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Alkaline phosphatase labeled SERS active sandwich immunoassay for detection of *Escherichia coli*

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ABSTRACT

In this study, a sandwich immunoassay method utilizing enzymatic activity of alkaline phosphatase (ALP) on 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for *Escherichia coli* (*E. coli*) detection was developed using surface enhanced Raman spectroscopy (SERS). For this purpose, spherical magnetic gold coated core-shell nanoparticles (MNPs-Au) and rod shape gold nanoparticles (Au-NRs) were synthesized and modified for immunomagnetic separation (IMS) of *E. coli* from the solution. In order to specify the developed method to ALP activity, Au-NRs were labeled with this enzyme. After successful construction of the immunoassay, BCIP substrate was added to produce the SERS-active product; 5-bromo-4-chloro-3-indole (BCI). A good linearity ($R^2 = 0.992$) was established between the specific SERS intensity of BCI at 600 cm^{-1} and logarithmic *E. coli* concentration in the range of 1.7×10^1–1.7×10^6 cfu mL^{-1}. LOD and LOQ values were also calculated and found to be 10 cfu mL^{-1} and 30 cfu mL^{-1}, respectively.

1. Introduction

Detection of pathogenic bacteria is one of the most crucial issue for food safety and human health [1]. Over the years, *Escherichia coli* (*E. coli*) has considered major pathogenic bacteria and may results death-causing diseases [2]. Therefore, studies on developing reliable, sensitive and rapid methods for detection of *E. coli* are on the increase [3,4]. Several techniques are being developed for specific detection of foodborne pathogens [5]. Among them, automated or modified conventional assays [6], biosensors [7], immunological methods [8], nucleic acid based assays [9] can be considered as rapid methods. Nanoparticles with their large surface area allow a great number of biomolecules to be immobilized and consequently scales up the number of reaction sites available for interaction with the target species [10,11]. In addition, excellent electronic and optical properties of nanoparticles improve the sensitivity and response time of biosensors [12,13]. Nanoparticles with magnetic properties allow to improve the immunomagnetic separation (IMS) method which offers a rapid alternative to selective enrichment of the pathogens [14,15]. Due to these advantages, nanoparticles are frequently used in the development of biosensors combined with immunoassays [16–18].

Surface enhanced Raman spectroscopy (SERS) is a very useful technique that enables the development of rapid and sensitive methods of *E. coli* detection [19–23]. Additionally, SERS could be used for molecular identification of analytes, namely DNA, enzymes, proteins, biomarkers and microorganisms [24–28]. Usage of SERS coupled with enzymes in the development of pathogen detection assays is reported in the literature since SERS enables monitoring of the enzymatic reactions by following the signal specific to the SERS active product [29–32] Immobilization of enzymes to the surface of nanoparticles provides an increment in the sensitivity of the system [33–35].

Alkaline phosphatase (ALP) is one of the most commonly used biomarkers in enzyme immunoassays and affinity sensing methods for monitoring proteins, nucleic acids, enzymes and bacteria [36–41]. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) is the substrate of ALP, which has no specific SERS signal while after the enzymatic activity of ALP, BCIP is hydrolyzed to 5-bromo-4-chloro-3-indole (BCI) and inorganic phosphate [36]. The product of ALP enzyme, BCI, is used as a label in SERS based enzyme activity detection methods and immunoassays [42,43].

In the present study, ALP enzyme immobilized nanoparticles were used to develop a SERS-based biosensor for *E. coli* detection. For this purpose, spherical gold coated magnetic nanoparticles (MNPs-Au) were synthesized and modified with *E. coli* antibody for the further IMS process. In the meantime, gold nanorods (Au-NRs) were labeled with ALP enzyme and also modified with *E. coli* antibody. *E. coli* were

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captured and separated from the medium through the application of an external magnetic field on antibody modified MNPs-Au. Afterwards, ALP labeled and antibody modified Au-NRs were added to the specifically captured bacteria in order to form the sandwich immunoassay. Finally, the substrate of ALP enzyme, BCIP, is added. Subsequent to enzymatic hydrolysis of the substrate, nanoparticle enhanced SERS signal specific to BCI is monitored with the increasing *E. coli* concentration that was captured with the developed sandwich immunoassay.

