Sensors and Actuators B 259 (2018) 492–497

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

High sensitivity gram-negative bacteria biosensor based on a small-molecule modified surface plasmon resonance chip studied using a laser scanning confocal imaging-surface plasmon resonance system

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ARTICLE INFO

Article history:
Received 10 October 2017
Received in revised form 5 December 2017
Accepted 7 December 2017
Available online 16 December 2017

Keywords:
Biosensor
LSCI-SPR
Gram-negative bacteria
Small molecule

ABSTRACT

The highly sensitive and rapid detection and distinction of gram-negative bacteria are essential for the early diagnosis and accurate medical treatment of bacterial infection. In this work, we investigated a new selective and sensitive method of detecting gram-negative bacteria based on a small molecule modified sensor chip by a laser scanning confocal imaging-surface plasmon resonance (LSCI-SPR) system. The small molecule (polymyxin B) strongly interacted with bacteria cell membrane. SPR signals changed with SYTO 9 stained bacteria concentrations, and fluorescence images were recorded at the same time. Results showed that *Escherichia coli* were distinguished clearly with a limit of detection (LOD) of 1.0×10^2 CFU/mL for *Escherichia coli*. Moreover, a good linear relationship was observed between bacteria concentrations and SPR signals from 1.0×10^2 CFU/mL to 1.0×10^6 CFU/mL with a linear coefficient R^2 of 0.99146. All these results indicated that this small-molecule modified sensor can be used for quantitative detection of known gram-negative bacteria and qualitative distinction of unknown gram-negative bacteria within 10 min each sample in real-time.

1. Introduction

Pathogenic bacteria are causative agents of serious infections and threaten public safety worldwide [1–4]. To make precision early diagnosis and use accurate medicine, it is necessary to develop real-time, quantitative, sensitive and rapid methods of identification and discrimination bacteria species. There have been a lot of methods of bacterial classification of gram-positive and gram-negative bacteria. The Gram stain is almost always the "first stage criteria" in the preliminary identification of bacteria species by the different structure of their cell walls, but the Gram stain is not an infallible tool because it is time-consuming and inaccurate [5]. Moreover, classical microbiological technique (CMT) depends on the culture source and the growth rate of the pathogen and is thus time-consuming [6]. The situation is similar to polymerase chain reaction (PCR) [7,8], Whereas enzyme-linked immunosorbent assay (ELISA) is rapid and simple but requires specific enzyme labeling and complex methods and has limited application[9]. Thus, an effective method for the early diagnosis and successful medical treatment of bacterial infection with the potential to be miniaturized into a low-cost, portable system is urgent to develop.

Surface plasmon resonance (SPR) is a widely used technique for the in situ and real-time measurements of biosensor applications [10–12]. The highly sensitive optical reflectivity of gold to dielectric changes in the environment enables the SPR sensor to detect traces of contaminants in water, food, air, and label-free biochemical assays [13–15]. In our previous work, we have developed a laser scanning confocal imaging (LSCI)-SPR system and used it to detect the interactions of ion-ssDNA [16], antibody-antigen [17], large molecule-biological cell interaction [18] and small molecule-biological cell [19].

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https://doi.org/10.1016/j.snb.2017.12.042
0925-4005/

SPR technology has been used to detect pathogenic microorganisms [20]. Baccar et al. developed the SPR immunosensor for *E. coli* using antibody, and the limit of detection (LOD) is 1.0×10^3 CFU/mL [21]. Nguyen et al. combined SPR and PCR into an inline all-in-one platform for pathogenic-bacteria detection to allow reusability of the device and further miniaturization of the whole system [22]. Yilmaz et al. combined SPR and QCM using synthetic receptors based on whole-cell imprinting [23].

In this paper, a small molecule (polymyxin B) that strongly interacts with a gram-negative bacteria cell membrane was modified on the gold film of an SPR chip [24,25]. The small molecule can overcome the disadvantages of antibodies and other large molecules. For example, small molecules are difficult to inactivate, the modification method is simple, and the test conditions are easy to reach [19,26,27]. The interaction between small molecule and bacteria can alter the cell-membrane structure and render bacteria cell permeable [28]. The binding of bacteria stained with the cell-membrane permeable SYTO 9 green-fluorescent nucleic acid stain to small molecule (polymyxin B) induced changes in SPR signals and fluorescent images [29]. Results showed that this method can distinguish gram-negative bacteria from common pathogens, including gram-negative bacteria, gram-positive bacteria, and fungi. Quantitative detection results were obtained through gram-negative bacteria real-time monitoring in sodium chloride injection by using an LSCI-SPR system. Our method can be a supplement to Gram stain and be used for early clinical diagnosis and provide effective supporting data for appropriate treatment programs. Moreover, this specific small-molecule modification can inspire a new method of SPR sensor-chip design.

2. Experiment

2.1. Materials and reagents

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* AS1.2031, *Klebsiella pneumonia* AS1.1736, *Staphylococcus aureus* ATCC 6538, *and Candida albicans* ATCC 2091 were purchased from Antibaterial Material Testing Center of Technique Institute of Physics and Chemistry, Chinese Academy of Sciences. Sodium chloride injection was purchased from Shanghai Qidu pharmaceutical Co., Ltd. 3-Mercaptopropionic acid (3-MPA), 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and polymyxin B sulfate were purchased from TCI Development Co., Ltd. (Shanghai, China). SYTO 9 was obtained from Molecular Probes. The ultrapure water (18.2 MΩ × cm) used in all experiments was purified using a Millipore Milli-Q gradient system.

