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Bifunctional linker-based immunosensing for rapid and visible detection of bacteria in real matrices

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ABSTRACT

Detection of pathogens present in food and water is essential to help ensure food safety. Among the popular methods for pathogen detection are those based on culture and colony-counting and polymerase chain reaction (PCR). However, the time-consuming nature and/or the need for sophisticated instrumentation of those methods limit their on-site applications. We have developed a rapid and highly sensitive immunosensing method for visible detection of bacteria in real matrices based on the aggregation of AuNPs without requiring any readout device. We use biotinylated anti-bacteria antibodies as bifunctional linkers (BLs) to mediate the aggregation of streptavidin-functionalized gold nanoparticles (st-AuNPs) to produce visually recognizable color change, due to surface plasmon resonance (SPR), which occurs in about 30 min of total assay time when the sample is mildly agitated or within three hours in quiescent conditions. The aggregation of st-AuNPs, which produces the indication signal, is achieved very differently than in visual detection methods reported previously and hence affords ultrahigh sensitivity. While BLs can both bind to the target and crosslink st-AuNPs, their latter function is essentially disabled when they bind to the target bacteria. By varying the amount of st-AuNPs used, we can tailor the assay effectiveness improving limit of detection (LOD) down to 10 CFU mL^{-1} of *E. coli* and *Salmonella*. Test results obtained with tap water, lake water and milk samples show that assay performance is unaffected by matrix effects. Further, in a mixture of live and autoclaved *E. coli* cells our assay could detect only live cells. Therefore, our BL-based immunosensor is suitable for highly sensitive, rapid, and on-site detection of bacteria in real matrices.

1. Introduction

On-site and real-time detection of deleterious substances such as pathogens, toxins and allergens present in food and water is essential to ensure the safety of human health (Committee, 2014; Ec, 2007). To address this issue, detection methods which are rapid and simple enough to be performed in out-of-laboratory settings, by even untrained persons, have long been needed (Rotariu et al., 2016; Vaisocherová-Lísalová et al., 2016). Thus, indication principles producing visually recognizable signal are considered the most suitable (Priyadarshini and Pradhan, 2017; Safavieh et al., 2014; Valderrama et al., 2015).

Though several visible-detection methods have been reported, poor limit of detection (LOD) has hampered their widespread use (Safavieh et al., 2014; Singh et al., 2017; Yoo and Lee, 2016). Biosensing process can be thought of as comprising two sequential steps: target recognition and signal production. Since acknowledged methods allowing specific recognition of biological targets are rather limited, emerging techniques to improve the LOD have focused on improving signal production, or more specifically *signal amplification* upon target recognition (Yoo and Lee, 2016).

Detection methods based on *target amplification* strategy such as polymerase chain reaction (PCR) for identification of genetic substances (e.g., DNA) are highly selective and sensitive (Singh et al., 2017; Yoo and Lee, 2016). However, unfortunately, such methods are not suitable

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for testing food and biological products. When detection methods based on the identification of genetic substances are applied to natural or pasteurized food or other biological products, dead cells or their fragments commonly contained in samples lead to faulty results (Simpson and Lim, 2005). Furthermore, target amplification methods for the detection of biochemical contaminants such as toxins, allergens, pesticides, which are of major concern to the food industry, are not yet available.

Accordingly, there have been efforts to develop biosensing techniques based on signal amplification strategies (Guo et al., 2013). Among these, visible indication principle employing colorimetric signal generation via facilitated agglomeration of nanoparticles (NPs) is the most popular, given its rapidity and simplicity (Priyadarshini and Pradhan, 2017). However, because considerable amount of target is necessary to elicit visually recognizable colorimetric signal for the indication, the LOD of such methods is not low enough for practical applications (Yoo and Lee, 2016). Therefore, limitations in sensitivity, rapidity, and on-site applicability exist in current methods. For example, among the lowest reported LOD is 10^2 CFU mL^{-1} and total assay time is more than 6 h (Raj et al., 2015; Prasad et al., 2011). We developed a novel concept of using bifunctional linkers (BLs), which maybe also known as switchable linkers, to enable signal amplification utilizing agglomeration of NPs (Lim et al., 2012; Hahn et al., 2017). A BL is a crosslinker bridging two NPs, thus it can crosslink many NPs leading to their large-scale aggregation. However, BLs should be designed in such a way its crosslink function is lost, or essentially switched off, in the presence of target. Hence, when the target is present, aggregation of NPs does not occur at any appreciable extent. By employing BLs, some desideratum for visible detection could be available without taking away the advantages of conventional visible detection based on the aggregation of NPs. Herein we evaluated the practical applicability of BLs-based signal amplification strategy for highly sensitive detection of bacterial targets in environmental water and food samples.

2. Materials and methods

2.1. Materials

Gold(III) chloride trihydrate ($HAuCl_4{\cdot}3H_2O$) and bovine serum albumin(BSA) were purchased from Sigma-Aldrich (St. Luis, MO, USA). Streptavidin (from *Streptomyces avidini*) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Biotin-labeled *Samonella* sp. group antibody and *Escherichia coli* (*E.coli*) O + K antibody were purchased from Genetex (Irvine, CA, USA). Phosphate buffered saline (PBS) was purchased from EMD Millipore (Darmstadt, Germany).

