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Aptamer-functionalized localized surface plasmon resonance sensor for the multiplexed detection of different bacterial species

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Abstract

A localized surface plasmon resonance (LSPR)-based sensor with an immobilized aptamer ligand was developed and used for the label-free and accurate detection of bacteria through observing the changes in the peak extinction intensity. The ability of this biosensor to recognize pathogenic bacteria was analyzed and conditions were optimized with different probe concentrations, incubation time for aptamer immobilization, and incubation time for cell binding. A single LSPR-based sensor was used to successfully detect and identify three different bacterial species as proof-of-concept experiments; in all cases, the sensor showed a detection limit of 30 cfu per assay. Furthermore, the sensor system could clearly identify

various target bacterial species in a multiplexed mode with high specificities on a single chip. The label-free bacteria sensor developed by combining LSPR and aptamers will be useful for diagnosing various infectious diseases through a single convenient assay.

Highlights

- The aptamer-immobilized LSPR sensor was developed for bacteria detection.
- The optimal condition for aptamer binding on LSPR chip was determined.
- The optimal condition for bacteria detection was determined.
- Rapid, accurate, and multiplexed detection of different bacteria is possible.

Keywords

Aptamer; Bacterial cell detection; Localized surface plasmon resonance sensor; Label free

1. Introduction

Bacteria, which are part of a very large group of single-celled organisms, exist in all human habitats. Some of these bacteria are causative agents of various infectious diseases and can often cause death if not treated properly, in particular in immnuo-compromised patients. Pathogenic bacteria can infect humans, animals, and plants through the intake of food, water, and air, while can be transferred through the contact with infected surface or liquid. Thus, the rapid and accurate detection of pathogenic bacteria is of great importance in all areas related to health and safety, including clinics, food industries, environment, and defence. This has prompted the need for accurate assay systems for detecting bacteria, which could facilitate the use of early antibiotic interventions to treat infectious diseases associated.

Molecular diagnostic methods, which typically rely on the detection of nucleic acids (DNA or RNA) from clinical and environmental samples, can effectively detect bacteria with high sensitivity and specificity. In particular, polymerase chain reaction (PCR)-based assays have been demonstrated successful for the detection of small numbers of bacterial cells present in some samples [1,2]. However, these assays are time-consuming and require preparation of the target DNA, limiting their widespread use as diagnostic platforms. Thus, there has been an urgent need for new strategies for the rapid and simple detection of bacterial species with high sensitivity and specificity. Recently, various platforms for the direct detection of bacterial cells have been developed through the use of different sensing approaches, including the assessment of electrical signals [3,4], electrochemical sensing [5-8], surface-enhanced Raman scattering (SERS) [9-11], fluorescence signals [12,13], localized surface plasmon resonance (LSPR) [14], and optical sensing [15,16].

Among these sensing strategies, LSPR is considered a promising method for label-free and sensitive detection of biomolecules, as it displays an intensity change and/or a shift of the single sharp spectral extinction peak when an unmodified analyte binds to a local dielectric surface [17-20]. Another exciting technique that can be used for sensing cells is the employment of nucleic acid ligands known as aptamers. Compared with the antibodies that have traditionally been used for cell sensing, aptamers have the benefits of high affinity and specificity, long-term stability, easy synthesis and modification, low cost, and a wide spectrum of target analytes that can be designed [21]. These distinctive advantages of aptamers can potentially employ for the efficient detection of pathogenic bacteria.

Interestingly, however, despite such superb sensing capabilities of LSPR-based strategies and advantages of aptamer ligands, there has been no report, to our knowledge, on the use of LSPR-based bacterial cell sensor that employs aptamer ligands.

Here, we describe the label-free and accurate detection of bacteria using an aptamer-immobilized LSPR-based sensor. In this system, a multispot gold-capped nanoparticle array (MG-NPA) chip is composed of a dielectric layer comprising a thin gold (Au) layer on silica (Si) nanoparticles (NPs)-absorbed glass slide. Using this sensor system, we successfully detected and identified three different bacterial species in a multiplexed mode. Combination of LSPR sensing platform with aptamer provides unique advantages. First, the well-defined MG-NPA structure used in this study results in sensitive and reproducible LSPR peaks [22-24]. Second, functionalization of specific aptamer ligands on the MG-NPA structure surface allows the simple and rapid detection of bacterial species without the need for any modification or purification process of the test samples. Third, the use of multiple spots with different aptamers on a single chip allowes us to simultaneously identify different bacterial species in a single assay. Therefore, the aptamer-immobilized LSPR-based sensor system developed in this study will be useful for convenient, rapid and accurate identification of bacterial species, including pathogenic bacteria, by simple assay.

