Design and Function of Fluorescent Silica Nanoparticles for Bacteria Detection

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(Manuscript received March 23, 2018; accepted May 10, 2018)

Abstract

Food safety is one of the concerned issues. As a result, the managements to protect consumers adequately from foodborne illness are to be required. The standard method for specific pathogen detection is culture method. However, conventional methods based on culture have disadvantages of time-consuming, which might cause infectious diseases to spread rapidly. Therefore, rapid and simple methods for bacteria detection have been attracting much attention in this research area. Recently, we developed dipicolylamine (dpa)-modified fluorescent silica nanoparticles (FSiNP) for bacteria detection. In this study, we prepared two FSiNPs (Bt/dpa-HCC/FSiNP and B/FSiNP) whose surfaces were modified with dipicolylamine or phenyl boronic acid. Cu-Bt/dpa-HCC/FSiNP formed aggregates with both S. aureus and E. coli, whereas B/FSiNP formed aggregates with S. aureus selectively. Bt/dpa-HCC/FSiNP could examine the existence of bacteria in water and B/FSiNP could detect either S. aureus or E. coli. These results demonstrated that surface-functionalized silica nanoparticles could detect bacteria in water within 10 min.

Keywords: Silica nanoparticles, Biosensor, Fluorescent response, Boronic acid, Dipicolylamine

1. Introduction

Nowadays, one of the worldwide important issues is to save the safety of food and water. About 0.6 billion people suffer from foodborne illness all over the world by a year1). The standard method of pathogen detection is culture method2). However, it takes about five days to determine the species of bacteria. This time-consuming detection of bacteria cause sometimes serious problem for diagnosis and medical treatment. To solve this problem, many bacterial detection methods are developed; ELISA, PCR etc.3-5) These methods can obtain accurate results. However, they require expensive institutions and technical knowledge6-9). Thus, it is desired to develop low cost, rapid and easy bacterial detection methods.

Dipicolylamine is known to coordinate with various metal ions10). Moreover, dipicolylamine coordinated with metal ions is able to bind with phosphoric acid derivatives11-13). Previously, we synthesized coumarin derivative as fluorophore site and dipicolylamine as recognition site10). It is known that dpa-HCC coordinated with metal ions can detect phosphoric acid derivatives and increase fluorescent intensity. B.D. Smith et al. developed bis-type dipicolylamine probes possessing fluorophore site14). These probes coordinated with zinc ions and recognized the phosphoric acid derivatives of the bacterial cellular membrane. These probes made it capable to stein and destroy bacteria.

Phenylboronic acid forms ester binding with cis-diol of saccharides under basic condition15). Thus, the saccharide sensors which use phenylboronic acid are attracting much
Moreover, phenylboronic acid is known to connect with saccharide chains on the surfaces of bacteria. Fluorophore-doped silica nanoparticles have three advantages: 1) it is easy and needs low costs to synthesize silica nanoparticles, 2) silica nanoparticles can have many functions because the process of modifying its surfaces is simple, 3) the fluorescent emission can be stable because fluorophore is included in silica nanoparticles. Previously, we reported fluorescent silica nanoparticles (FSiNPs) modified with metal-dipicolylamine complex (M-dpa-HCC) for *Staphylococcus aureus* (*S. aureus*) detection. Among the M-dpa-HCC/FSiNP complexes, Cu-dpa-HCC/FSiNP formed large aggregates with *S. aureus* in 10 min, which were easily observed by the naked eye. Herein, we develop two types of fluorescent silica nanoparticles (FSiNP) and evaluate bacteria recognition function (Scheme 1). One is betaine modified on the surface FSiNP (Bt-dpa-HCC/FSiNP), which can connect with phosphate acids on bacteria surface and betaine can disperse fluorescent silica nanoparticles in water. The other is phenyl boronic acid modified on fluorescent silica nanoparticles (B/FSiNP), which can connect with sugar chains on bacteria surface.

