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A multicolor time-resolved fluorescence aptasensor for the simultaneous detection of multiplex *Staphylococcus aureus* enterotoxins in the milk

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ABSTRACT

Food safety is one of the most important public health issues worldwide. Foodborne illnesses caused by *Staphylococcus aureus* enterotoxins (SEs) commonly occur, affecting both developing and developed countries. In this study, multicolor lanthanide-doped time-resolved fluorescence nanoparticles labeled with aptamers were used as bioprobes, and graphene oxide (GO) was employed as a resonance energy acceptor. Based on the "turn down" strategy, the simultaneous detection of multiplex SEs was realized in a homogeneous solution. Under the optimal conditions, the developed method exhibited high sensitivity and selectivity to three serological types of enterotoxins, including type A, B, C1, with limits of detection below 1 ng mL^{-1}. The application of this bioassay in milk analysis with no sample dilution was also investigated, and the results of recovery rates covered from 92.76% to 114.58%, revealing that the developed method was accurate. Therefore, this detection aptasnesor can be a good candidate for multiplex analysis and screening with simple and effective operations.

1. Introduction

In recent years, food poisoning incidents caused by the ingestion of food contaminated with enterotoxins from foodborne pathogens have had a common occurrence worldwide. Food poisoning caused by *Staphylococcus aureus* enterotoxins (SEs) accounts for a large number of bacterial foodborne illnesses (Pinchuk et al., 2010) and is characterized by a short incubation period (2–8 h) and includes nausea, violent vomiting, abdominal cramping, with or without diarrhea (Argudin et al., 2010). SEs consist of a group of five classical serological types (SEA, SEB, SEC1/2/3, SED, SEE) and other new types (SEG–SEV, except for SER) of heat-stable, small (26–30 kDa) protein exotoxins secreted by certain strains of *S. aureus* (Argudin et al., 2010; Naomi Balaban, 2000). Moreover, various foods can be frequently contaminated by SEs, such as meat, poultry, egg, milk and dairy products, and salads despite being heat- or acid-treated (Ortega et al., 2010). In some cases, some SEs act as super antigens that cause a severe allergic and autoimmune response as well as toxic shock syndrome. These effects indicate that poisoning by SEs is not only a common food safety problem that affects public health but also a potential biochemical weapon (Molenda et al., 2008). As a result, the development of high-efficiency or high-throughput, sensitive, and practical detection methods for SEs is critical to prevent and control food contamination and the deliberate addition of adulterants.

Currently, common detection methods of SEs available in laboratories and commercially are based on molecular biological methods (mainly involving polymerase chain reactions (PCR)) and immunological techniques (Hennekinne et al., 2010). However, PCRs detect only gene-encoding enterotoxins in strains of *S. aureus* isolated from food samples instead of the SEs themselves and require complicated handling procedures, strict laboratory conditions, and professional operators (Fusco et al., 2011; Monday and Bohach, 1999). Immunoassay-based methods have been developed in diverse detection modes, such as ELISA (Fey et al., 1984), surface

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plasmon resonance (SPR) (Naimushin et al., 2002), electrochemical (Sun et al., 2012) and piezoelectric (Salmain et al., 2011) immunosensors, and hydrogel biochips (Rubina et al., 2010). Nevertheless, antibodies are typically obtained from experimental animal or hybridoma cells, which is a costly and time-consuming process. Moreover, antibodies proteins are susceptible to the effects of environment, leading to poor analysis performances. Partial antibodies of SEs, such as hapten of serological types S and T, are difficult to harvest, thus no immune-based assays are currently available (Ono et al., 2008). SEs usually exist in complex biological samples, including food matrices and clinical and environmental samples, thus the pretreatments are usually cumbersome and complicated. Traditional immunofluorescence assays cannot avoid the impacts of unstable optical fluorophores (e.g., fluorescent dyes and materials) and autofluorescence from the sample background. In the coming decades, the advantages of emerging aptamer-based and time-resolved fluorescence techniques have shown up. Zhang et al. (2013) designed an time-resolved fluorescent aptasensor for direct detecting adenosine deaminase (ADA). The detection limit of this aptasensor was lowered to 2 U L^{-1} and without any need of sample clean-up process, which was sensitive and convenient for ADA quantification in human serum. Huang et al. (2012) reported a sensitive and label-free strategy for thrombin detection by using aptamer and Tb-complexes and gold nanoparticles (GNPs), obtaining a detection limit of 0.14 nmol L^{-1}.

Aptamers, with high-affinity binding to given targets, are proposed as alternative and innovative recognition elements. In particular, aptamer-based biosensors possess the following advantages over biosensors using natural receptors such as antibodies and enzymes: high specificity and affinity for a wide range of targets, high reproducibility and purity via commercial synthesis, high chemical stability, and great flexibility in the design of novel biosensors (Zhou et al., 2014). Lanthanide-doped (Ln^{3+}-doped) inorganic nanoparticles (NPs) have been suggested as a promising new class of fluorescent probes. The new-type time-resolved fluorescence from Ln^{3+}-doped NPs rather than conventional lanthanide ion chelates not only exhibits high chemical stability, high resistance to photobleaching, low cytotoxicity, and favorable biocompatibility but also and more importantly has an especially simple and controlled synthesis, multicolor doping, large effective Stokes shifts, and long luminescence lifetimes. These features can help avoid the overlap between the excitation and emission spectra of the fluorophore itself (inner filter effect) and the emission from the biological matrix and the autofluorescence from untreated bio-samples based on time-resolved fluorescence detection (Liu et al., 2013).