2. Experimental

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Co. Glass slides were purchased from Matsunami Glass Ind., Ltd, Japan. Si NPs were purchased from Polysciences Inc. NAP-5 column was purchase from GE healthcare Co. Reference bacteria were obtained from the

Korean Collection for Type Cultures (KCTC; Korea) and the American Type Culture Collection (ATCC; USA), and cultured according to the provided instructions (Table S1 in Supporting information). Oligonucleotides were synthesized at Genotech (Korea). The sequences of the bacterial species-specific aptamers are as follows: *Lactobacillus acidophilus*-specific aptamer (Lac-apt), 5'-AGCAGCACAGAGGTCAGATGTAGCCCTTCAACATAGTAATATCTCTGCATTCTGTGTGCCTATGCGTGCTACCGTGAA-$(CH_2)_3$-SH-3' [25]; *Salmonella typhimurium*-specific aptamer (Sty-apt), 5'-TATGGCGGCGTCACCCGACGGGGACTTGACATTATGACAG-$(CH_2)_3$-SH-3' [26]; and *Pseudomonas aeruginosa*-specific aptamer (Pae-apt), 5'-CCCCCGTTGCTTTCGCTTTTCCTTTCGCTTTTGTTCGTTTCGTCCCTGCTTCCTTTCTTG-$(CH_2)_3$-SH-3' [27].

2.2 Preparation of the LSPR-based Sensor

Glass slide (76 mm × 26 mm × 1 mm) was treated by ultrasonication for 10 min in acetone, ethanol, and ultrapure deionized (DI) water and then deposited subsequently with chromium (5 nm in height) and gold (40 nm in height) by using an E-beam evaporator (MHS 1800; MooHan Vacuum Co., Korea) at a base pressure of 4×10^{-6} Torr (1^{st} Au layer deposition process on a glass slide, see Figure 1a). For forming multiple spots on a single chip, a multi-spot (3 nm in diameter) silicon-rubber mask was attached to the surface of the Au-deposited glass slide. The slide with the mask was then treated with 1 mM 4,4'-dithiobutyric acid (DDA) for 1 h, and then incubated with Si NPs (100 nm in diameter) activated by 1% (v/v) 3-aminopropyltriethoxy silane (γ-APTES) and 400 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 1 h (Silica nanoparticle deposition process, see Figure 1a). The Si

NP-modified slide was then rinsed thoroughly with DI water to remove the excess surface modified nanoparticles, and subsequently dried at room temperature (RT). Finally, a top Au layer (30 nm in height) was deposited onto the Si NP-modified substrate using the E-beam evaporator (2^{nd} Au layer deposition process, see Figure 1a).

2.3. Functionalization of the sensor surface using aptamers

The thiolated aptamers were treated with 1 M dithiothreitol (DTT) and purified using NAP-5 column. The sensor surface was exposed to 10 μM aptamer in phosphate buffered saline (PBS) at RT for 4 h under humidified condition, and then washed with 0.1% (w/v) sodium dodecyl sulfate (SDS) for 5 min, rinsed with DI water, and dried under a nitrogen stream.

2.4. Detection of bacterial species

The samples with different bacterial species were dropped and incubated on different spots of the aptamer-functionalized sensor chip at RT for 1 h under humidified condition to prevent the evaporation of the samples. The slide was washed with PBS for 5 min and dried under the nitrogen stream. The LSPR spectra from the chip were measured at ambient conditions.

Instrumentation

The optical measurement system used to assess the LSPR spectra consisted of a light source (wavelength range, 360-2000 nm), spectrophotometer (wavelength range, 200-1100 nm) and an optical fiber probe bundle (fiber core diameter, 300 m; wavelength range, 250-900 nm) as a spectroscopy system, purchased from Ocean Optics (USA). The optical properties of the LSPR sensor were assessed across a wavelength range of 400 to 750 nm, using the optical

measurement system at RT. Scanning microscopic images of particles and cells were obtained using Hitachi SEM (S-4800; Hitachi, Japan).

