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Selective and High Dynamic Range Assay Format for Multiplex Detection of Pathogenic *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Legionella pneumophila* RNAs Using Surface Plasmon Resonance Imaging

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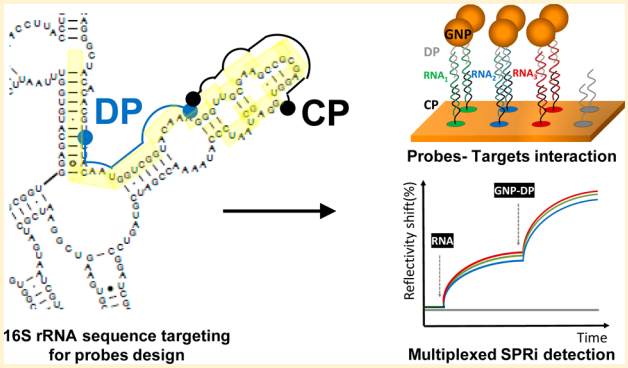
Supporting Information

ABSTRACT: Due to its well-characterized and highly conserved structure, as well as its relative abundance in metabolically active cells, bacterial 16S rRNA sequence plays an important role in microbial identification. In this work, a biosensing strategy has been developed for simultaneous detection of 16S rRNA analytes of three pathogenic bacterial strains: *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Surface plasmon resonance imaging (SPRi) was used as a detection technique coupled with DNA probe sandwich assemblies and gold nanoparticles (GNPs) for signal amplification. The targets 16S rRNA were selectively captured at the interface of the biosensor by surface-bound DNA probes through a hybridization process. GNP-grafted DNA detection probes were then introduced and were hybridized with a defined 16S rRNA region on the long DNA−RNA sandwich assemblies, resulting in a significant increase of the SPR signal. The results demonstrated the successful implementation of this strategy for detecting 16S rRNA sequences in total RNA mixed samples extracted from the three pathogenic strains at a concentration down to 10 pg mL^{-1} with a large dynamic range of 0.01−100 ng mL^{-1} and high selectivity. Since no particular optimization of the probe design was applied, this method should be relatively easy to adapt for quantification of a wide range of bacteria in various liquids.



A crucial aspect for preventing the spread of diseases by controlling water and air quality, improving patient care or limiting nosocomial infections, is the rapid and sensitive detection of pathogenic bacteria.[1−6] *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* are among the most common pathogens that contaminate water and hospital settings as well as food products, respectively.[3−5] *L. pneumophila* is the main cause for Legionnaires' disease, a form of severe pneumonia that might be fatal in certain cases.[7,8] *P. aeruginosa* can cause serious pathogenicity and host tissue damage once in contact with patients in healthcare units.[9,10] *S. typhimurium* is responsible for salmonellosis, a zoonotic disease of considerable importance.[11,12] Nowadays, several bacterial identification methods are available, including traditional approaches of plating and culturing, biochemical staining, microscopy, and flow cytometry.[3,11−16] Approaches based on immunoassays, polymerase chain reaction (PCR), and sequencing have also shown promising potential as highly sensitive tools for bacterial identification.[17−21] However, due to their long procedural times and considerable cost, most of these techniques still lack practical applicability within the context of on-site analysis or diagnostic routines. With the exception of PCR, these methods are unable to detect different bacterial strains simultaneously. Therefore, there is a need for rapid, easy-to-use, multiplexing platforms that allow accurate and simultaneous pathogen detection and identification.

