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Direct, label-free, selective, and sensitive microbial detection using a bacteriorhodopsin-based photoelectric immunosensor

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ABSTRACT

A photoelectric immunosensor using purple membranes (PM) as the transducer, which contains photoactive bacteriorhodopsin, is here first demonstrated for direct and label-free microbial detection. Biotinylated polyclonal antibodies against *Escherichia coli* were immobilized on a PM-coated electrode through further surface biotinylation and bridging avidin or NeutrAvidin. The photocurrent generated by the antibody-coated sensor was reduced after incubation with *E. coli* K-12 cultures, with the reduction level increased with the culture populations. The immunosensor prepared via NeutrAvidin exhibited much better selectivity than the one prepared via avidin, recognizing almost none of the tested Gram-positive bacteria. Cultures with populations ranging from 1 to 10^7 CFU/10 mL were detected in a single step without any preprocessing. Both AFM and Raman analysis confirmed the layer-by-layer fabrication of the antibody-coated substrates as well as the binding of microorganisms. By investigating the effect of illumination orientation and simulating the photocurrent responses with an equivalent circuit model containing a chemical capacitance, we suggest that the photocurrent reduction was primarily caused by the light-shielding effect of the captured bacteria. Using the current fabrication technique, versatile bacteriorhodopsin-based photoelectric immunosensors can be readily prepared to detect a wide variety of biological cells.

1. Introduction

Biological transducers are natural biosensors produced by living organisms (Stieve, 1983). Compared with inorganic transducers, which are commonly employed in artificial biosensors, biological ones are more eco-friendly and more likely to be adopted in devices with fewer interfacial challenges that may otherwise arise due to the incompatibility between biological recognition elements and inorganic transducers. Of these natural photosensors, bacteriorhodopsin (BR) residing in the purple membrane (PM) of *Halobacterium salinarum* is the simplest and most studied. This retinal-containing protein uses solar energy (wavelength 500-650 nm, λ_{max} at 568 nm) to pump protons unidirectionally from the cytoplasmic (CP) to the extracellular (EC) side, accompanied by the production of multiple spectrally distinct intermediates as well as a photovoltaic force which can be readily converted into electrical impulses (Chen et al., 2016; Hampp, 2000; Wagner et al., 2013). BR is considered an idea transducer because its photoinduced properties vary in magnitude with environmental conditions. For example, BR-based photoelectric biosensors for anesthetics, ions, light intensity, pH, and radiation monitoring have been devised (Ahmadi and Yeow, 2011; Hong, 2006; Kim et al., 2013; Miyasaka and Koyama, 1993; Palazzo et al., 2014). Recently, another new photoelectric biosensor was proposed based on the energy transfer between BR and a receptor-functionalized fluorophore and on the premise that the binding of targeted chemicals or biological molecules altered or quenched the emission spectrum of the fluorophore (Friedrich et al., 2013; Knoblauch et al., 2014). Quantitative detection using this sophisticated method, however, has yet to be demonstrated.

Microbial detection is important for public and environmental health protection, as well as for biomedical, cosmetic, and food industries. Despite tremendous progress in biological science and technology in the past, methods for easy, low-cost, rapid, and sensitive detection of microorganisms remain a pressing demand for both developing and developed countries. Considered as alternatives to laborious and slow, classical culture-based methods, which are also poor for slow-growing or viable but nonculturable microorganisms, powerful modern molecular and other non-culture-based techniques have developed rapidly over the last two decades (Braga et al., 2013; Rastogi and Sani, 2011; Singh et al., 2009; Syed, 2014; Zourob et al., 2008). Some of the molecular techniques based on antibodies, bacteriophage, or nucleic acids have achieved microbial detection limits at or below single-digit colony forming units (CFU)/mL (Obeidat et al.,

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2012; Ripp et al., 2008; Zourob et al., 2008), whereas expensive labels or complicated procedures are often involved, preventing their widespread use. With advanced nanotechnology, new-generation electrochemical biosensors with similar high sensitivities have recently been devised (Barreiros dos Santos et al., 2013; Hernández et al., 2014; Zelada-Guillén et al., 2009), opening the door to direct, fast, label-free, and simple microbial detections.

Considering that BR photoelectric activity correlates positively with incident light intensity (Miyasaka and Koyama, 1993) and that most bacteria scatter light at around 600 nm, a novel BR-based photoelectric immunosensor is first devised in this study to directly and quantitatively detect microorganisms in a single step. Using a previously developed durable PM-coated photoelectric chip (Chen et al., 2014) as the transducer, the immunosensor chip was prepared by first surface-biotinylating the solvent-accessible residues of the PM patches deposited on the electrode and then anchoring the biotinylated antibodies against *Escherichia coli* through bridging avidin or NeutrAvidin, a deglycosylated form of avidin. By this construct, *E. coli* cells were able to be immunocaptured on the electrode in very close proximity to PM, thus forming an effective shield to block part of the incident light and resulting in a reduction of BR photocurrents. Because the transient (differential) photocurrent of the PM-coated chip was generated solely from illuminated BR, the photocurrent reduction can be totally attributed to the binding of the captured cells. Compared with common electrochemical and optical detection methods, this BR-based immunosensor had signals with the least interference from either background molecules or measuring environments. Moreover, due to the popular use of the (strept)avidin-biotin complex, this new biosensor technology can be easily adapted to sensing a great variety of microorganisms, providing a universal platform for microbial detection in different applications.