Results and discussion

A schematic representation of bacterial species detection using the LSPR-based sensor is shown in Fig. 1a. First, the MG-NPA chip was fabricated by stepwise deposition of Au and Si NPs on a glass substrate (see Experimental Section and Fig. 1b). The dielectric structure of the chip yields a sharp and simple LSPR peak and shows sensitive responses upon binding of biomolecules [22-24]. The stepwise deposition of well-arranged Si NPs on the substrate produces a stable and reproducible LSPR peaks [22-24]. Next, the chip was functionalized with an aptamer, to enable subsequent binding reactions with the corresponding bacterial species. Aptamer-functionalized surface does not require any pre-treatment of test samples, allowing the simple and rapid detection of bacterial cells. Upon incubation of the chip with a sample, the presence of the target bacterial cells in the sample could be determined by observing a change in the LSPR peak. The LSPR-sensing process can be generally visualized by observing a red shift in the wavelength and/or a change in the extinction intensity of the peak. Unlike other noble metal structures, MG-NPA chip exhibits a clearer change in the peak extinction intensity compared to the wavelength shift upon binding of the target biomolecule [22-24].

To determine the optimal assay condition, the aptamer concentrations and incubation times were varied for aptamer immobilization and for cell-aptamer interaction on chips. First, the effect of varying conditions on aptamer immobilization on chips was

investigated by using an aptamer specific to *Lactobacillus acidophilus*. As shown in Fig. 2, the LSPR peak intensity was changed by varying the aptamer concentration (50, 25, 10, 5, 1, 0.5, and 0.1 μM) and incubation time (1, 2, 3, 4, 5, and 6 h). The change in LSPR peak intensity was maximized and saturated upon incubation of the chip with 10 μM aptamer for 4 h, indicating that the maximum binding of aptamer on the sensor surface was achieved under these conditions (Fig. 2a and b). In order to confirm the aptamer-binding capacity of the chip, the chip was sequentially treated with aptamer (Lac-apt) and its complementary DNA-attached Au NPs, and subjected to scanning electron microscope (SEM) analysis (see Fig. 2c and Supporting information). The Au NPs (~10 nm) were found to be densely and evenly bound on the Au chip surface. The number of aptamers on the spot of the chip was calculated following the method reported previously (see Supporting information)[28]. These data demonstrate that the chip was successfully functionalized with the utilized aptamer.

Next, the incubation time for cell-aptamer binding was optimized. The chip treated with 10 μM aptamer for 4 h was incubated with *L. acidophilus* (10^9 cfu mL^{-1}) for different times (20, 40, 60, 80, 100, and 120 min). As seen for the aptamer-bound chip, the cell-aptamer interaction on the chip also induced a change in the peak extinction intensity (Fig. 2d and e), which increased with the incubation time up to 1 h and became saturated thereafter (Fig. 2d). Cells captured by the aptamer-functionalized chip were also confirmed by SEM analysis (Fig. 2f). The bacterial cells went through the fixation process with 2.5% glutaraldehyde during the preparation of SEM specimen (see Supporting information). The fixation process can effectively maintain cell morphology [29]. Fig. 2 shows that an aptamer concentration of 10 μM per assay, an aptamer

incubation time of 4 h, and cell binding time of 1 h are optimal. Thus, all subsequent experiments were performed under this condition.

As a proof-of-concept multiplex detection of bacterial species, the sensitivity and specificity of the assay were determined using three different bacteria, *L. acidophilus, S. typhimurium,* and *P. aeruginosa*, under the optimal condition determined above. *S. typhimurium* is a representative food-borne pathogen; together with *E. coli* O157:H7, they cause an estimated 76 million illnesses and nearly 5,000 deaths annually in the US [26]. *P. aeruginosa* is among the pathogens that cause nosocomial infections with increasing threat, and thus significantly increases the mortality risk of inpatients [27]. The detection limit of the assay was determined by changing the number of target bacterial cells per volume (cell concentration hereafter) over a range of 10^9 to 10^1 cfu mL^{-1} (upper panels of Fig. 3). The extinction intensity showed a logarithmic increase with respect to cell concentration over a range of 10^9 to 10^4 cfu mL^{-1} (lower panels of Fig. 3). The SEM images confirmed that the changes in LSPR peak intensities reflected the concentrations of bacterial cells bound to the chip surface (Fig. S1 in Supporting information). Interestingly, the slopes of these plots displayed subtle variations, indicating that the binding affinities between the different aptamers and their target cells were slightly different. The K_d values previously reported for these aptamers were 13 ± 3 nM for the *L. acidophilus*-specific aptamer [25] and 17.27 ± 5 nM for the *P. aeruginosa* specific aptamer [27]. Thus, our results suggest that the LSPR sensor enables the quantitative detection of bacterial cells. Considering the assay volume (3 µl), this sensing system can detect 30 colony forming unit (cfu) of bacteria in an assay. This detection limit is comparable to or slightly higher than other sensing methods including

