high decrease for *E. coli*-MIP indicates that its long-term stability was weaker than *S. aureus*-MIPs. The repeatability of the method revealed an average result of 1.35 ± 0.25 nm for *E. coli*-MIP and 1.19 ± 0.08 nm for *S. aureus*-MIPs (Figure S12C,D). The proximity of the measurements and their low standard deviations indicate that the detection approach is consistent and reliable regarding the repeatability for multiple experimental setups.

FDTD Modeling of AuNP-Modified MIP Sensor Surfaces. The electromagnetic behavior of the AuNP-modified MIP sensor surfaces was modeled by using the FDTD method. This method is the commonly utilized numerical method in plasmonics and can be used to comprehend the LSPR response in various nanostructure sizes, shapes, and surroundings. The primary goal of this section was to analyze the spectral behavior of AuNPs in response to bacterial binding. To simulate the interactions between MIPs and AuNPs, they were integrated into a model based on the previously determined DLS diameter results: 25 nm for AuNPs and 76 nm for MIP. Figure 5A demonstrates the expected size and geometric form of the AuNPs-modified MIP sensor in the cross-sectional plane schematic of the FDTD model. Accordingly, the AuNPs-modified MIPs were placed on a 2 nm thickness of PLL layer over a polystyrene (PS) substrate and were modeled as a homogeneous slab dispersed in AuNPs. AuNPs were modeled in the stacked form, where their added diameters (3×25 nm) were approximately equal to the height of the PS-MIP slab (76 nm) that they were dispersed in 2 nm mesh size and was set as the resolution of the FDTD model. The presence of *E. coli* and *S. aureus* was modeled by a change in surrounding refractive index (RI). Initially, the surrounding RI was set to 1.33 to model an

aqueous solution. The AuNPs-modified MIP sensor was illuminated with a TFSF source, which is advantageous for the analysis of scattering from periodically arranged AuNPs. The absorption cross-section was calculated by placing six planar monitors around the simulated model, which collected field profiles in the frequency domain and integrated them to determine the absorption cross-section. Through this analytical approach, the absorption spectra revealed a resonance at 527 nm. For calculating the electric field distribution over the AuNPs-modified MIPs at 527 nm, the solution RI was set to 1.33 (Figure 5B). The distribution of the electric field is localized near the AuNP surfaces due to localized surface plasmons, accompanied by scattered fields in all directions, leading to power absorption at the AuNP surfaces. This local field enhancement aided in the improvement of spectral shifts induced by molecular recognition. This was achieved since the sensing region was close to the AuNP surface. To model bacterial binding on the MIPs, we considered potential changes in the RI in the close vicinity of MIPs. This strategy was chosen because the RI of biomolecules changes linearly with increasing their concentration in a solution.⁶⁴ Assuming the MIPs capture bacterial outer membrane proteins, the surrounding RI gradually increased from 1.451 to 1.521, corresponding to the approximate change in the RI value for captured bacterial outer membrane proteins (Figure 5C). The normalized absorption cross-section red-shifted as the RI of the MIP-target complex increased. When the RI reached 1.521, the resonance red-shifted by approximately 5 nm (Figure 5D). Considering the data presented in Figures 2H and 3H, the wavelength shifts from the lowest to highest concentration would potentially increase 0.05 of RI. On the other hand, this RI change might be caused by both bacteria concentration and complex environment medium where bacteria grow.

CONCLUSIONS

In conclusion, our developed plasmonic detection platform, utilizing bacteria-MIPs decorated with AuNPs, demonstrates a robust and versatile system for simultaneously detecting two distinct bacteria, *E. coli* and *S. aureus*, within a single diagnostic assay. The synthesized bacteria-MIPs exhibited high selectivity, confirming their ability to discriminate between bacteria with Gram-specific differences. The integration of the MIPs–AuNP complexes into a 96-well plate format provided a scalable and efficient method for the concurrent detection of two bacterial strains on the same platform. The platform displayed an impressive detection range of 1×10^5 to 1×10^9 cfu/mL, with low LOD values (4.8×10^4 cfu/mL for *E. coli* and 4.2×10^4 cfu/mL for *S. aureus*), showcasing its sensitivity and potential for practical applications. UTI is generally caused by microorganisms that is present in a high concentration, usually $\geq 10^5$ cfu/mL.⁶⁵ Moreover, the platform's performance was evaluated in simulated infection environments using artificial urine and artificial serum spiked samples, demonstrating its capability to detect the target bacteria with high sensitivity and reliability. The amalgamation of molecular imprinting technology, plasmonic enhancement, and a well-established 96-well plate format presents our diagnostic technique as a promising solution for bacterial detection. With their high sensitivity and accuracy, our plasmonic sensors can provide bacterial identification in a relatively short turnaround time (1 hour).

