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Rapid detection of *Cronobacter sakazakii* in dairy food by biofunctionalized magnetic nanoparticle based on nuclear magnetic resonance

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ABSTRACT

In order to ensure the safety of infant formula powder in China, a rapid and sensitive detection method for food-borne bacteria is urgently needed. We have developed a reliable immunoassay based on nuclear magnetic resonance for the specific detection of *Enterobacter sakazakii* in dairy samples with biofunctionalized magnetic nanoparticles. This method is able to detect *Cronobacter sakazakii* in milk powder and cheese samples at 1.1 to 11 MPN using the most-probable-number (MPN) assay, within an incubation period of less than 2 h. Longer incubation time (>4 h) or higher pH (>7) will decrease the sensitivity of this method. This method does not fit for the detection of bacteria at higher concentrations (>1100 MPN). This method has great potential to becomea useful tool for the rapid detection of bacterial contaminations in food, environmental and agricultural samples.

1. Introduction

Cronobacter sakazakii, which was formerly known as "yellow-pigmented *Enterobacter cloacae*", is a motile, peritrichous, Gram-negative food-borne pathogen. Since its first report in 1958, this bacteria has been shown to be responsible for severe necrotizing enterocolitis (Klostranec & Chan) (NEC), bacteraemia and meningitis, with case fatality rates ranging between 40 and 80% as reported (Bowen & Braden, 2006; Friedemann, 2009; Yan et al., 2012). This bacteria has also been reported to associate with life-threatening infections in low-birth-weight babies (Farmer Iii, Asbury, Hickman, & Brenner, 1980). Traditional methods for the identification and quantification of *C. sakazakii* generally require multiple steps of enrichment and biological tests, which can take as many as 6–7 days to finish. In recent years, a number of rapid methods have been developed to detect *C. sakazakii* (Almeida et al., 2009; Iversen, Lancashire, Waddington, Forsythe, & Ball, 2006; Liu et al., 2012), such as multiplex polymerase chain reaction (PCR) assay (Chen, Song, Brown, & Lampel, 2010; Hassan et al., 2007), infrared spectroscopy (Lin et al., 2009), fluorescence in situ hybridization (Almeida et al., 2009), DNA microarray (Wang et al., 2009), and sandwich enzyme-linked immunosorbent assay (Park et al., 2012). Although these methods can achieve high specificity and low minimum detection limit for the detection of *C. sakazakii*, they are excessively expensive and complicated to be utilized in industrial settings. Therefore, it is necessary to develop a rapid, sensitive, accurate, and cost-efficient detection assay in the dairy industry.

With the development of nanotechnology, superparamagnetic nanoparticles, conjugated with oligonucleotides, small molecules, peptides and antibodies, have been attracting attention because of their inherent magnetic features and potential applications in the

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medical, environmental and food sciences (Kaittanis, Nath, & Perez, 2008; Merkoci, 2010; Yang et al., 2011). Noticeably, the utilization of these nanoparticles also offers a unique alternative for the detection of bacterial targets to improve detection sensitivity, and reduced preparation time with smaller samples (Esteban-Fernández de Ávila, Pedrero, Campuzano, Escamilla-Gómez, & Pingarrón, 2012; Kim, Josephson, Langer, & Cima, 2007; Perez, Josephson, O'Loughlin, Högemann, & Weissleder, 2002; Perez, Simeone, Saeki, Josephson, & Weissleder, 2003). Mechanistically, this technology is based on nuclear magnetic resonance-based magnetic relaxation switches (MRSw). In the presence of molecular targets with changes in the spin–spin relaxation time of water (T2), magnetic nanoparticles (NPs) are switched from a dispersed state to a clustered state. This switch (MRSw) has been shown to be able to detect nucleic acid and protein targets with high sensitivity (Josephson, Perez, & Weissleder, 2001; Josephson, Tung, Moore, & Weissleder, 1999; Kaittanis, Naser, & Perez, 2007; Kaittanis, Santra, Asati, & Perez, 2012).

The utilization of MRSw biosensor has been explored in a series of practical applications, including the detection of viruses and bacteria. A report on the detection of microcystin-LR in the water of Tai Lake (Jiangsu Province, China) based on the relaxation of magnetic nanoparticles has achieved a detection range of 1–18 ng/g (Ma et al., 2009). Koh and his colleagues demonstrated that the limit value of "projected sensitivity concentration" could be significantly reduced to 1.4×10^{-4} nm when using a high-sensitivity MRSw biosensor to detect the influenza antibodies (Koh, Hong, Weissleder, & Josephson, 2008). Kaittanis and colleagues established immuned magnetic beads probes to identify *Mycobacterium avium* spp. *Paratuberculosis* in milk and blood samples and demonstrated that the detect limit reached as low as 15.5 MPN (Kaittanis et al., 2007). Using cationic-magnetic beads as sensors, *C. sakazakii* can be detected at 1–5 MPN/500 g of infant milk formula in less than 24 h (Mullane et al., 2006).

In this paper, we report a novel method for the rapid detection of *C. sakazakii* in dairy products with high sensitivity. We constructed a *C. sakazakii* specific NMR biosensor by coupling a polyclonal rabbit anti-*C. sakazakii* antibody to superparamagnetic iron oxide nanoparticles. We found that as the number of bacteria increased in the solution, the increased *C. sakazakii* epitopes interacted with more magnetic nanoparticles in the solution, therefore causing substantial changes in the T2 value. This simple method has yielded high detection sensitivity for *C. sakazakii* and thus can potentially be developed into a rapid detection tool for infectious food-borne bacteria.