2. Experimental

2.1. Materials and Reagent

Absolute ethanol (99%) was acquired from Merck KgaA (Darmstadt, Germany). Pierce avidin was obtained from Thermo-Scientific (Rockford, IL). Sodium hydroxide (NaOH), Na_2HPO_4, and KH_2PO_4 were obtained from J.T. Baker (Deventer, The Netherlands). Biotinylated anti-*E. coli* polyclonal antibodies were obtained from Fitzgerald Inc. (North Acton, MA,). Albumin from bovine serum (BSA), potassium chloride (KCl), sodium chloride (NaCl), 5-bromo-4chloro-3 indolyl phosphate disodium salt, 11-mercaptoundecanoic acid (11-MUA), hexadecyltrimethylammonium bromide (CTAB), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), perchloric acid ($HClO_4$), gold (III) chloride solution ($HAuCl_4$), hydroxylamine hydrochloride, iron(III) chloride ($FeCl_3$), iron(II) sulfate heptahydrate (Fe_2SO_4), L-ascorbic acid (AA), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), Tris hydrochloride, alkaline phosphatase, *N*-hydroxy-sulfosuccinimide sodium salt (NHS), 2-morpholinoethane-sulfonic acid monohydrate (MES), sodium borohydride ($NaBH_4$), and Tween 20 were obtained from Sigma-Aldrich (Steinheim, Germany). Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) was purchased from Acros Organics (Geel, Belgium).

2.2. Solutions and Buffers

Phosphate Buffer Saline (PBS) buffers (67 mM and pH 7.4) were prepared with Na_2HPO_4, KH_2PO_4, NaCl and KCl. PBST was prepared by adding 0.05% Tween 20 (v/v) to PBS. 2-Morpholinoethanesulfonic acid buffer (MES; 0.05 M) was adjusted to pH 6.5. EDC and NHS solutions (0.2 M EDC and 0.05 M NHS), and avidin solution (0.5 mg mL^{-1}) were prepared by using 0.05 M MES buffer. In order to perform the enzymatic reactions, 0.05 M $MgCl_2$ was added to Tris-HCl buffer (0.1 M and pH 9.8).

2.3. Synthesis of Au-NRs and MNPs-Au

2.3.1. Synthesis of Au-NRs

Gold nanorods were prepared using a seed-mediated growth technique [23]. Seed solution was prepared by mixing 7.5 mL of 0.1 M CTAB and 250 μL of 0.01 M $HAuCl_4$ solutions. Once mixed, 600 mL of 0.01 M ice-cold $NaBH_4$ was added rapidly to the resulting solution, and allowed to stand for 5 min to form the seed solution. To prepare rod-shaped gold nanoparticles, 4.75 mL of 0.1 M CTAB, 1 mL of 0.01 M $HAuCl_4$, and 60 μL of 4×10^{-3} M $AgNO_3$ were mixed, and a dark orange color was obtained as the resulting color. When the dark orange color was observed, 250 μL of 0.1 M AA was added dropwise to the resulting solution to obtain the stock solution. The solution turned colorless after adding 250 μL of AA. Then, 5 mL of seed solution was added to the stock solution. The final mixture was stirred for a few seconds and left to stand for 3 h at room temperature to obtain nanorods.

