Biosensors and Bioelectronics 79 (2016) 121–129

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Specific and selective electrochemical immunoassay for *Pseudomonas aeruginosa* based on pectin–gold nano composite

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ARTICLE INFO

Article history:
Received 3 October 2015
Received in revised form
24 November 2015
Accepted 4 December 2015
Available online 9 December 2015

Keywords:
Pseudomonas aeruginosa
Hydroquinone
Hydrogen peroxide
Phosphate buffer
IgG-HRP
CCLP-Au NPs

ABSTRACT

In this report, we have successfully fabricated an immunosensor for detection of *Pseudomonas aeruginosa* in water. The monoclonal antibody was immobilized on the surface modified with CCLP (Calcium Cross-Linked Pectin)–Au NPs (gold nanoparticles)/Glassy Carbon Electrode. The building of the immunosensor was evaluated in each step by cyclic voltammetry (CV) and impedance spectroscopy (EIS). The electrochemical detection was done based on the anti rabbit IgG HRP (Horseradish Peroxidase) which binds to the immune complex and the response was observed using Hydro Quininone (HQ) and Hydrogen peroxide (H_2O_2) in PB (Phosphate Buffer) electrolyte. From the results, the sensitivity range is from 10^1 to 10^7 CFU/ml and LOD is calculated as 9×10^2 CFU/ml. The developed immunosensor also have high selectivity, stability, reproducibility and reusability.

1. Introduction

Pseudomonas aeruginosa is a bacterium with ubiquitous occurrence in the environment; it is readily recovered from the faeces of animals, feeds, water and foodstuffs. The bacterium is involved in the aetiology of mastitis of humans and animals. *P. aeruginosa* strains causing septicemia have been isolated from patients with cancer, HIV infections or malignant aematological lesions and from patients treated in intensive gynecological or surgical units of hospitals. Oligotrophic bacteria such as pseudomonas are capable of reproducing and forming biofilms under conditions that are usually considered as nutrient restricted. Such organisms are found in low nutrient environments such as drinking water, groundwater, and surface water (Emde and Finch, 1991) attaining CFU densities of 10^6–10^7 cells per ml in distilled water (McFeters et al., 1993). Due to its ability to survive on a wide range of nutritional sources and its tolerance to various physical conditions; it has been recognized as an emerging opportunistic human pathogen and found on moist surfaces; medical equipment, like breathing machines, catheters, etc., are one of the leading causes of cross infections, especially in patients who have been hospitalized for more than a week (Fazeli et al., 2012; Seki et al., 2013; Tam et al., 2010). It is a Gram-negative, rod-shaped, strictly aerobic, oxidase-positive, catalase-positive bacterium that produces diffusive and/or insoluble pigments (Palleroni, 1992). This bacterium does not require organic growth factors. It has traditionally served as a surrogate or indicator for the presence of other opportunistic pathogens. Now, *P. aeruginosa* is considered to be an opportunistic pathogen in patients with low general or local resistance against infections (Morais et al., 1997) and its presence is unacceptable because it has been implicated in waterborne and food borne diseases; it is now considered to be a primary infectious agent (Choi et al., 2013). Current detection techniques involve time-consuming plate counting culture methods. Polymerase chain reaction (PCR) has also emerged as a rapid and accurate diagnostic technique (Motoshima et al., 2007). Consequently, bacterial identification has been performed with the use of many techniques, viz., colorimetry, fluorimetry, electrochemistry, flow optometry and mass spectrometry (mainly MALDI-MS) (Qin et al., 2003; Jothikumar et al., 2014; Metters et al., 2014; Sismaet et al., 2014; Davis, 2014; Braga et al., 2013). In a recent study, a highly specific and sensitive Genome Exponential Amplification Reaction (GEAR) assay for the detection of *E. coli* has been designed (Qin et al., 2003). Although the detection of bacteria has been widely explored with the use of various sensing materials and sophisticated instrumentation techniques, it is still a challenge to create a sensitive, speedy, and inexpensive method. Therefore, a cost-effective alternative diagnostic technique for rapid pathogen identification is the need of the hour to prevent the occurrence of extensive pathogenic outbreaks.

Recently, electrochemical biosensors have been demonstrated for the bacterial detection (Tawil et al., 2014; Ronkainen, 2010). Electrochemical sensing strategies have also provided a method to measure the chemical marker2-aminoacetophenone, which is

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http://dx.doi.org/10.1016/j.bios.2015.12.006
0956-5663/

indicative of *P. aeruginosa* infection, and its implementation in breath-sensing devices is proposed (Jothikumar et al., 2014). Similarly, electrochemical identification of *P. aeruginosa* via the addition of amino acids to up regulate pyocyanin production has also been reported (Metters et al., 2014). Such detection schemes primarily utilize the electrodes modified with biological recognition elements such as enzymes to generate the electrochemical signal when attached elements react with the specific target analytes/bacteria. The assembly of receptor coupled-lipid mono/bi layer on electrode (Ahmed et al., 2014; Thet et al., 2011) and redox liposome (Wilkop et al., 2007; Jenkins and Olds, 2004; Kim et al., 2006) triggered response by the bacteria/toxins were also studied. The principles of electrochemical immunosensors are well known (Skladal, 1997) and their potential for detection of pathogens was realized as well (Lin et al., 2013; Yang et al., 2014; Matric et al., 2012). Horseradish Peroxidase is an electrochemically active label with good solubility and stability; it enhances the catalytic activity of the substrates but also can reduce the nonspecific adsorption between electrode surface and label (Wang et al., 2014).

