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Full Length Article

Fluorometric sensing of endotoxin based on aggregation of CTAB capped gold nanospheres

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ABSTRACT

Gold nanoparticles (GNPs) of different sizes were used to carry out comparative fluorometric detection study on endotoxin. At excitation wavelength, 308 nm the GNPs exhibited strong emission intensity at wavelength 421 nm with varying intensities at 215.122, 234.965 and 262.551 for 15 nm (GNPs-I), 30 nm (GNPs-II) and 40 nm (GNPs-III), respectively. The different sizes of GNPs were interacted with endotoxin to study the effect of GNPs size on endotoxin detection. The electrostatic interaction between GNPs (GNPs-I, II, and III) and endotoxin led to the enhancement of the fluorescence intensities. The sensitivity of endotoxin detection was improved significantly by decreasing the size of the GNPs to 15 nm (GNPs-I). The endotoxin detection limit using GNPs-I was theoretically calculated to be 0.56×10^{-9} M using the formula 3SD/slope, and it was able to detect lower levels of endotoxin when compared to GNPs-II or GNPs-III. The GNPs-I showed excellent selectivity for endotoxin detection with the optimized pH and volumetric ratio. Most importantly the optimized size was successfully used to detect endotoxin in real samples (milk samples and fruit juices) with a recovery rate of 98–105%.

1. Introduction

The food contaminant endotoxin bound to the cell wall of *Escherichia coli* is commonly termed as lipopolysaccharide (LPS). The endotoxin is highly responsible for causing various diseases in the human body like fever, vomiting, diarrhea, a decrease in blood pressure, sepsis or septic shock, inflammatory response and disseminated intravascular coagulation [1,2]. The endotoxin comprises of three major parts that are O-antigen, core oligosaccharide, and Lipid A. The Lipid A domain is held responsible for inducing toxicity in human beings, which is released into the blood stream after lyses of the bacterial cells by the human immune system. The endotoxin is released into the surrounding medium during the growth of the bacteria cell. Therefore, there is a growing urge to detect endotoxin in food before the consumers consume it. Every year 150,000 cases have been reported in the US due to life threatening health condition (septic shock), which was induced by endotoxin [3,4].

Several techniques like ELISA [5–8], PCR [9–12] and culture based assays [13,14], long-range surface plasmon-enhanced fluorescence spectroscopy (LRSP-FS) [15], modified SDS phage method and inductively coupled plasma mass spectrometry (ICPMS) are currently available for detecting the gram-negative bacteria *E. coli* as such or endotoxin present in the food The above available techniques needed costly instruments and were time-consuming. Endotoxin as such was also quantified earlier through the Limulus Amebocyte Lysate (LAL) assay. The LAL assay also has many disadvantages due to the false affirmative response due to molecules having structures similar to the carbohydrate [3,16].

Over the recent years, gold nanoparticles (GNPs) have been used extensively for detection of *E. coli* as such or endotoxin in food. The wide usage of GNPs was mainly due to the simple preparation method and functionalization of the nanoparticle with antibodies, aptamers or chemicals having exceptional spectral features and good stability characteristics. Detection of *E. coli* was colorimetric detected using gold nanoparticles functionalized with borohydride [17], mercaptoethylamine [18], aptamers [19], antibodies [20] and cysteine [21] by observing the change in the surface plasmon resonance band. Rod-shaped gold nanoparticles were also synthesized and functionalized with 11-Mercaptoundecanoic acid and Cystamine dihydrochloride to detect efficiently *E. coli* bacteria [22,23]. Recently *E. coli* endotoxin (lipopolysaccharide) was also detected using gold nanoparticles capped with cysteamine [16] and with gold nanorods capped with CTAB and polymer [24]. Fluorometric detection of *E. coli* was also carried using gold nanoclusters capped with lysozyme by

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observing the fluorescence enhancement of the aggregated gold nanoclusters [25].

To the best of our knowledge, this is the first report on the fluorometric detection of *E. coli* endotoxin using different sizes of CTAB capped GNPs (15 nm, 30 nm, and 40 nm) prepared using the seed-mediated process. The effect of GNPs size, pH and interaction volume ratio was optimized for detecting endotoxin, and the optimized GNPs size 15 nm (GNPs-I) were further used to study the selectivity of the developed method. The endotoxin detection limit using GNPs-I was 0.56×10^{-9} M, and it was able to detect lower levels of endotoxin when compared to 30 nm (GNPs-II) or 40 nm (GNPs-III). The efficiency of the developed sensor probe was checked successfully by detecting endotoxin in real samples (milk samples and fruit juices) with the optimized GNPs size 15 nm (GNPs-I).

2. Experimental

2.1. Apparatus

UV–visible spectrophotometer (UV-2600 Shimadzu, Tokyo, Japan) was used to record the absorption spectra from 200–800 nm. The fluorescence spectroscopy studies were also acquired using the fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer Agilent Technologies, G9800A). The fluorescence intensity for gold nanoparticles (GNPs) was recorded at $\lambda_{ex}/\lambda_{em}$=308/421 and the excitation and emission slits width were set at 10 nm and 20 nm. The mean hydrodynamic diameter of GNPs were measured using the Dynamic light scattering (90 Plus Particle Analyzer, Brookhaven Instruments Corporation, NY, USA) and the surface charge was obtained using the Nano Particle Analyzer (SZ100, Horiba Scientific, Japan). The size and morphological features of GNPs were studied using the high-resolution transmission electron microscope (JEOL JEM 2100 High-Resolution Transmission Electron Microscope) with accelerating voltage 200 kV.