electrical signals [3,4], electrochemical sensing [5,7-8], SERS [11] and optical sensing [15,16] which showed the detection limit of 10^1 to 3×10^4 cfu mL^{-1}. It should be noted that very small amount of sample volume (3 µl) was used in our study, as compared to other sensors, which generally require samples ranging from tens to hundreds of microliters. Thus, the use of a larger assay volume with, for example, a microfluidic flow cell could allow more efficient detection of bacteria. The detection limit of our assay could also be improved by employing the aptamer with higher affinity and specificity. The detection limit of the aptamer-LSPR sensor system developed here is quite impressive when compared with those of other optical sensors [8-13,16].

In order to determine assay selectivity which is important for multiplexed detection, the multi-spot LSPR chip was functionalized with three different species-specific aptamers (Lac-apt, Sty-apt, and Pae-apt) and cross-checked whether each corresponding bacterial species were bound specifically to their corresponding aptamers. As shown in Fig. 4, the highest LSPR peak intensities were observed with proper binding between each aptamer and its specific target bacterium. As negative controls, cell-free PBS solution and *E. coli* were used; these did not trigger any significant change in LSPR peak intensity. Thus, these results indicate that the aptamer-functionalized LSPR-based sensor could clearly identify various target bacterial species in a multiplexed mode with high specificities on a single chip.

In order to be useful in practice, a pathogen-diagnosing assay should be simple, sensitive, fast, and high-throughput. To date, aptamer-based assays for bacterial detection have employed electrical and optical sensors. These approaches have achieved high sensitivities, but are not amenable to high throughput detection in a single assay

[3,4,7]. In contrast, the aptamer-immobilized LSRP sensing assay developed here is rapid, simple, and most importantly, capable of simultaneously identifying different bacterial species in a single assay by employing various specific aptamers. Although relatively few aptamers have been developed for clinically important pathogenic bacteria, the future availability of aptamers will allow multiplex detection of many different bacterial species in a single assay, thereby increasing the potential usefulness of this assay in a wide range of fields, including pathogen diagnostics. Furthermore, although the LSPR signals reported herein were measured using a large home-made instrument, portable instruments with LSPR-based sensors can be employed, enabling the facile and mobile detection of pathogenic bacteria in point-of-care diagnostics.

4. Conclusions

In summary, we developed an easy-to-build, aptamer-immobilized LSPR-based sensor for the simple, rapid, sensitive, specific, and multiplex sensing of bacterial species. Using this sensor system, three different bacteria could be detected with high sensitivity and specificity as a proof-of-concept demonstration. It is thus expected that the use of an LSPR-based sensor coupled with bacterial species-specific aptamers will enable efficient diagnostics of different bacterial species including pathogenic bacteria.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found, in the online version, at http://XXX.XXX

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Figure legend

Fig. 1. Aptamer-immobilized LSPR sensor system. (a) Schematic representing the detection of bacterial cells using the aptamer-functionalized LSPR-based sensor; (b) Multispot gold-capped nanoparticle array (MG-NPA) chip. Each spot (diameter of 3 mm) on chip consists of Si NPs with dielectric structure formed by the deposition of Au layer.

