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Development of an aptasensor using reduced graphene oxide chitosan complex to detect *Salmonella*

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ABSTRACT

The development of accurate and rapid biosensors to detect pathogenic *Salmonella enterica* is an active area of interest with significant impact towards public health. An electrochemical aptasensor was developed using electrochemically-reduced graphene oxide-chitosan (rGO-CHI) composite as a conductive substrate to detect whole-cell *Salmonella enterica* serovar *Typhimurium*, a common serovar that causes foodborne infections in humans. A thiol-functionalized aptamer specific to *Salmonella* outer membrane protein was selected as the bio-recognition element and was immobilized on rGO-CHI using glutaraldehyde as the crosslinker. The sensitivity and selectivity of this aptasensor against *S. Typhimurium* was investigated using cyclic voltammetry and differential pulse voltammetry techniques. The rGO-CHI composite formed a conductive coating (4.5 A m^{-2}) which was stable to accommodate the buildup of activating agents without degrading. The developed aptasensor is specific to *Salmonella* and could distinguish between *Salmonella enterica* cells and non-*Salmonella* bacteria (*S. aureus, K. pneumonia* and *E. coli*). The aptasensor exhibited a low limit of detection of 10^1 CFU mL^{-1} for *S. Typhimurium*. The system was tested with artificially spiked raw chicken samples and the results were consistent with the sensitivity results obtained using with pure cultures. This shows the potential of the developed aptasensor in direct *Salmonella* detection in contaminated food.

1. Introduction

Salmonellosis is a foodborne disease caused by *Salmonella enterica,* an ubiquitous pathogen that is commonly found in poultry, eggs and vegetables [1]. *Salmonella* has over 2500 serovars, among these, *Salmonella Enteritidis* and *Salmonella Typhimurium* are the most common non-typhoidal serovars associated with human illnesses [2]. These *Salmonella* serovars are responsible for gastrointestinal diseases and can cause severe illness in immunocompromised people such as the elderly, children or generally people with low immune function [3]. In the United States, the annual cases of salmonellosis were about 140 million people, including 55 million children under the age of 5 years [4]. In Malaysia, an increase in food- and water borne *Salmonella* cases reported in the past decade (48.51 cases per 100,000 people) [5,6]. These reports showed that strains of *S. Typhimurium* are ubiquitous and multidrug resistant [5]. Hence, there is a need for the development of a point-of-care biosensor device for rapid and accurate detection of *Salmonella.* Many molecular methods have been developed for *Salmonella* detection, for instance PCR, multiplex PCR, real-time PCR, and NASBA [7,8]. These methods are commonly used for detection but pose several setbacks which could be improved to make detection easier. Generally, these methods require skills to handle nucleic acid fragments, purification steps, and have a complex working protocol [9,10]. Thus, biosensors could offer an alternative, a simpler and more affordable method for pathogen detection [11]. Electrochemical biosensors detect an analyte based on the change in response as a result of electrochemical interaction between the analyte and the electrode surface [12]. An optimal biosensor is one which has a high signal-to-noise ratio,

Abbreviation: rGO, reduced graphene oxide; CHI, chitosan; GLU, glutaraldehyde; MB, methylene blue; *S. Typhimurium*, *Salmonella Typhimurium*; ssDNA, single stranded deoxyribonucleic acid; CV, cyclic voltammetry; DPV, differential pulse voltammetry; CFU mL^{-1}, colony forming unit per mL

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a low detection limit of analyte and has the ability to measure a wide range of analyte concentrations [13].

Carbon sources such as graphene and glassy carbon are of great interest to researchers and have been used in the fabrication of sensing devices. The characteristic chemical inertness, high current density, and super hydrophobicity at the nanometer scale of reduced graphene oxide (rGO) make it a conductive platform in the development of biosensor [14]. rGO exhibits a wide electrochemical potential and minimal charge transfer resistance compared to graphite and glassy carbon [15]. The combination of rGO with chitosan (CHI) further enhances the properties of rGO by producing a suitable microenvironment for biosensors to provide effortless surface modification [11,16]. Studies conducted by Justin and He [17,18] showed that the pairing of these two substances increases mechanical and tensile properties and produces a strengthened canvas for layering on active ingredients.

The specificity of the biosensor in detecting the desired target exclusively is assured by the high affinity of the biosensing element to bind specifically with the target of interest. Aptamers, enzymes and antibodies are commonly used as recognition biomolecules in biosensors. Aptamers are unique because they are structurally defined as single-stranded nucleic acids but unlike traditional nucleic acids, their expediency is their ability to bind to a wide range of analytes like peptides, proteins, viruses and whole cells with high affinity and specificity [19,20]. Joshi et al. [21] documented a nucleic acid sequence complementary to the outer membrane protein of *S. Typhimurium* as a potential strain-specific detection target. The specificity of an aptamer binding to the target is determined *in vitro* by the SELEX process where a large library of nucleic acid sequences is incubated with the required analyte and after several filtering processes, desired nucleic acid sequences is determined and amplified [21].

Biosensors with aptamers as the recognition biomolecule are known as aptasensors [22]. Several aptasensors have been developed by researchers with different sensing platforms. Ma et al. [13] developed an electrochemical aptasensor using glassy carbon electrode modified with graphene oxide (GO) and gold nanoparticles to detect *Salmonella* with a low detection limit of 3 CFU mL^{-1}. Hernandez et al. also reported a potentiometric aptasensor using rGO and GO as a platform which had a detection limit of 1 CFU mL^{-1} for *Staphylococcus aureus* [23]. Zelada-Guillén et al. proposed another potentiometric aptasensor with carbon nanotubes platform and reported a low detection limit of 8×10^2 CFU mL^{-1} for *Staphylococcus aureus* [24]. Sheikhzadeh et al. also documented an electrochemical aptasensor using poly [pyrrole-*co*-3-carboxyl-pyrrole] copolymer which gave a broad detection range of 1×10^2 to 1×10^8 CFU mL^{-1} for *S. Typhimurium* [25].