2. Materials and methods

2.1. Reagents

Polyclonal rabbit anti-*C. sakazakii* antibody (6.82 mg/mL) was prepared by Shanghai Youke Biotechnology Co. Limited. 1-[3-(dimethylamino)-propy-3-ethylcarbodiimide HCL (EDC·HCL) and N-hydroxy-succinimide (NHS) were purchased from SinopHarm. Nutrient broth medium (Restaino, Frampton, Lionberg, & Becker, 2006) and blood agar were provided from Beijing Land Bridge Technology Co. Ltd. Chemical reagents and salts were supplied by Shanghai Ling Feng Chemical reagent Co. Ltd.

2.2. Synthesis of magnetic beads

The synthesis of superparamagnetic nanoparticals (Fe_3O_4) was implemented by a previously reported method (Ma et al., 2009). Synthesis of $Fe(acac)_3$ Precursor: according to literature, Ferrum (a) acetylaceton ate was synthesized. A 20 mL aqueous solution of $FeCl_3$ (3.25 g, 20 mmol) was added with 2, 4-pentanedione (12.3 mL, 120 mmol) under magnetic stirring. After 15 min, triethylamine (6.0 mL) was added to the above mixture. Red precipitates were filtered off to give a crude product. The product was recrystallized in a mixture of ethanol and water to yield pure $Fe(acac)_3$ crystals. The crystals were dried at 80 °C under vacuum and then stored at a desiccator before use.

Preparation of Fe_3O_4 nanoparticles: A slurry of $Fe(acac)_3$ (1.06 g) was dissolved in 15 mL oleylaminein and 15 mL phenylate in a 100 mL three-neck flask. The mixture solution was heated at 110 °C for 1 h, then was refluxed at 210 °C for 30 min, thereafter was continued to be heated to 300 °C and maintain 1 h. The above steps were all under a nitrogen atmosphere. The resulting mixture was cooled down to room temperature to form a black suspension. After centrifugation at 12,000 rpm for 10 min, the supernatant was removed and a black precipitate was obtained. The resulting black precipitate was washed with ethanol three times to acquire pure Fe_3O_4 NPs. Finally, the Fe_3O_4 NPs were dried in vacuum at 120 °C before use.

To empower the magnetic beads with broad compatibility to biomacromolecules, formation and functionalization of $Fe_3O_4@SiO_2$ nanocomposite particles steps were carried out. Igepal CO-520 (2.0 g, 4.75 mmol) was dispersed in cyclohexane (35 mL) by sonication until the mixture turned to limpidity. Dry Fe_3O_4 NPs (10 mg) were added to the above solution. The resulting mixture was stirred vigorously to disperse the NPs, and ammonium hydroxide (29.4%, 0.35 mL) was added to form a transparent and black solution of reverse microemulsion. TEOS (0.2 mL) and APS (35uL) were added, and the mixture was gently stirred. The reaction was continued for 48 h at room temperature. The resulting $Fe_3O_4@SiO_2$-NH_2 NPs were precipitated by addition of acetone and were collected by centrifugation at 12,000 rpm for 10 min. The collected $Fe_3O_4@SiO_2$-NH_2 NPs were dispersed in ethanol or water and purified by repeating the centrifugation and redispersion process. The size, distribution and morphology of the amino-modified silica-coated Fe_3O_4 were characterized by atomic force microscope (AFM).

2.3. Coupling the antibodies to the magnetic beads

The amino-modified silica-coated Fe_3O_4 was used to conjugate the antibody. According to a previous report (Ma et al., 2009), 1-p-3-ethylcarbodiimide HCL (EDC·HCL) and N-hydroxy-succinimide (NHS) were used to activate the carboxyl of the antibody. In particular, 200 mg EDC·HCL and 250 mg NHS were added into 0.1 mL anti- *C. sakazakii* -antibody (6.82 mg/mL). Then the mixture was diluted into 5 mL PBS (pH 7.4). After incubation at 4 °C overnight, 10 mg amino-modified silica-coated Fe_3O_4 was dispersed in 5 mL PBS (pH 7.4). Subsequently, 5 mL activated anti- *C. sakazakii* -antibody was poured into the upper mixture and left to react for 4 h at room temperature. Finally, the products were separated by magnetic separators and washed by PBS (pH 7.4) for three times, then suspended in PBS (pH 7.4) and kept at 0–4 °C.

In order to further estimate the protein immobilization capacity of the functional $Fe_3O_4@SiO_2$-NH_2 NPs, the bovine serum albumin (BSA) was taken as a control sample. A series of known concentrations of BSA were measured with ultraviolet spectrophotometer to get the OD_{280} values. A standard curve between known concentrations and OD_{280} values of BSA was obtained. 10 mg functional $Fe_3O_4@SiO_2$-NH_2 NPs were poured into a 5 mL system with BSA of unknown concentration activated by EDC·HCL and NHS. Then, the mixed solutions were left at room temperature for 4 h. The BSA immobilized by the magnetic beads was separated by magnetic separators. The amount of the BSA proteins immobilized by the magnetic beads was determined by measuring the absorption at 280 nm of the initial and the final supernatant.