2.3.2. Synthesis of Spherical MNPs-Au

Synthesis of MNPs-Au was performed in two steps. First, spherical iron nanoparticles were synthesized as mentioned in our previous work [44]. The Fe_3O_4 nanoparticles were prepared by coprecipitating Fe (II) and Fe (III). Fe (II)/Fe (III) ratio was kept as 0.5 in an alkaline solution. $FeCl_3$ of 1.28 M and $FeSO_4 \cdot 7H_2O$ of 0.64 M were dissolved in deionized water. This solution was then stirred vigorously until the iron salts were dissolved. Subsequently, a solution of 1 M NaOH was added dropwise into the mixture by stirring for 40 min. The precipitated magnetite, which was black in color, was collected on a permanent magnet and washed with deionized water. In order to obtain oxidized Fe_3O_4 nanoparticles, the resulting iron salts precipitate was first washed in 2 M $HClO_4$ and left for 3 h. The particles were then centrifuged for 20 min at 10000 rpm. Afterwards, the supernatant solution was discarded and the precipitate was washed three times with deionized water.

In the second step of the synthesis, Au shell coating procedure was carried out in the presence of CTAB in order to encapsulate iron nanoparticles with gold shells. The applied procedure is slightly different from our previous work [45]. Fe_3O_4 nanoparticles of 10 mg were suspended in 0.27 M EDTA solution that was prepared in 1 M NaOH. Afterwards, the solution was stirred in a sonicator for 5 min and then particles were centrifuged for 10 min at 10000 rpm. The resulting iron precipitate was mixed with 7 mL 0.1 M CTAB, 3 mL 0.01 M $HAuCl_4$ and 300 μL 1 M NaOH. Subsequent to vigorous stirring of this solution, 150 mg hydroxylamine hydrochloride was added and stirred for more 3 min. The obtained nanoparticle solution, which was dark red in color, was left for 24 h before use.

2.4. Microorganisms

Escherichia coli, *Salmonella enteritidis* and *Enterobacter aerogenes* were obtained from Refik Saydam National Type Culture Collections, Ankara, Turkey. The stock cultures were grown on LB Broth (Laboratorios Conda, Madrid, Spain) at 37 °C for 18 h. The cultures were serially diluted (10-fold steps) with PBS. Efficiency of the antibody's selectivity was calculated using plate count method on LB Agar (Laboratorios Conda, Madrid, Spain).

2.5. Instrumentation

Absorption spectra of both magnetic gold and rod-shaped gold nanoparticle solutions were recorded using an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA) with a photodiode array detector.

Transmission electron microscope (TEM) measurements were performed on a JEOL 2100 HRTEM instrument (JEOL Ltd., Tokyo, Japan). TEM samples were prepared by pipetting 10 mL of nanoparticle solution onto TEM grids and after leaving them to stand for 10 min, the solution is evaporated under vacuum.

DeltaNu Examiner Raman Microscopy system (Deltanu Inc., Laramie, WY) with a 785 nm laser source and a cooled charge-coupled device (CCD, at 0 °C) detector was used for *E. coli* detection. The optimized parameters were determined as 100 mW laser power, and 20 s acquisition time.

2.6. Design of Sandwich Immunoassay

Fig. 1 illustrates the main steps of the developed ALP labeled SERS active sandwich immunoassay.

MNPs-Au were treated with a solution of 0.05 M 11-MUA that was prepared in absolute ethanol and kept overnight at room temperature to form self-assembled monolayer (SAM). Afterwards, they were gathered through magnet assisting and washed three times with absolute ethanol and once with MES buffer. Through treating for 20 min with EDC-NHS solution (0.2 M EDC and 0.05 M NHS), free carboxyl groups present on the surface of SAM-modified MNPs-Au were activated and washing procedures were performed three times by using MES buffer. In order to bound biotin labeled antibody specifically to MNPs-Au, covalent attachment between activated carboxyl groups and avidin was utilized. Thereof, 0.5 mg mL^{-1} avidin solution prepared in MES buffer was mixed with MNPs-Au for 40 min and washed three times with MES buffer. Then MNPs-Au were treated for 1 h with 1 mg mL^{-1} BSA in