Biosensors, combined with DNA microarray technology, provide a promising tool for multiplexing detection of microorganisms.[22−24] Advantages of biosensor systems include high specificity and selectivity, rapid response times, and simplification of sample preparation steps. On the other hand, the use of DNA probes offers an alternative strategy to microbial detection. Such probes directed against ribosomal RNA (rRNA) sequences have already been shown to be effective.[23,25] More specifically, targeting the 16S rRNA (a component of the 30S small subunit of bacterial ribosomes[26]) that reflects the amount of viable cells in the sample could directly impact the selectivity of the design approach through

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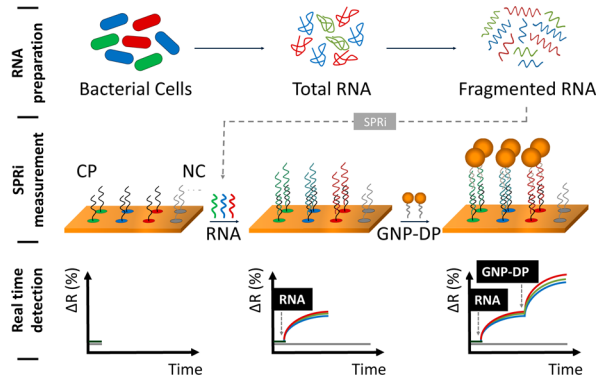
the selection of appropriate sequences.[25,27,28] Numerous biosensing techniques such as fluorescent microscopy,[29,30] electrochemistry,[31,32] impedance,[33] and surface-enhanced Raman spectroscopy[34] were used to detect 16S rRNA. With advantages including real-time and label-free detection, surface plasmon resonance (SPR) has offered promising avenues for time effective detection of a complex rRNA mixture.[25,35−37] Small et al.[25] were the first ones to describe the use of a DNA microarray for the detection of 16S rRNA with SPR. However, due to the rRNA secondary structure that prevents an optimal hybridization with the oligonucleotide probes, significant sensitivity limitation was observed compared to PCR-based assays. Biotinylated oligonucleotides were then applied near the position of the capture probe and contributed to reducing the structural interference and to increasing the hybridization efficiency.[25] A detection sensitivity of 14 μg mL^{-1} total RNA, representing 7.5 × 10^6 cells, was obtained. By optimizing the rRNA denaturation procedure, Nelson et al.[35] could lower the detection limit to 2 μg mL^{-1} 16S rRNA from *Escherichia coli*. To improve the sensitivity of SPR detection systems, which has limited its further application for bacterial identification in general, Joung et al.[38] used peptide nucleic probes (PNA) and gold nanoparticles for signal amplification by ionic interaction with 16S rRNA hybridized on the PNA-immobilized SPR sensor chip. This method resulted in a detection limit of *E. coli* rRNA of 58.2 ± 1.37 pg mL^{-1}. Recently, Foudeh et al.[37] used a near-infrared quantum dots (QDs) signal amplification strategy with a surface plasmon resonance imaging (SPRi) biosensor to detect *L. pneumophila* 16S rRNA with a limit of detection (LOD) down to 1 pM. In this study, we introduce a simple protocol for simultaneous, fast, and sensitive detection of three pathogenic species: *L. pneumophila, P. aeruginosa*, and *S. typhimurium* (Scheme 1). The proof-of-principle of SPRi-based probe gold nanoparticles (GNPs) for simultaneous detection of three different pathogens was shown by designing and assembling thiolated capture probes (CP) onto a microarray platform and by its subsequent specific hybridization with the target 16S rRNA of each bacterial strain present in the mixed total RNA sample.

Scheme 1. Illustration of the Assay Procedure



EXPERIMENTAL SECTION

Chemical and Reagents. GNPs with a diameter of 20 nm were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bis(*p*-sulfonatophenyl)phenylphosphine dihydrate dipotassium (BSPP); 6-mercapto-1-hexanol (MCH); potassium phosphate dibasic solution, 1 M, pH 8.9 (1 M K_2HPO_4); sodium chloride (NaCl); sulfuric acid (H_2SO_4); hydrogen peroxide (H_2O_2); ethanol; and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CH_3O-PEG-SH (MW 1200 Da) was purchased from Rapp Polymere GmbH (Tübingen, Germany). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). SSPE buffer (20× buffer is 3.0 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA at pH 7.4.) was purchased from Invitrogen (Carlsbad, CA, USA).