![Scheme 1 Structure of Cu-Bt-dpa-HCC/FSiNP and B/FSiNP.](image)

2. Experimental
2.1 Reagents and chemicals
2-{4-[2-(Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), acetonitrile (Luminasol), sodium hydroxide, sodium nitrate, cyclohexane, 1-hexanol, 30% ammonia hydroxide and acetic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 7-hydroxycoumarin-3-carboxylic acid (1.072 g, 5.09 mmol) were dissolved in 30 mL of acetonitrile and refluxed for 2 h. 7-hydroxycoumarin-3-carboxylic acid (1.072 g, 5.09 mmol) dissolved in 30 mL of acetonitrile was added to the mixture. The reaction mixture was stirred for 2 h and filtrated to obtain highly viscous yellow liquid. The viscous liquid was dissolved in chloroform and extracted by water/chloroform. The organic layer was washed with deionized water and dried in vacuo to give a yellow solid (1.31 g, 3.13 mmol, 62.1%). 1H NMR spectra were measured with a Lambda GX-500 (JEOL Ltd., Tokyo, Japan) at 300 K. Elemental analysis was performed with a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer (PerkinElmer, Inc., MA, USA). All pH values were recorded with a Horiba F-52 pH meter (HORIBA, Ltd., Kyoto, Japan). UV-Vis absorption spectra were measured with a Hitachi U-3900 UV-Vis spectrophotometer (Hitachi High-Technologies, Co., Tokyo, Japan) equipped with a Peltier thermocontroller with a 10-mm quartz cell at 25 °C. Fluorescence spectra were measured with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Technologies, Co., Tokyo, Japan) equipped with a Peltier thermocontroller with a 10-mm quartz cell at 25 °C.

2.2 Apparatus
2.2.1 Synthesis of dpa-HCC
2,2'-dipicolylamine (1.025 g, 5.04 mmol) and formaldehyde (37% aqueous solution, 0.441 mL, 5.04 mmol) were dissolved in 30 mL of acetonitrile and refluxed for 2 h. 7-hydroxycoumarin-3-carboxylic acid (1.072 g, 5.09 mmol) dissolved in 30 mL of acetonitrile was added to the mixture. The reaction mixture was stirred for 2 h and filtrated to obtain highly viscous yellow liquid. The viscous liquid was dissolved in chloroform and extracted by water/chloroform. The organic layer was washed with deionized water and dried in vacuo to give a yellow solid (1.31 g, 3.13 mmol, 62.1%). 1H NMR spectra were measured with a Lambda GX-500 (JEOL Ltd., Tokyo, Japan) at 300 K. Elemental analysis was performed with a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer (PerkinElmer, Inc., MA, USA). All pH values were recorded with a Horiba F-52 pH meter (HORIBA, Ltd., Kyoto, Japan). UV-Vis absorption spectra were measured with a Hitachi U-3900 UV-Vis spectrophotometer (Hitachi High-Technologies, Co., Tokyo, Japan) equipped with a Peltier thermocontroller with a 10-mm quartz cell at 25 °C. Fluorescence spectra were measured with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Technologies, Co., Tokyo, Japan) equipped with a Peltier thermocontroller with a 10-mm quartz cell at 25 °C.

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2.3.2 Synthesis of fluorescent silica nanoparticles (FSiNP)
The fluorescent silica nanoparticles (FSiNP) was synthesized by a Stöber method, using tetraethyl orthosilicate (TEOS) as the precursor in the presence of [Ru(bpy)3]2+ as fluorescent core of FSiNP. Then, the surface of FSiNP was modified by 3-aminopropyltrimethoxysilane (APS). The synthetic procedure is shown as follows; Cyclohexane (22.5 mL), Triton X-100 (5.4 mL), 1-hexanol (5.4 mL) and 20 mM [Ru(bpy)3]2+ (1.5 mL) were mixed and sonicated by homogenizer. The mixture was stirred for 1 h at room temperature. Tetaethyl orthosilicate (TEOS, 0.60 mL) and 30% ammonia hydroxide (1.80 mL) was added and stirred for 24 h. The reaction was stopped by adding acetone and the reaction mixture was suspended. The suspension was centrifuged and washed by acetone, ethanol and water to obtain the products (FSiNP).
2.3.3 Synthesis of APS modified FSiNP (FSiNP-APS)  
FSiNP (60 mg) were dispersed in methanol (10 mL). 3-aminopropyltriethoxysilane (APS, 240 mg) dissolved in methanol (10 mL) were added to this solution. Acetic acid (3 mL) and water (7 mL) were added to this solution and refluxed for 24 h. The solvent were centrifuged and washed by methanol and water. The precipitate was dried in vacuo and obtained the products (FSiNP-APS).