2. Materials and methods

2.1. Materials

E. coli K-12, *Bacillus subtilis* subsp. *spizizenii, Lactobacillus acidophilus, Pseudomonas aeruginosa, and Staphylococcus aureus* were all obtained from the Bioresource Collection and Research Center in Taiwan. The preparations of PM and papain-digested PM as well as the sources of other materials are described in the Supplementary materials.

2.2. Chip preparation and cell detection

Following the procedure previously reported (Chen et al., 2014), PM was first modified with EZ-Link sulfo-NHS-LC-LC-Biotin to yield b-PM, a biotinylated form of PM, and then affinity-immobilized onto ITO glass that had been aminated with 3-aminopropylphosphonic acid (APPA), using oxidized avidin as the linker. For antibody immobilization, the b-PM chip was first modified with 0.24 mM EZ-Link Iodoacetyl-PEG2-Biotin at pH 7.2 and room temperature, and then coated with 2 mg/mL avidin and 50 μg/mL biotin-conjugated anti-*E. coli* antibodies at 4 °C in sequence. To improve chip selectivity, 0.1 mg/mL NeutrAvidin was used instead to capture the biotinylated antibodies. After a brief rinse, the antibody-coated b-PM chip was incubated with 10 mL of either bacterial samples or the blank cell-binding buffer (10 mM phosphate buffer, pH 7.4) with shaking at room temperature, washed with the same buffer, and then subjected to photocurrent measurements. The b-PM coated chip was mounted in a cuvette filled with the electrolyte (10 mM KCl, pH 8.5) and front-illuminated with an 80-mW green CW laser (beam diameter: 3 mm). A platinum bar was used as the counter electrode and the generated photocurrent was real-time measured with a current amplifier (Keithley, Model-428) and recorded with an oscilloscope. All measurements were taken with the same instrument parameter settings. The total photocurrent density of the chip was defined as the difference between the maximum light-on ($I_{peak-on}$) and the minimum light-off ($I_{peak-off}$) values of its photocurrent density response to an on-and-off irradiation cycle comprising a 2–3 min continuous illumination and then a 2–3 min light interruption. The total photocurrent density of an antibody-coated b-PM chip after being incubated with a bacterial sample was compared with the value of another control chip incubated with only the blank cell-binding buffer and the reduced percentage was defined as the photocurrent reduction level. The photocurrent reduction levels of pure stationary-phase cultures ($1–10^7$ CFU/10 mL) whose concentrations had been determined by the traditional plate count method were used to prepare the calibration curve.

Atomic force microscopy (AFM) was carried out in air and at room temperature, using a Bruker Dimension Icon Scanning Probe Microscope operated in PeakForce mode and equipped with a Bruker ScanAsyst-Air probe. Raman spectra were obtained using a UniNano Tech UniG2D Raman Spectroscope with a 50 mW 532 nm CW laser as the light source. The quartz crystal microbalance (QCM) experiment is detailed in the Supplementary materials.

3. Results and discussion

3.1. Surface fabrication

We recently reported a robust and unidirectional PM fabrication method illustrated in the upper part of Fig. 1, where b-PM, a biotinylated PM whose EC side had been modified with a NHS-ester of biotin, was affinity-immobilized on amine-functionalized ITO glass using oxidized avidin as the linker (Chen et al., 2014). In this PM orientation, a much higher photocurrent could be obtained than in the other direction (Wang et al., 1997). To maximize the light-shielding effect of captured microorganisms, a close spatial arrangement between the captured cells and the immobilized BR molecules was achieved by first conjugating cell-specific antibodies on top of this b-PM chip, again through (strept)avidin-biotin interactions and then immunocapturing the cells. To achieve this, as shown in the lower part of Fig. 1, we used an iodoacetyl derivative of biotin, EZ-Link Iodoacetyl-PEG2-Biotin, to modify the exposed CP side of the immobilized b-PM patches so that avidin can be subsequently attached to adsorb biotin-conjugated antibodies. PM conjugatability with both iodoacetyl and NHS-ester derivatives of biotin was first investigated in solution and confirmed by labeling the resulting biotinylated PMs with fluorescent streptavidin conjugates. The large red clumps appearing in Fig. S1a,b evidence successful biotinylation for both cases. Although iodoacetyl derivatives are generally considered to be a specific modifier for cysteines, they can also react with histidines, methionines, and amines in the order of decreasing reactivity, especially when sulfhydryl groups are absent (Gurd, 1967). Because native BR contains neither cysteine nor histidine residues (Khorana et al., 1979) and there are 3 methionines located on the PM surface, Met32 and Met163 on the CP side as well as Met68 on the EC side, PM biotinylation by the iodoacetyl reagent was possible. Moreover, this biotin reagent also labeled another papain-digested PM (Fig. S1d) which lacked Met68 due to the prior cleavage of the Leu66-Gly72 loop between helices B and C by papain (Ovchinnikov et al., 1979), implying the biotinylation on the CP side. Therefore, it is possible that biotin can be conjugated on the exposed CP side of b-PM patches through this iodoacetyl reagent if the patches had been immobilized with their EC side facing the solid support.

Successively layering b-PM on supports through (strept)avidin-biotin interactions was subsequently investigated using a QCM sensor to demonstrate the surface biotinylation of immobilized b-PM patches as well as to simulate the further attachment of biotinylated molecules. First, an alternate layer-by-layer fabrication process comprising, in sequence, surface biotinylation of a b-PM coted QCM crystal by the iodoacetyl biotin reagent, streptavidin adsorption, and b-PM deposition was repeated several times with periodic measurements of the