2.2. Instrument

The LSCI-SPR experimental setup was similar to that described in our previous work with the fiber spectrometer changed to monochromatic and the detector, a photomultiplier tube (H10723-20, Hamamatsu, Japan) [19]. A 60 × objective (Nikon CPlan Apochromatic; N.A. 1.40, W.D. 0.13 mm) was used for imaging with 3.9 nm Cr and 49 nm Au film deposited onto K9 ($n = 1.51630$) by thermal evaporation method successively. The sizes of the gold films were $30 \times 25 \times 2$ mm^3. The gold films had many (111) planes and few (222) planes [19]. SYTO 9 was excited with a sapphire solid laser at 488 nm with 50% intensity ratio and M pin hole. The side of the slide with Au film was immersed in a flow cell with a 0.13 mm-thick bottom, and the volume of the flow cell is 0.3 mL. The blank side was attached onto the prism using a refractive index matching liquid. The prism, substrate, and flow cell were fixed on the stage of the inverted microscope of the laser scanning confocal microscope (Nikon C1 Si, Japan) system. The focal plane was positioned on the gold film to detect the fluorescence image when pathogenic bacteria were injected into the flow cell. The LED (MCWHL2-2C, USA) was collimated and polarized by transverse-magnetic polarization using a Glan Taylor prism with the 5.0×10^{-5} extinction ratio. The samples were then injected into the flow cell by using a spring pump (Harvard 33 Twin Syringe Pump, USA). LSCI-SPR instrument integrated a wavelength-dependent SPR with a laser scanning confocal microscopy can successfully monitor the process of species interaction in real-time and in situ by combining the advantages of the two techniques (i.e., identifying the interaction process of the small molecule with gram-negative bacteria and imaging).

Atomic force microscopy (AFM) measurements were conducted using the DI Multimode SPM from Veeco Systems. Fourier transform infrared spectroscopy (FTIR) was recorded with Excalibur 3100. X-ray photoelectron spectroscopy (XPS) analysis of the substrate was conducted on a Thermo Scientific X-ray photoelectron spectrometer (ESCALAB 250Xi, UK) with Al Kα radiation.

2.3. Sensor-chip fabrication and characterization

Biosensor substrates were rinsed sequentially with ethanol for 10 min, 50% ethanol ultrapure water solution, and ultrapure water. Cleaned chips were stored in ultrapure water. Fig. 1 shows the LSCI-SPR senor-chip modification process and sensing principle. The conditions of concentration of adsorbate and immersion time were investigated as Table S1. Treated substrates were soaked in 20 mM 3-mercaptopropionicacid (3-MPA) solution for 12 h at 25 °C to form a self-assembled monolayer on the gold surface through Au-S bond because the sulfide bond of 3-MPA can adhere firmly onto gold [30–33]. The sensor chip was rinsed twice with ultrapure water to remove the nonspecific adsorption of 3-MPA. The coverage of 3-MPA on the gold film was estimated to be about 403 ng/cm^2 by the method used in our previous work [16,34,35]. Bare gold film showed only the peak of CO_2 in air (Fig.S1a). The FTIR spectra of 3-MPA covered gold film exhibited absorption peaks at 1697 cm^{-1} (Fig.S1b), which corresponded to the characteristic peak of carboxyl.

The carboxyl group on 3-MPA was activated by injecting a mixed solution containing NHS (5 mg/mL) and EDC (5 mg/mL) for 0.5 h. In this process, carboxyl was activated with EDC/NHS. The sensor chip was rinsed twice with ultrapure water, and then 520 mg of polymyxin B sulfate was added to 20 mL of ultrapure water and dissolved completely before soaking the sensor chip into it for 3–4 h. The activated carboxyl reacted with the amino-group of polymyxin B to form amide bond. The sensor chip was rinsed twice with ultrapure water and stored in ultrapure water at 4 °C before use. The coverage of polymyxin B on the gold film was estimated to be about 558 ng/cm^2 by the method used in our previous work [16,34,35]. The most characterized peaks of polymyxin B at 1680-1630 (amide I) and 1570-1510 (amide II) cm^{-1} [36]. The peaks at 1650.1 and 1535.4 cm^{-1} (Fig. S1c) proved that polymyxin B assembled on the gold film.

The XPS spectrum peak of the final sensor chip can be ascribed to Au, S, C, N, and O elements (Fig. S2c), which conformed to the elements of Au-3-MPA-polymiyxin B. The XPS peaks of bare gold film showed only Au, C, and O (Fig. S2a). Fig. S2b shows the peak at about 160 eV belonging to S_{2p} spectrum, which further demonstrated that 3-MPA was successfully modified onto gold film. Fig. S2c shows the peak of N better than Fig.S2b. These results clearly indicated that polymyxin B closely assembled the sensor chip.

2.4. Detection measurements

SPR signal data were collected with LabVIEW program, which also recorded the reflectance intensity (I) fixed on a point of the resonance peak with the maximum slope. Relative reflectance