2.2. Materials and reagents

Hydrogen tetrachloroaurate hydrate ($HAuCl_4$) was procured from SRL Pvt. Ltd (India). Trichloroacetic acid, cetyltrimethylammonium bromide (CTAB) and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (India). Ascorbic acid was brought from SD Fine Chemicals Ltd. (India). Interfering agents like sodium chloride, monosodium phosphate, sodium carbonate, dipotassium phosphate, citric acid, sucrose, chitosan (50 kDa), glucose, ovalbumin (OVA, 44 kDa), bovine serum albumin (BSA, 66.4 kDa), polyvinyl alcohol (PVA, 160 kDa), aspartic acid, glutamic acid and Tween-20 were purchased from Himedia Laboratories Pvt Ltd. (India). The endotoxin from *E. coli* 055: B5 was procured from Sigma-Aldrich (India). The molecular weight of endotoxin was found to be 10 kDa from the previously available reports [16]. The endotoxin was dispersed in phosphate buffer (10 mM, pH=7.2) and Millipore water (Millipore, Milford, MA) was used throughout the experiments. All the glasswares were washed completely with aqua regia and cleaned with Millipore water before use.

2.3. Gold nanoparticle preparation

The GNPs were prepared according to Jana et al. but with some slight modifications [26,27]. In brief, the gold seeds were synthesized by quick addition of freshly prepared 0.6 ml of $NaBH_4$ (0.1 M) to 20 ml aqueous solution mixture containing $HAuCl_4$ (2.5×10^{-4} M) and trisodium citrate (2.5×10^{-4} M) with rapid stirring. The immediate change in color to pink indicated the formation of the particle. The gold seeds were left undisturbed and were used for the synthesis of GNPs after a period of two hours. At room temperature the trisodium citrate cannot reduce the $HAuCl_4$ therefore; here citrate only serves as a capping agent to the gold seeds.

The stock growth solution was prepared by adding solid 3 g of cetyltrimethylammonium bromide (0.08 M) in 200 ml aqueous solution of $HAuCl_4$ (2.5×10^{-4}). The mixture was heated to 45 °C to obtain a clear orange color. The stock growth solution was then cooled down to room temperature before use. Three different sized GNPs (15, 30 and 40 nm) were synthesized in 100 ml beakers. 15 nm (GNPs-I) were prepared by rapid addition of 0.1 ml of ascorbic acid (0.1 M) to 18 ml of growth solution. Next, 2 ml of the as-synthesized gold seeds was added with rapid stirring. The aqueous solution slowly turned wine red, which indicated the formation of GNPs-I. The solution was kept in stirring for another ten min. The GNPs-I was further used as seeds to synthesis GNPs-II after 30 min of preparation. 30 nm (GNPs-II) and 40 nm (GNPs-III) were synthesized similarly to GNPs-I. 0.1 ml of ascorbic acid (0.1 M) was added to 18 ml of growth solution and finally 2 ml from GNPs-I or GNPs-II, respectively was added to the mixture with rapid stirring. The aqueous solutions turned dark red confirming the formation of GNPs-II and GNPs-III, respectively. Here, the GNPs-II was used as seeds for the synthesis of GNPs-III. The GNPs-I, II, and III were stable at room temperature due to the availability of CTAB in the solution. Excess of CTAB was then further removed by centrifugation and dispersing the pellets in Milli-Q water.

2.4. Fluorometric detection of endotoxin

The pH and interaction volume ratio of GNPs and endotoxin was studied for detecting endotoxin in phosphate buffer (10 mM; pH=7.2). Detection of endotoxin was carried out efficiently by interacting 500 μl of different concentrations of endotoxin (0.5×10^{-9}–50×10^{-9} M) with 500 μl of GNPs-I (pH 4–5) for ten min. Similarly detection of endotoxin was also carried out using GNPs-II and GNPs-III. Different endotoxin concentration (400 μl) ranging from 1×10^{-9}–80×10^{-9} M and 10×10^{-9}–90×10^{-9} M was interacted with GNPs-II (600 μl) and GNPs-III (600 μl), respectively. The fluorescence intensity of GNPs-I, II and III was studied using fluorescence spectrophotometer at 421 nm before and after interaction with endotoxin. All the experiments were performed out in triplicates, and the statistical significance of the recorded results were checked using one-way ANOVA test in the excel sheet. The error limit for all the experiments was set up to ±5%.

Selectivity study was carried out for endotoxin detection by interacting GNPs with 1 mM sodium chloride, monosodium phosphate, sodium carbonate, dipotassium phosphate, citric acid, sucrose, chitosan, ovalbumin (OVA, 44 kDa), bovine serum albumin (BSA, 66.4 kDa), polyvinyl alcohols (PVA, 160 kDa), aspartic acid, glucose, ascorbic acid, glutamic acid, and Tween-20.

2.5. Real sample pre-treatment for endotoxin detection

Different milk samples (liquid milk, milk powder, and raw milk) are pre-treated before detecting the presence of endotoxin [28]. The pre-treatment method was mainly adopted to eliminate the calcium salt of phosphoprotein (casein) from the milk samples. A mixture containing 1.2 ml of chloroform and 2 ml of trichloroacetic acid (10%) was added to the milk samples, which separated out the calcium cations from the milk along with the insoluble phosphoprotein. The milk samples were then vigorously vortexed, sonicated for 15 min and then centrifuged for 10 min at 13,000 rpm to completely settle down the available protein content. The supernatant was then collected and then again centrifuged for 1 min at 3000 rpm after setting the pH to 7.0 with the