Considering our work and previous studies, the advantages of MIP-based plasmonic platforms for bacteria sensing can be discussed in three key areas. First, MIPs, often referred to as

“artificial antibodies”, offer rapid, large-scale production within days, require no specialized storage conditions, and provide versatility in target recognition with a high surface-to-volume ratio. In addition, MIPs can be produced for different targets. Through a high surface-volume ratio, MIPs offer more binding points and can denote multiple antigen-recognition sites. Conventional batch fabrication, on the other hand, has limitations such as the need for a high volume of reagents and aggregation issues on the course of production, hindering their yield efficiency. The recent innovations like microreactor platforms have addressed these issues, synthesizing trillions of MIPs with low-volume reagent consumption (2 times improvement in reagent volume) for a short period of time (5–30 min).⁴³ Second, the integration of MIPs into plasmonic bacterial sensing leverages their random synthesis process, which targets multiple bacterial antigens and surface markers, enhancing capture efficiency. The combination of MIPs with plasmonic systems, particularly AuNPs, allows for efficient tethering or encapsulation of AuNPs within the MIP matrix, providing intrinsic localized surface plasmon signals for in situ analyte detection. Plasmonic sensors, in addition, offer rapid, label-free, and real-time detection, since the plasmonic signals respond instantly when the target binds. Alongside these assets, MIP-based plasmonic sensors can be adapted to recognize a variety of substances, enhancing their versatility for multiple applications. Moreover, this integration offers rapid, label-free, and real-time detection capabilities with enhanced sensitivity due to the high surface area-to-volume ratio of AuNPs.⁶⁶ Lastly, the practical applications of this platform are numerous, such as high-throughput screening capability using a 96-well plate format, simultaneous detection of multiple samples or target types, and easy integration into existing laboratory workflows, especially in clinical and environmental monitoring studies.⁶⁷ The method's adaptability allows for the identification of various bacteria by modifying the MIP composition, enhancing its utility across microbiology and biotechnology applications. Unlike fluorescence or enzymatic assays, MIP-based plasmonic sensors can detect bacteria directly, eliminating the need for extra labeling steps.⁶⁸ This simplifies the assay procedure and manipulations, minimizes the assay cost, and reduces potential interferences from labeling steps.⁶⁹ In our study, we employed two model organisms with distinct Gram characteristics to demonstrate this versatility. This approach, combining the specificity of MIPs with the sensitivity of plasmonic sensing, offers a powerful tool for rapid and accurate bacterial detection in fields such as food safety, clinical diagnostics, and environmental monitoring without the need for additional labeling steps, thus simplifying procedures and reducing costs.

In the literature, our platform for bacterial detection stands out as a unique and advanced approach compared to existing methods. Shan et al., for instance, developed a technique for detecting *E. coli* O157:H7 in bacterial mixtures using imprinted polymers, specifically bacterial cell-imprinted polypyrrole and nafion complexes on AuNPs-coated microplates.⁷⁰ While there are numerous colorimetric platforms that utilize AuNPs for bacterial detection, our approach is distinctive in its use of MIPs as the recognition element.^{30,71–74} In addition, our sensor surfaces offer dual functionality that represents a significant advancement in the field. These surfaces not only enable bacterial recognition through bacteria-imprinted polymeric nanoparticles, but also incorporate AuNPs to impart plasmonic properties to the platform. This combination

enhances both the specificity and the sensitivity of bacterial detection. Furthermore, a key advantage of our method is its ability to detect multiple target bacteria using 96-well plates, demonstrating its versatility, multiplexing-on-a-plate and high-throughput screening. This feature is particularly valuable in clinical and environmental monitoring applications, where numerous analyses are necessary. The integration of MIPs with plasmonic sensing offers several benefits over traditional methods. MIPs provide high specificity and stability, while the plasmonic properties of AuNPs allow for rapid, label-free detection. This combination results in a sensitive and selective platform for bacterial detection that can be easily adapted to recognize various bacterial species or strains. When compared to the performance metrics of isothermal titration calorimetry and surface plasmon resonance (SPR) these gold standard methods provide much more detail of the binding kinetics between the bacteria and sensor surface. However, these gold-standard methods require specialized and expensive instruments. Instead, our method utilizes a standard plate reader which is a more common instrument in any biochemistry and biomedicine laboratories, and this feature would expand our methods utility and possible deployability in detecting *E. coli* and *S. aureus*. In sum, our research represents a significant step forward in the development of accurate and efficient bacterial detection platforms. By combining the specificity of MIPs with the sensitivity of plasmonic sensing, we have created a versatile tool with broad implications for disease diagnosis and monitoring. This approach addresses the growing need for rapid, sensitive, and selective detection of pathogenic bacteria in fields such as food safety, clinical diagnostics, and environmental monitoring. Bacterial diversity encompasses significant variability in the cell wall structure, surface antigens, and pathogenicity. This study serves as a proof-of-concept to demonstrate the sensor's capability to simultaneously detect two distinct bacterial species: *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). Their selection highlights the sensor's ability to differentiate bacteria based on cell wall composition and recognition patterns. Expanding the analysis to include species with unique cell wall structures, such as *Mycobacterium* sp., or extremophiles could further enhance understanding and broaden the sensor's applicability. Potential studies will explore these possibilities in the future. In conclusion, this research represents a significant step forward in the development of accurate and efficient bacterial detection platforms, with broad implications for disease diagnosis and monitoring.

■ ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study are available from the corresponding author on reasonable request.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsanm.4c06252>.

Experimental section; preparation of bacteria solutions; calculation of AuNPs concentration; spectral measurements and data analysis; results and discussion; characterization of MIPs, NIPs, and AuNPs; preparation of AuNP-modified MIP sensor surfaces; characterization of AuNP-modified MIP sensor surfaces; and *E. coli* and *S. aureus* detection assays (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Ö.E.: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing-review and editing, visualization. Y.S.: conceptualization, methodology, formal analysis, investigation, data curation, writing-original draft, writing-review and editing, project administration. Y.A.: conceptualization, methodology, writing-review and editing, visualization. F.I.: conceptualization, methodology, resources, writing-original draft, writing-review and editing, supervision, funding acquisition.

Notes

The authors declare no competing financial interest.

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