2.3.4 Synthesis of dpa-HCC modified FSiNP (dpa-HCC/FSiNP)  
FSiNP-APS (106.0 mg) and dpa-HCC (42.7 mg) were dispersed in methanol (30 mL). 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (29.7 mg) was added to the reaction mixture and stirred for 16 h at room temperature. The mixture was centrifuged and the crude was washed by methanol and water. The precipitate was dried in vacuo and obtained the products (dpa-HCC/FSiNP).

2.3.5 Synthesis of betaine and dpa-HCC modified fluorescent silica nanoparticle (Bt/dpa-HCC/FSiNP)  
The dpa-HCC/FSiNP (42 mg) was dispersed in methanol (30 mL). Betaine (58 mg) and DMT-MM (0.16 g) was added to the solution and stirred for 24 h at room temperature. The mixture was centrifuged (10,000 rpm, 10 min) and the crude was washed by methanol and water. The precipitate was dried in vacuo and obtained the products (Bt/dpa-HCC/FSiNP).

2.3.6 Synthesis of p-phenylboronic acid modified fluorescent silica nanoparticle (B/FSiNP)  
FSiNP-APS (34 mg) and p-phenylboronic (43 mg) acid were dispersed in methanol (30 mL). DMT-MM (110 mg) was added to the reaction mixture and stirred for 24 h at room temperature. The mixture was centrifuged and the crude was washed by methanol and water. The precipitate was dried in vacuo and obtained the products (B/FSiNP).

2.4 Observation by fluorescent microscopy  
Cu-Bt-dpa-HCC/FSiNP, Cu-dpa-HCC/FSiNP or B/FSiNP (0.1 mg/mL) was dispersed in water. Each solution was incubated with bacterial (S. aureus and E. coli) solution and stirred for 10 minutes at 2,000 rpm. Then, each mixture was observed by fluorescent microscopy.

2.5 Measurement of Fluorescent intensity and Turbidity  
To investigate the dispersibility of dpa-HCC/FSiNP and Bt/dpa-HCC/FSiNP in water, eight silica nanoparticles’ solutions were prepared; \([\text{dpa-HCC/FSiNP}]= 1.0, 0.5, 0.25, 0.125 \text{ mg/mL}\). The fluorescent intensity and turbidity were measured at 0 and 10 min after mixing. And also, for quantitative evaluation of bacterial detection function, the turbidity and fluorescent intensity was measured. The Bt-dpa-HCC/FSiNP, dpa-HCC/FSiNP and B/FSiNP was dispersed in water. The concentration of Bt-dpa-HCC/FSiNP or dpa-HCC/FSiNP solution is 0.25 mg/mL and that of B/FSiNP solution is 0.2 mg/mL. Probe solution (750 μL) and bacterial solution (750 μL) were mixed. The mixture were shaken for 10 min at 2,000 rpm. After shaking, the mixture left to stand for 60 min. The change of turbidity and fluorescence intensity was measured.