Oligonucleotide Sequences. To target the 16S rRNA of three different bacterial species, *L. pneumophila* (M59157), *P. aeruginosa* (PAO1), and *S. typhimurium* (SL1344), pairs of specific DNA probes were designed to be complementary to sequences within the target V8 regions (Supporting Information, Table S1 and Figure S1). Using the method previously described for *L. pneumophila*,[37] the specificity of these probes was confirmed by submitting the sequence to the Check Probe program of the Ribosomal Database Project. Synthetic 60 bp RNA from *L. pneumophila*'s 16S rRNA, synthetic 16S rRNA of *Legionella israelensis* for specificity analysis, and the negative control sequences were synthesized by Integrated DNA Technology (Coralville, IA, USA).

Surface Functionalization. Thiolated oligonucleotide probes were first grafted on gold-coated prisms (Horiba Scientific-GenOptics, Orsay, France) as self-assembled monolayers (SAMs). The prism gold surface was cleaned by piranha treatment. Droplets of approximately 200 nL of K_2HPO_4 buffer (1 M, pH 8.9) containing a mixture of 20 μM thiol-modified DNA probes and 10 μM PEG solution were deposited on the surface. After overnight drying, prisms were rinsed with deionized water and dried under nitrogen stream. Prior to the SPR measurements, the functionalized prisms were blocked with PEG (150 μM) and kept for 60 min at room temperature. Prism surfaces were then rinsed with deionized water and dried under nitrogen stream. GNP functionalization was performed according to the protocol described by Melaine et al.[39,40]

RNA Sample Preparation. *Sample Collection.* Bacteria were cultured using the following media and conditions: *L. pneumophila* (M59157) was first cultured on CYE plate at 37 °C for 3 days. The colonies were then suspended in AYE broth at an OD_{600} of 0.1; *P. aeruginosa* (PAO1) and *S. typhimurium* (SL1344) were first cultured on NA (Nutrient Agar) plate at 37 °C for 24 h and then suspended in Nutrient Broth Medium (Difco) until the cultures reached exponential phase (OD_{600} of 0.4 and 0.6 respectively); *Campylobacter jejuni* (81176) were first cultured in Mueller−Hinton (Difco) plates for 48 h at 42 °C and then in Mueller−Hinton broth at 42 °C until the culture reached exponential phase (OD_{600} of 0.4).

RNA Extraction. Samples for RNA extraction and colony-forming-unit (CFU) counts were collected from each replicate when the cultures reached exponential phase. Cells in each sample were centrifuged at 10 000 rpm for 2 min and RNA was extracted using TRIzol reagent (Ambion) according to the manufacturer's protocol. The RNA was then treated with Turbo DNase (Ambion) for 30 min and purified by standard acid phenol extraction. The final purified RNA yield was measured using a NanoDrop spectrophotometer (Thermo Scientific). For RNA fragmentation, a protocol previously described was used.[35] Briefly, total RNA extract was fragmented according to the fragmentation kit provided by the manufacturer (Ambion). The final solution was incubated at 75 °C for 15 min, followed by the addition of blocking solution (EDTA). The samples were stored in ice prior to use.

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SPRi Measurements. SPRi detection was performed using a scanning-angle SPRi instrument (model SPRi-Lab+, Horiba, France). The SPRi apparatus, equipped with an 800 nm LED source, a CCD camera, and a microfluidic flow cell, was placed in an incubator at 25 °C (Memmert Peltier, Rose Scientific, Canada). Reflectivity signals were acquired upon the stabilization of the baseline, and measured values were averaged over the replicates of each spot series and plotted as a function of time. Binding events with both the probes and the controls were monitored with at least three spots for each experiment. At each step, the substrate was washed with buffer, and the difference in the reflected intensity (ΔR (%)) was measured by taking into account the difference between the initial and final buffer signals. Successive hybridizations were followed by surface regeneration using 50 mM NaOH.