In this study, we proposed an innovative strategy by using a thiol-aptamer-immobilized rGO-CHI composite as a conductive platform for the detection of foodborne pathogens. The rGO-CHI aptasensor was prepared using rGO and CHI composite decorated onto glassy carbon electrode *via* electrodeposition. Thiol-aptamer was used in this study to ensure glutaraldehyde (GLU) formed binding with one aldehyde end of GLU and other aldehyde end formed binding with amino group from CHI (Fig. 1). This avoids competitive binding by forming heterobifunctional crosslinkages to ensure successive layering of active agents [26]. Sensitivity and selectivity studies were carried out to evaluate the ability of the prepared aptasensor to quantify trace amount of *Salmonella* in pure cultures and in food samples.

2. Materials and method

2.1. Materials and reagents

Graphite powder, Sulfuric acid (H_2SO_4) 98%, Phosphoric acid (H_2PO_4) 85%, Hydrochloric acid (HCl) 37%, Potassium Permanganate ($KMnO_4$), Hydrogen Peroxide (H_2O_2), Chitosan (CHI) moderate molecular weight, Glacial Acetic acid, Sodium Nitrate, Potassium Ferricyanide, Potassium Ferrocyanide, Phosphate Buffer Saline (PBS) 10 X, Potassium Chloride (KCl), Sodium Hydroxide (NaOH), Methylene Blue (MB), Glutaraldehyde (GLU) 25% were purchased from Sigma Aldrich. Serovars of *Salmonella enterica* used were *Salmonella Enteritidis*, *Salmonella Weltevreden*, *Salmonella Typhi* and *Salmonella Typhimurium* (model organism) while non-*Salmonella* bacterial species used included *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae.* The DNA sequence of the thiolated aptamer (recognition biomolecule) is: 5′(C12)-SH- TAT GGC GGC GTC ACC CGA CGG GGA CTT GAC ATT ATG ACA G-3′ [27]. Optimization with different premodified aptamers was done using amine- and blank aptamer: 5′(C12)-NH_2- TAT GGC GGC GTC ACC CGA CGG GGA CTT GAC ATT ATG ACA G-3′ and 5′(C12) TAT GGC GGC GTC ACC CGA CGG GGA CTT GAC ATT ATG ACA G-3′.

2.2. Apparatus

Morphological studies of rGO-CHI composite were done using Scanning electron microscopy (SEM) (Quanta FEG 650, FEI, USA) and Transmission electron microscopy (TEM) (LEO LIBRA 120, Carl Zeiss, Oberkochen, Germany). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were carried out using PGSTAT302N electrochemical workstation (Metrohm AG, Switzerland). Ultrasonic bath sonicator was used to exfoliate GO. All bacterial culture work was performed in the Biosafety cabinet (ESCO Streamline SC2-6A1). For the production of GO slurry, industrial centrifuge (HITACHI CR22N, Japan) and homogenizer (IKA RW20 digital Homogenizer) were used and the setup was installed in a fumehood (LABCONCO Protector Laboratory Hood). Fourier transform infrared (FTIR), RAMAN and X-ray diffraction (XRD) studies were done using FTIR Spectrometer (Bruker IFS 66V/S, USA), RAMAN Spectrometer (Renishaw inVia Raman microscope, UK) and X-ray diffractometer (Bruker d8 ADVANCE), respectively.

2.3. Preparation of graphene oxide

Graphite powder was oxidized into GO based on the modified Hummers method [11,28]. Graphite powder (5 g) was dissolved in 150 mL of acid mixture 115.5 mL 98% sulfuric acid (H_2SO_4) and 35.5 mL 85% phosphoric acid (H_3PO_4) and then placed in an ice bath with constant stirring for 1 h. Potassium permanganate ($KMnO_4$) (30 g) was added in batches to the acid mixture until the solution turned dark green. The mixture was then stirred vigorously for 1 h. The speed of stirring was reduced and the mixture was left to oxidize for three days until a dark brown paste was observed. The paste was then diluted with 250 mL of distilled water and 10 mL hydrogen peroxide (H_2O_2) and left for 10 min as the color of the solution changed from dark brown to yellow. The mixture was allowed to settle until the acid layer and GO layer were separated. The upper acid layer was then carefully decanted without washing off the GO layer. Hydrochloric acid (HCl) (1 M) was added into the GO paste and homogenized. The diluted GO solution was centrifuged at 12000 RPM for 30 min and supernatant was discarded and this washing was repeated three times. Further washing was carried out six times with distilled water. The GO slurry was then dried in the oven to produce dry flakes of GO. Flakes of GO were pulverized using mortar and pestle to obtain fine GO powder.

2.4. Preparation of reduced graphene oxide-chitosan (rGO-CHI) platform via electrodeposition

Prior to electrodeposition, GCE was cleaned by polishing with 0.3 μm and 0.05 μm alumina powder followed by ultrasonication in ethanol and distilled water [29]. The GO and CHI solution was made by mixing 1 mg of GO powder in 1 mL of 0.2% (*w/v*) chitosan solution with 30 min ultrasonication. The cleaned GCE was then immersed into 10 mL GO-CHI solution (1 mg/mL) with continuous slow stirring and chronoamperometry was done to electrodeposit GO-CHI. Electrodeposition was carried out at fixed potential of − 1.0 V for 400 s (Fig. 1, step 1) [29].