Pectin (poly galacturonic acid) is a good candidate for forming a supra molecular polymer composite because it has two hydroxyl groups and one carboxylic group which lead to multiple hydreogen bonds that form a flexible and robust film. Remarkably, pectin contains –OH and –COOH functional groups which can be used to support the nanoparticles (Zakharova et al., 2012; Jonassen et al., 2013; Thakur et al., 1997; Shi and Gunasekaran, 2008). Recently, nanomaterials and nanoparticles come into the focus of scientists as advantageous tools for preparation of electrochemical biosensing layers with potentially enhanced performance. Gold nanoparticles (Au NPs) have played powerful role in nanoscience and nanotechnology due to its high stability, biocompatibility, excellent electron conductivity and unique surface chemistry. Specific size and morphology of the Au NPs have been the focus of intensive research because of its potential applications in the field of electronic, optical, optoelectronic and magnetic devices (Shan and Tenhu, 2007; Zhang et al., 2006). Au NPs binding towards amino group and enhances as an electroactive label by accelerating the electron transfer. However, thus pectin acts as the stabilizing agent in Au NPs and very few reports for chemical synthesis of other nanoparticles (Khazaei et al., 2013; Ngenefeme et al., 2013). Au NPs binding towards amino group and enhances as an electroactive label by accelerating the electron transfer. Thus, it amplifies electrochemical signal and retains the biological activity of antibody via improving the analytical performance of immunoassay (Kuang et al., 2010; Kavosi et al., 2014; Ambrosi et al., 2010; Lv et al., 2014; Pondman et al., 2013).

In this work, we developed an immunosensor platform containing CCLP-AuNPs with immobilized antibody over the glassy carbon electrode. The electrochemical signaling is solely dependent on the presence of HRP tagged in secondary antibody which catalyses the HQ and Hydrogen peroxide for the redox reaction. In addition, the cyclic voltammetry and impedance spectroscopy were also used to record and confirm the development of the immunosensor for the rapid, stable, specific, sensitive and reusable system for the detection of the pathogenic *P. aeruginosa.* This is the first detailed report about the development highly selective and sensitive immunoassay for *P. aeruginosa.*

2. Experimental

2.1. Chemicals

Auric chloride, pectin from citrus peel, hydroquinone, disodium hydrogen phosphate, monosodium dihydrogen phosphate and bovine serum albumin (BSA) were purchased from Sigma Aldrich, India. Concentrated HCl, H_2O_2 were of analytical grade purchased from Merck, India. Pottassium chloride, potassium hexacyanoferrate and potassium ferricyanide were of laboratory reagent purchased from Himedia, India. Calcium chloride (fused) was purchased from Lobachemic India. IgG-HRP was purchased from Genie, India. All the reagents used for the experiments were prepared in doubly distilled water.

2.2. Apparatus

Cyclic voltammetry (CV) and Linear Sweeping Voltammetry (LSV) was carried out with a Biologic Scientific Instrument (SP-150), electrochemical workstation. Electro Impedance Spectroscopy (EIS) was carried out using CHI Instrument (6206e), electrochemical analyzer. Conventional three electrode systems were used at room temperature with Glassy Carbon Electrode (GCE) 3 mm diameter as a working electrode, Ag/AgCl (saturated KCl) is used as reference electrode and platinum wire as counter electrode for the present work. Prior to use, the Glassy Carbon Electrode (GCE) was polished by 0.3 μm and 0.05 μm alumina slurry followed by rinsing with water, ethanol and water, in turn. The 0.1 M phosphate buffer solution (pH 7.0) prepared from disodium hydrogen phosphate and monosodium dihydrogen phosphate was used to prepare electrolyte for CV and LSV. Surface morphological study of the film was studied by using FESEM. Indium tin oxide (ITO) thin film coated glass electrodes was used for surface morphological studies. The UV-Absorbtion studies were performed using Agilent Technologies (82357B). Measurements of the pH were made with a EUTECH Instrument's pH meter.

2.3. Preparation of electrochemical immunosensor assay

First the CCLP-Au NPs modified electrode was prepared as per the protocol followed in our earlier report (Rajkumar et al., 2014). The film was prepared from the solution containing 6 mg/ml of $CaCl_2$, 3 mg/ml pectin and 0.1 mM $HAuCl_4$ by cyclic voltammetry method. Before deposition, the above solution was well sonicated about 30 min to get a homogeneous solution. The CCLP-Au NPs film was electrochemically deposited in the potential range 1.4 V and −1.2 V for 10 cycles at 50 mV/s (Fig. S1). Then, the CCLP-Au NPs modified electrode was dried. Then the anti Ps was drop casted on CCLP-Au NPs modified electrode and kept for 15 min for drying.

Then, as prepared GCE/CCLP-Au NPs/anti Ps was treated with 0.1% BSA for 10 min to block the redundant sites on the surface of CCLP-Au NPs. Then, the modified film GCE/CCLP-Au NPs/anti Ps was dipped and incubated with the antigen (Ps) for 15 min and rinsed with 0.1 M PB (pH 7.0). Finally, 10 μl of Au tagged IgG-HRP was drop casted onto the GCE/CCLP-Au NPs/anti Ps/Ps and incubated for 30 min and electrode was washed with 0.1 M PB (pH 7.0) for three times. The sandwich structure of GCE/CCLP-Au NPs/anti Ps/Ps/Au-IgG-HRP was formed. The voltammetric measurements of the above film were analyzed in PB containing 2.5 mM HQ and 1 mM H_2O_2.

2.4. Sensor regeneration and reproducibility

After using the immunosensor for analysis which can be reused by removing the antigen capture step, 100 mM glycine–HCl buffer pH 2.5 was flowed for 10 min (until a steady baseline (CCLP-Au NPs /anti Ps/GCE) was reached). The stability of the formed immunocomplex exists for 12 h and reproducibility ($n=6$) was analyzed by storing the immunosensor at 4 °C.