Statistics. The lower detection limit was defined as the smallest concentration of an analyte, calculated as the blank signal plus or minus three standard deviations. All data were expressed as the mean ± standard deviation (SD). In the graphics, the gray line, named NC, represents the SPRi signal obtained for negative control probes. The yellow line, named Au, represents the SPRi signal measured on the bare gold spots where no DNA functionalization was performed.

RESULTS AND DISCUSSION

Principle of the SPRi Assay. A previously developed protocol[37] for the bacterial strain *L. pneumophila* (LP) was used to target the known V8 regions of *S. typhimurium* (ST) and *P. aeruginosa* (PA). 16S rRNA sequences were used to design a set of specific capture and detection probes. With the aim of making this approach easy to implement, the V8 regions of the 16S rRNA sequences for the different bacterial strains were systematically selected without further optimization (Figure 1).

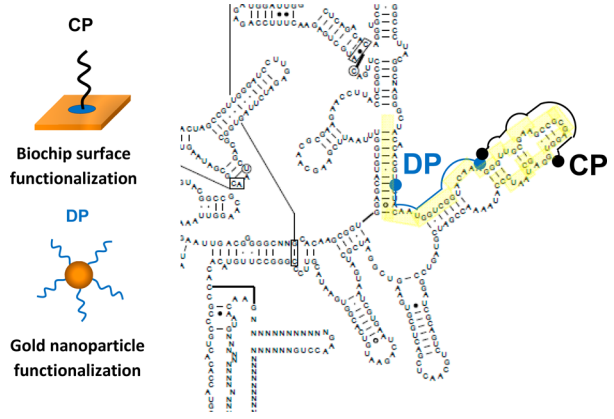

Figure 1. Illustration of the probe strategy design. Here, the 16S rRNA sequence of *Pseudomonas aeruginosa* is used as an example. The secondary structure of the 16S rRNA (on the right) was obtained from the Ribosomal Database Project Web site. For the other strains and more details, please see the Supporting Information. CP, capture probe; DP, detection probe.

The probe sequences, as well as the negative control, are listed in Table 1. The principle of the detection assay is presented in Scheme 1. Thiolated CP, containing the sequence complementary to the target 16S rRNA of each bacterial strain, was immobilized on the sensor surface by a self-assembled monolayer (SAM) method. In the presence of target 16S rRNA, the sequences specifically hybridized and induced a reflectivity variation. With the addition of DP immobilized on gold nanoparticles via their 5′-thiolated extremity, another hybridization reaction occurred at the second targeted region of the 16S rRNA sequences. This resulted in an increase of the SPR signal due to combined amplification modes of DNA−RNA−DNA sandwich assemblies and GNPs.

Validation of the Detection Conditions. Since signal amplification plays a key role for increasing SPR biosensor sensitivity,[37,38] we first determined the signal amplification parameter using synthetic 16S rRNA from *L. pneumophila* as a model organism to validate our approach. The most commonly used amplification method takes advantage of the unique optical properties of spherical gold nanoparticles in the size range of 13−20 nm. As such, gold nanoparticles of 20 nm, functionalized with DPs, were used as signal amplification agents upon the formation of DNA−RNA−DNA sandwich structures on the sensor surface. The SPR signal amplification was assessed by comparing the SPR measurements obtained through the introduction of DP alone and DP immobilized on GNPs. The measurements were repeated three times on the same chip (Figure 2).