2.2. Sample preparation

Real matrices chosen for testing were tap water, lake water, and whole milk. Tap water was obtained directly from a faucet in our laboratory, and lake water samples were from lake Mendota (Madison, WI, USA), which is general use recreation water. Store-bought pasteurized whole milk served as food matrix. Before using these samples, we confirmed the absence of target organisms, *E.coli* and *Salmonella,* by testing with selective media such as *E.coli* and coliform selective medium from 3M film and *Salmonella* selective medium, XLD medium. These samples were spiked with known amounts of *E.coli* and *Salmonella* and used in our assay for determining the amount of target bacteria present.

2.3. Synthesis of gold nanoparticles and streptavidin functionalization

Aqueous gold nanoparticles (AuNPs), 13 nm in diameter, were synthesized according to a published procedure (Turkevich et al., 1951) and colloidal gold was synthesized by reducing $HAuCl_4{\cdot}3H_2O$ aqueous solution reacting with sodium citrate at ebullition as previously reported (FRENS, 1973). Deionized (DI) water was used in all experiments. In an Erlenmeyer flask equipped with a hot plate, 10 mL of 1 mM $HAuCl_4{\cdot}3H_2O$ was brought to a rolling boil. 1 mL of 38.8 mM citrate sodium was added to 10 mL boiling solution of 1 mM $HAuCl_4{\cdot}3H_2O$. The solution turned dark brown within 10 s; the final color change to burgundy occurred 50 s later, which was cooled down to room temperature. This solution of colloidal AuNPs was characterized by an absorbance of 0.43 and wavelength is 520 ± 0.5 nm. The size and distribution of AuNPs were characterized through transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively (Fig. S1A).

AuNPs were coated with streptavidin according to a published method (Grabar et al., 1995) with slight modifications. 600 μL of colloidal AuNPs (wavelength: 520 ± 0.5 nm abs.: 0.43) was added to 200 μL of streptavidin (0.2 mg mL^{-1} in borate buffer). The mixture was incubated at room temperature and centrifuged several rounds in Eppendorf 5415 C centrifuge (Eppendorf NA, Hauppauge, NY) at 10,000 rpm for 30 min to remove any excess streptavidin present and was restored in 1% (w v^{-1}) of BSA dissolved in PBS. Streptavidin can be capped on the anionic-charged AuNPs by strong electrostatic adsorption (Grabar et al., 1995; Presnova et al., 2014).

2.4. Bacteria culture and plate counting

E. coli (ATCC 11775T) cultures were grown 24 h in Lysogeny broth (LB) at 37 °C. The relationship between UV–Vis absorption spectra at 660 nm of wavelength and increasing population of bacteria in LB was monitored and verified by conventional plate-culture method. A sample with 1×10^8 CFU mL^{-1} of *E. coli* at exponential growth phase was picked and diluted in PBS to fit expected concentration of *E. coli*. The concentrations of *E. coli* sample before performing the detection were also verified by plate culture.

Salmonella typhimurium (S. typhimurium) (ATCC 19585) was grown in nutrient broth medium at 37 °C for 10 h and its population was verified by conventional plate-culture method using xylose lysine deoxycholate (XLD) agar. A sample with 1×10^9 CFU mL^{-1} of *S. typhimurium* was diluted in PBS to fit expected concentration. Further, these cultures were used for spiking water and milk samples used for testing.

3. Results and discussion

3.1. Concept of switchable linker and two-step assay

We designed experiments to determine the biosensor effectiveness by detecting target bacteria present in real matrices using streptavidin-coated AuNPs (st-AuNPs) and biotin-labeled bacteria antibodies (b-Abs), which served as BLs. Thus, target recognition occurs via antibody-antigen immunoreaction (function 1) and signal indication occurs via the aggregation of st-AuNPs (function 2), due to the well-known biotin-streptavidin binding reaction (Weber et al., 1989) (Fig. 1**A**), which brings about visible color change due to localized surface plasmon resonance (SPR). In general, when BLs bind to the target, they lose the ability to bridge st-AuNPs, and when BLs bridge NPs they do not participate in the immunoreaction.

The target recognition and signal indication steps in our assays are illustrated in Fig. 1**B, D** and Fig. 1**C, E**, respectively. The amount of BL needed to cause aggregation of st-AuNPs depends on the 'number' of target organisms in the system. And, for a given number of target organisms and st-AuNPs, there is a certain range of BL quantity, which leads to aggregation sufficient for visible color change, known as the range exhibiting visual color change (REVC). Therefore, we can identify three regions of BL: the REVC (Region 2) and one below (Region 1) and one above (Region 3) as shown in Fig. 1**C**. In Regions 1 and 3, unlike in Region 2, SRP is unchanged because st-AuNPs are not aggregated (Fig. S1B). In Region 2, st-AuNPs form large aggregates (Fig. S1 **C**) leading to