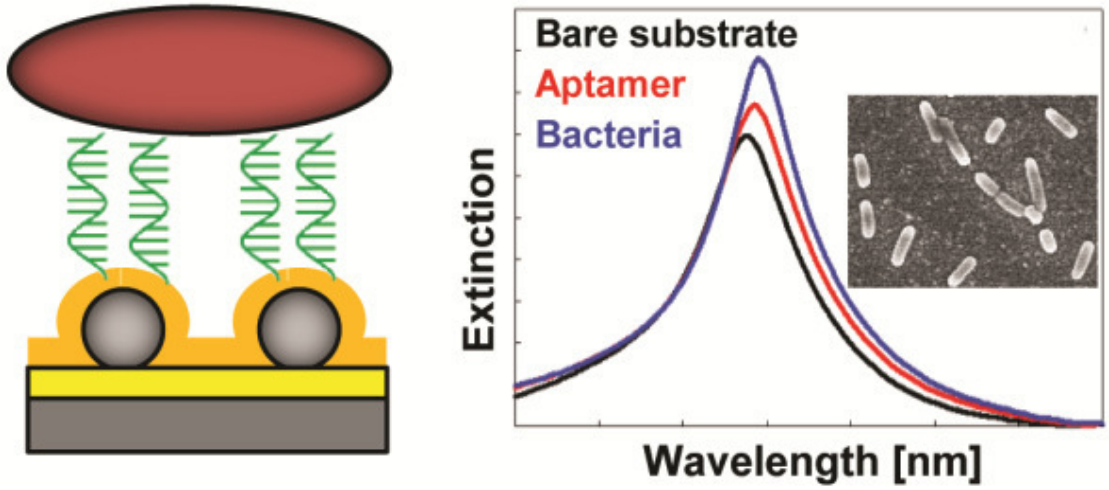
Fig. 2. Optimal conditions for cell sensing using the aptamer-immobilized LSPR-based sensor system. (a) Effect of aptamer concentration on LSPR optical properties. MG-NPA chips were incubated with different concentrations of the *Lactobacillus acidophilus*-specific aptamer (Lac-apt; 0.1, 0.5, 1, 5, 10, 25, and 50 μM); (b) Effect of aptamer incubation time on LSPR optical properties. Aptamers (10 μM) were incubated with the chip for various times (1, 2, 3, 4, 5, and 6 h); (c) Scanning electron microscope (SEM) images obtained (left panel) before and (right panel) after the immobilized aptamer on a chip was bound with its complementary DNA-attached Au NPs (see Supporting Information for a detailed method); (d) Effect of cell incubation time on LSPR optical properties. *L. acidophilus* (10^9 cfu mL^{-1}) was incubated with the aptamer-immobilized chip for various times (20, 40, 60, 80, 100, and 120 min); (e) LSPR spectrum profiles obtained from the aptamer-immobilized chip after binding of the target cells; (f) SEM images of bound cells (10^9 cfu mL^{-1} of *L. acidophilus*) on

a chip.

Fig. 3. The detection limits of the LSPR-based cell sensor. The detection limit was determined by observing the increases in LSPR peak intensities in response to different cell concentrations (10^1 to 10^9 cfu mL^{-1}) of (a) *L. acidophilus*, (b) *S. typhimurium*, and (c) *P. aeruginosa*. The upper panels show the LSPR spectrum profiles. The lower panels show the plots of the changes in LSPR signal intensity versus the target cell concentration. The data was obtained from three measurements, and the error bars represent standard deviations. Lac-apt, *L. acidophilus*-specific aptmaer; Sty-apt, *S. typhimurium*-specific aptamer; Pae-apt, *P. aeruginosa*-specific aptamer.