The introduction of DP led to an increase of SPR signal ($\Delta R \sim 4.6\% \pm 0.32$), which is attributed to the hybridization process with the specific region of target 16S rRNA sequences. For the same experimental conditions, the use of DPs immobilized on GNPs caused a 3-fold increase of the SPR signal ($\Delta R \sim 12.01\% \pm 0.24$). This amplification in SPR signal with DP functionalized GNP is the result of the combined effect of locally optical index changes, provided by the GNP mass and the plasmonic coupling between the biosensor and the GNP surfaces.[42] To achieve higher detection sensitivity, other experimental parameters such as the surface chemistry were investigated, optimized, and finally implemented in the selected protocol.[39] Under optimal experimental conditions, the lowest detectable concentration of *L. pneumophila* 16S rRNA was found to be 1 pM,[39] which is comparable or superior to other reported methods for RNA detection using SPR biosenors.[25,35,37,38]

Multiplex Detection of Total RNA Isolated from Three Pathogenic Bacteria. Total RNA from *L. pneumophila*, *S. typhimurium*, and *P. aeruginosa* bacterial cultures, isolated according to a protocol developed by Nelson et al.,[35] was employed for analysis with SPRi. The use of RNA fragmented samples was expected to affect the sensor performance either by nonspecific adsorption, by cross-hybridization, or by limited accessibility to the region in the target 16S rRNA.[35] A serial dilution of a solution containing a mixture of the three fragmented RNAs was exposed to the SPR microarray that contains capture probes specific to each of LP, ST, and PA species and a negative control (NC). Figure 3a shows the sensograms obtained with SPR amplification detection. The introduction of target RNA at different concentrations led to a proportional increase of the SPR signals. At the highest RNA concentration (30 ng, for a total sample volume of 300 μL), the most significant reflectivity variation was found for the ST probes (9.33%), corresponding to a percent reflectivity change of 0.31% ng^{-1}.

For LP probes, the variation in percent reflectivity was 0.25% ng^{-1}, while the one for PA was 0.21% ng^{-1}. In all three cases, the initial amounts of specific reflectivity changes were subtracted from the reflectivity change recorded for the negative control probes (0.027% ng^{-1}).

The plot of SPR reflectivity variation (%) versus total RNA concentration (Figure 3b) shows a linear calibration curve for

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Table 1. DNA Sequences for Capture Probes (Sensor Surface Immobilized) and Detection Probes (GNP Surface Immobilized) Designed To Be Complementary to the 16S rRNAs of *L. pneumophila*, *P. aeruginosa*, and *S. typhimurium*[a,b]

		DNA probes	
symbol	phylogenic specificity	capture probes (CP)	detection probes (DP)
LP	*L. pneumophila* M59157	HS-T_{10}-CAGGTCGCCCCTTCGCCGCC	CTCTGTATCGGCCATTGTAGCTTTTT- SH
PA	*P. aeruginosa* PAO1	HS-T_{10}-CACCTCGCGGCTTGGCAACC	CTTTGTACCGACCATTGTAGCTTTTT-SH
ST	*S. typhimurium* SL1344	HS-T_{10}-CAATCCGGACTACGACGCAC	TTTATGAGGTCCGCTTGCTCTTTTTT-SH
NC	negative control	HS-$T1_0$-TCAATGAGCAAAGGTAT	

[a]Based upon the database of rRNA sequences available at the Ribosomal Database Project.[41] [b]The negative control sequence (NC) is also presented.

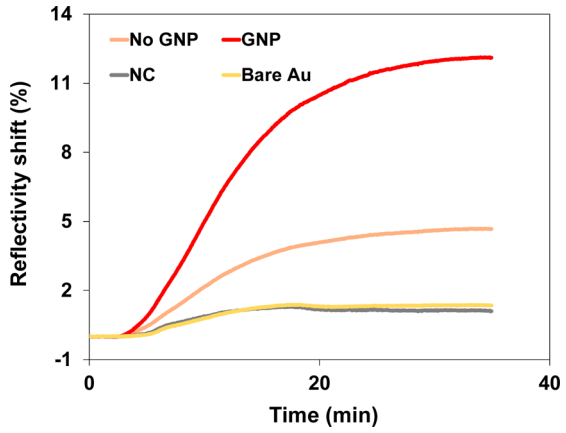

Figure 2. Real-time SPRi kinetic curves for the detection of *L. pneumophila* 16S rRNA sequence (50 nM) without (orange curve) or with GNP (100 pM) as amplification agents (red curve). The introduction of DPs immobilized on GNPs led to a 3-fold increase of the SPR signal ($\Delta R \sim 12.01\% \pm 0.24$).