Currently, array-based techniques have been available for multi-analyte detection at the surface of a solid interface, which allow the simultaneous analysis and quantification of multiple targets (Taitt et al., 2002). Despite the advantage of spatial resolution, immobilization and washing operations are inevitable for array-based biosensors, thus greatly limiting the effectiveness of applying this technology. In a previous study by our group, graphene oxide (GO) was employed as a time-resolved fluorescence quencher and played an excellent part in the homogeneous assay (Huang et al., 2015b). With a wide range of wavelengths (approximately 300–700 nm) in the absorption spectrum, which almost overlaps with the fluorescence spectra of various fluorescent materials including time-resolved fluorescent Ln^{3+}-doped NPs, graphene oxide exhibits great potential in simultaneous detection through homogeneous multiplexed fluorescence resonance energy transfer (multiplexed-FRET) systems.

Herein, a new time-resolved fluorescence aptasensor was designed for the simultaneous detection of multiple SEs. Based on the currently reported aptamers against SEs, three SEs (SEA, SEB, SEC1) were selected as multiple analytes, and their aptamers were used as recognition elements to capture target enterotoxins and trigger the quenching of multicolor time-resolved fluorescence signals. The developed aptasensor has been demonstrated to show high-throughput, sensitivity, and convenient performances in the simultaneous detection of multiple SEs.

2. Experimental section

2.1. Materials and instruments

Gadolinium (III) chloride hexahydrate ($GdCl_3 \cdot 6H_2O$), europium (III) chloride hexahydrate ($EuCl_3 \cdot 6H_2O$) and dysprosium (III) chloride hexahydrate ($DyCl_3 \cdot 6H_2O$) were purchased from Sigma-Aldrich Co. LLC (USA). Terbium (III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) was purchased from Aladdin Chemistry Co. Ltd. (China). Glutaraldehyde aqueous solution (25% in V/V) and the chemicals used to prepare the buffers and solutions were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Avidin was purchased from Sigma-Aldrich Co. LLC (USA). Polyethylenimine (PEI) (ca. 30% in water) was purchased from TCI Development Co., Ltd. (China). 96-well microplates (300 mL $well^{-1}$) were obtained from Corning (USA). SEs (SEA, SEB and SEC1, SEC2, SEC3) were supplied by Beijing Biomai (Beijing, China). SED and SEE were purchased from the Academy of Military Medical Sciences (Beijing, China). RIDASCREEN® SET A, B, C, D, E ELISA kit was purchased from R-Biopharm Analysis Systems Trading Co. Ltd. (Beijing, China). Solutions were prepared with ultrapure water from a Millipore water purification system.

The SEs aptamers (DeGrasse, 2012; Huang et al., 2014, 2015a) (as shown in Table S1) were synthesized by the Shanghai Sangon Biological Science & Technology Company (Shanghai, China) and were purified using high-performance liquid chromatography.

The powder X-ray diffraction (XRD) pattern was measured using a D8 Avance instrument (Bruker AXS Ltd., Germany). The morphologies of the nanomaterials were identified using a JEM-2100HR transmission electron microscope (TEM, JEOL Ltd., Japan). Time-resolved fluorescence emission spectra were recorded using a SpectraMax M5/M5e microplate reader in time-resolved mode spectra scan (Molecular Devices, USA). The transient decays of $KGdF_4$:Eu^{3+} NPs were recorded on an Edinburgh Instruments FLS920 spectrofluorimeter (Edinburgh, England). The X-ray photoelectron spectroscopy (XPS) was measured for elemental and chemical bonds analysis of GO by a Thermo Scientific ESCALAB 250Xi (Thermo Scientific, USA). The detection results were collected using a SpectraMax M5/M5e microplate reader in time-resolved mode Pot read (Molecular Devices, USA). All of the toxin-related operations were carried out in a 1300 Series Class II, Type A2 Biological Safety Cabinet (Thermo Fisher Scientific Inc., USA).

2.2. Synthesis of GO

GO was prepared from graphite powder by a modified Hummer's method (Hummers and Offeman, 1958). The detailed procedure for the synthesis of GO was described in a previous paper (Huang et al., 2015b).

2.3. Synthesis of Ln^{3+}-doped $KGdF_4$ NPs

$KGdF_4$ NPs doped with Ln^{3+} (Ln = Eu, Tb, Dy) were synthesized based on Ju's method (Ju et al., 2012) with some modifications. Briefly, a well-stirred transparent solution of 20 mL of ethylene glycol containing 2.0 mmol KCl, 1.0 mmol $GdCl_3$, required amounts of $LnCl_3$ (0.1 mmol Eu^{3+}, 0.1 mmol Tb^{3+}, and 0.01 mmol Dy^{3+}), and 1 mL of aqueous PEI was mixed with another well-stirred transparent solution of 20 mL of ethylene glycol with 6.0 mmol