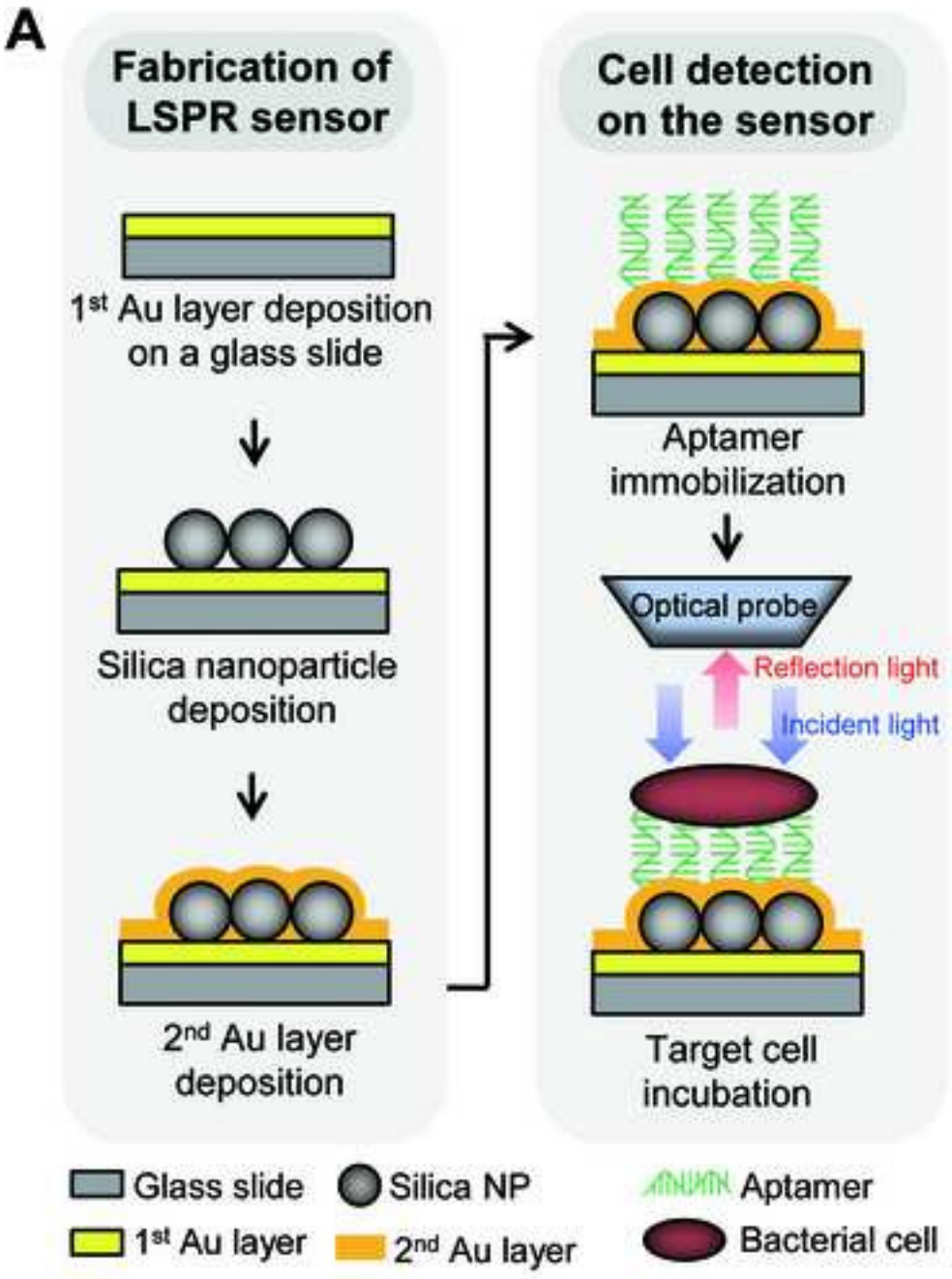
Fig. 4. Selective binding of bacterial cells to the aptamers immobilized on the LSPR sensor. (a) Design of the MG-NPA chip developed for the selectivity assay. Each species-specific aptamer was immobilized on each spot of chips and subsequently10^9 cfu mL^{-1} of different bacterial cells were applied to spots; (b) A change in the extinction intensity of the peak at each spot could be used to assess the stringency of affinity. Cell-free PBS solution and *E. coli* were applied to chips as negative controls. The data were obtained from three independent measurements, and the error bars represent standard deviations.

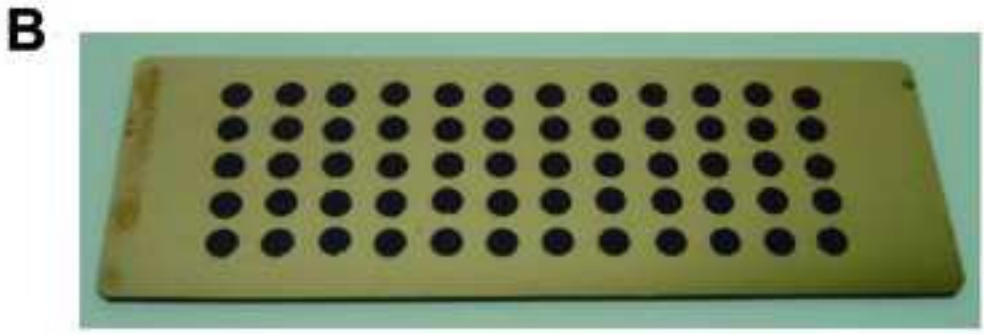
Graphical Abstract



Aptamer-immobilized LSPR biosensor system was developed for the rapid, specific, accurate, and multiplexed detection of different bacterial species.