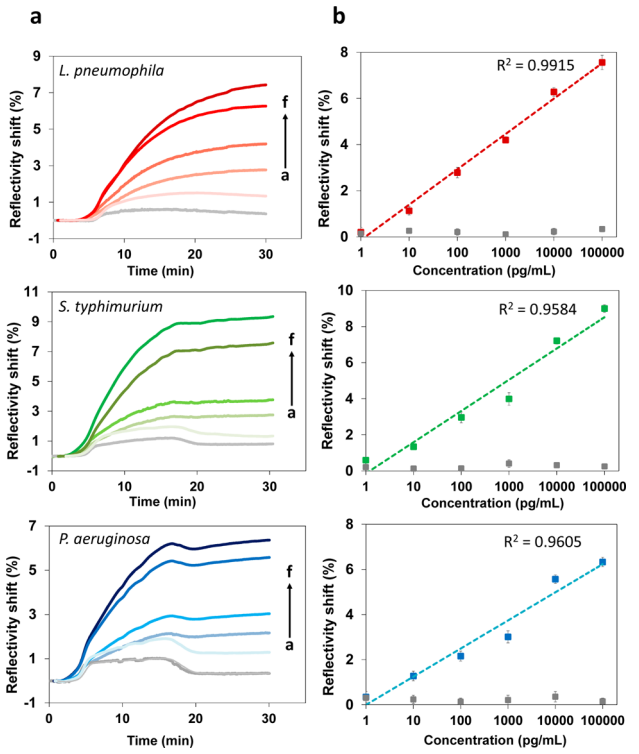

Figure 3. (a) SPR sensograms for amplified simultaneous detection of bacterial RNA from LP, ST, and PA strains, at 1, 10, 100, 1000, 10 000, and 100 000 pg mL^{-1} (from a to f). The introduction of target RNA at increased concentrations led to a proportional augmentation of the SPR signals. (b) Calibration curves for amplified detection of bacterial RNA from LP (red curve), ST (green curve), and PA (blue curve) strains, at 1, 10, 100, 1000, 10 000, and 100 000 pg mL^{-1}. The general dynamic detection range is 0.01−100 ng mL^{-1}. All data expressed as mean ± standard variation (n = 3).

the three species. While RNA concentration over 1000 pg mL^{-1} induced a difference in the total reflectivity change between the three strains, at the lower concentrations, i.e., 1, 10, 100, and 1000 pg mL^{-1}, variation in the SPRi signal showed similar trends. Based on the 3σ rule, the lowest detectable concentration of RNA was determined to be 10 pg mL^{-1} and the dynamic detection range was 0.01−100 ng mL^{-1}. Interestingly, all designed CPs and DPs exhibit high selectivity with their targets with minimal nonspecific binding even without any specific optimization step for each probe (Supporting Information). Notably, the detection limit of the developed SPR biosensor shows an improvement of 2 orders of magnitude compared to the one obtained with an SPR detection without any amplification method.[25,35] The performance of the current assay is also comparable to other reported optical techniques for the detection of total bacterial RNA.[37,38] This result can be attributed to the advantageous features of GNP signal amplification that complement the sandwich detection strategy.

Specificity of the Developed Sensor. The specificity of the SPR biosensor was investigated by exposing the microarray to a sample containing RNA targets (0.1 μg mL^{-1}) from five different bacterial species, including LP, ST, and PA, but also *C. jejuni* (CJ) and *L. israelensis* (LI). As a first step, RNA from LP, ST, and PA was introduced in the SPRi system and the signal was recorded according to the response obtained on their respective CP spots. The chip was then regenerated to remove the bound 16S rRNA sequences. Total RNA from CJ was then introduced and the signal from all the CP spots was recorded and averaged. After the chip regeneration, total RNA from LI was finally introduced and the same measurement was performed. NC and bare gold (Au) signals presented in Figure 4 correspond to the mean reflectivity variation measured on these spots for all the detection steps. As shown in Figure 4, the SPR signals from the specific RNA targets LP, ST, and PA provided a remarkable increase in SPR signal (~8-fold) as compared with other target signals while successively exposed to the sensor surface.