Figure 1


A
Fabrication of LSPR sensor
1st Au layer deposition on a glass slide
Silica nanoparticle deposition
2nd Au layer deposition
Cell detection on the sensor
Aptamer immobilization
Optical probe
Reflection light
Incident light
Target cell incubation
Glass slide
Silica NP
Aptamer
1st Au layer
2nd Au layer
Bacterial cell


B

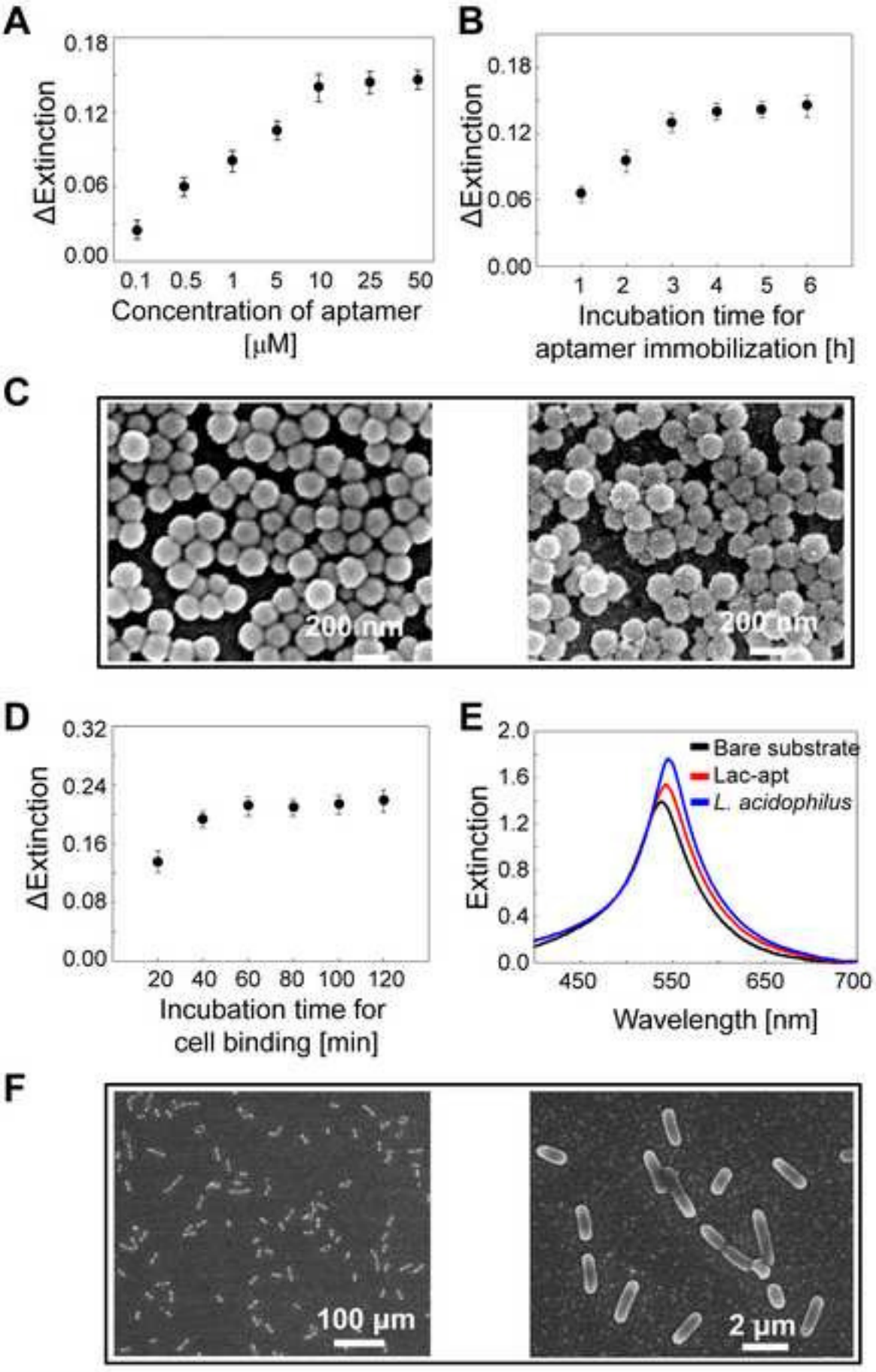


Figure 3