In that case, the average SPRi signal obtained from the three specific probes is slightly distinguishable from the signal of the negative control probes NC and the bare gold. These results indicate that the current biosensor displays excellent specificity for the simultaneous detection of, at least, three different bacterial RNAs (ST, LP, PA). This could be due to the high affinity of the designed probes with the targeted 16S rRNA regions.[27]

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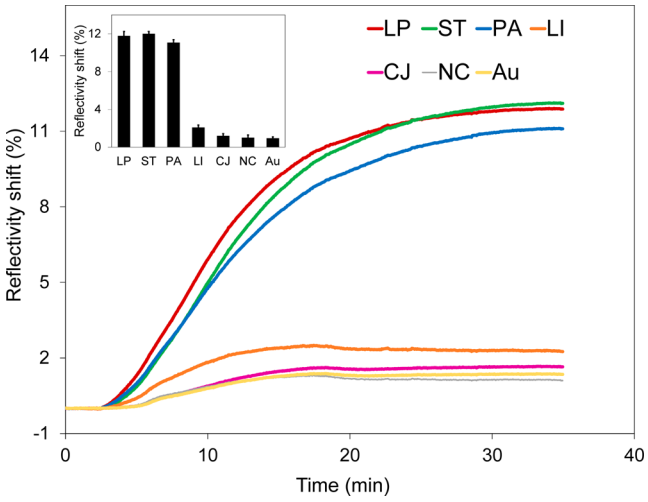

Figure 4. Comparison of SPR signals obtained upon introduction of a mixed RNA sample (0.1 μg mL^{-1}) of three specific bacterial strains: *L. pneumophila* (LP), *S. typhimurium* (ST), and *P. aeruginosa* (PA). The average signal obtained from the specific probes upon the successive introduction of *C. jejuni* (CJ) and *L. israelensis* (LI) RNA targets in the regenerated chip is also shown. Only the three RNA targets LP, ST, and PA give a significant increase of the SPR signal (~8 times) as compared with other targets CJ and LI while successively exposed to the sensor surface. CJ and LI signals are slightly distinguishable from the negative control (NC) and bare gold (Au) signals. All data expressed as mean ± standard variation (n = 3).

CONCLUSIONS

In this work, we introduce an adaptable and simple methodology based on an SPR microarray with gold nanoparticles as a signal amplification strategy for multiplex detection of pathogens. We successfully demonstrated the use of a multiplex assay to selectively and rapidly detect 16S rRNA from total RNA extracts of three different bacterial strains. The 16S rRNA can be targeted in the known V8 region without further optimization of the designed probes. Using SPR imaging and gold nanoparticle based signal amplification, target RNA with a concentration as low as 10 pg mL^{-1} can be simultaneously detected in a mixed sample, in less than 1 h. The developed system shows high specificity and selectivity toward the targets, as well as a large dynamic range of 0.01−100 ng mL^{-1}. Although the current developments provide a viable alternative to conventional detection techniques, further efforts are clearly needed to address simple implementation, automation, and reusability of the material for on-site or point-of-care applications. Going forward, a potential combination of this generic method with newly emerging microfluidic tools may have a significant impact on this issue.[43] This approach could then be extended to detect and quantify a wider range of microbial RNA sequences and for high throughput assays.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01942.

Details of DNA sequences selected for probe design; sensograms obtained for individual detection of 16S rRNA of *Salmonella typhimurium* and *Pseudomonas aeruginosa* (PDF)

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Notes

The authors declare no competing financial interest.

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