3. Results and Discussion

3.1 Modification effect of betaine upon response to bacteria  
To investigate the dispersibility of dpa-HCC/FSiNP and Bt/dpa-HCC/FSiNP in water, the turbidity and fluorescent intensity were measured at 0 and 10 min after mixing (Fig. 1). At 1.0 mg/mL, the amount of precipitated dpa-HCC/FSiNP was larger than that of Bt-dpa-HCC/FSiNP. Since Bt-dpa-HCC/FSiNP showed the similar dispersibility between 0.25 mg/mL and 0.125 g/mL, we considered that Bt-dpa-HCC/FSiNP exhibited well dispersability at 0.125 mg/mL. This result can be explained by positive charge of betaine. Due to that betaine is quaternary amine, Bt-dpa-HCC/FSiNP possesses the fixed positive charge on the surface at any pH conditions. This resulted in the stable dispersibility of Bt-dpa-HCC/FSiNP in water. Based on the positive charge, Bt-dpa-HCC/FSiNP had the hydrophilic surface and showed stable dispersibility.

We also evaluated the bacterial response of Cu-dpa-HCC/FSiNP and Cu-Bt-dpa-HCC/FSiNP probes with fluorescence spectra (Fig. 2). \(F_1\) and \(F\) are the fluorescent intensities at 450 nm before and after the addition of bacteria. The fluorescent intensity of Cu-Bt-dpa-HCC/FSiNP increased by the addition of both S. aureus and E. coli. It has been reported that dipicolylamine fluorescent probe coordinated with Cu\(^{2+}\) and Cu-dpa complex formed coordinate bond with phosphoric acids, and showed fluorescence emission\(^{25}\). This result suggested that Cu-Bt-dpa-HCC/FSiNP and Cu-dpa-HCC/FSiNP was bound with phosphoric acid moiety on bacterial surface and the fluorescent intensity increased.

![Fig. 1](image-url)  
(a) The ratio of \([\text{OD}_{700}(10 \text{ min})]-\text{OD}_{700}(0 \text{ min})]\) to \(\text{OD}_{700}(0 \text{ min})\) in Cu-dpa-HCC/FSiNP or Cu-Bt-dpa-HCC/FSiNP in water. (b) The ratio of \(|F(10 \text{ min})-F(0 \text{ min})|\) to \(F(0 \text{ min})\) in each Cu-dpa-HCC/FSiNP or Cu-Bt-dpa-HCC/FSiNP concentration in water at 25 °C, \(\lambda_{\text{max}} = 443\) nm (\(\lambda_{\text{ex}} = 380\) nm).
The interaction between bacteria (S. aureus and E. coli) and silica nanoparticle was observed by fluorescent microscopy. We also investigated the effect of modifying betaine on dpa-HCC/FSiNP influences the silica nanoparticle’s bacterial detecting function. The fluorescent microscopy images are shown in Fig. 3. Both Cu-Bt-dpa-HCC/FSiNP (Fig. 3 (A), (B)) and Cu-dpa-HCC/FSiNP (Fig. 3 (C), (D)) formed aggregates with bacteria. This result indicated that these probes detected the phosphoric acid groups on bacterial surface. The size and number of aggregates with Cu-Bt-dpa-HCC/FSiNP and bacteria (Fig. 3 (A), (B)) were larger than that of Cu-dpa-HCC/FSiNP (Fig. 3 (C), (D)). By modification of betaine, the nanoparticles’ dispersibility was increased and the frequency of interaction between silica nanoparticles and bacteria was also increased.

3.2 Bacteria response of B/FSiNP

Figure 4 showed the time-dependent changes of turbidity and fluorescent intensity at 593 nm by the recognition of B/FSiNP towards bacteria. The turbidity and fluorescent intensity of B/FSiNP decreased with S. aureus, not with E. coli within 15 min. This result indicated that B/FSiNP and S. aureus formed aggregates and precipitated in water. Phenylboronic acid is known to bind with saccharides and bacteria possess various saccharides on the membrane surface of bacteria. These results suggested that phenylboronic acid on B/FSiNP bound with saccharide chain on the bacterial surfaces.

Fluorescent microscopy images of the bacteria (S. aureus and E. coli) recognition of B/FSiNP were shown in Fig. 5. B/FSiNP formed aggregate with S. aureus, not with E. coli at pH 7.4. This result indicated that phenylboronic acid on silica nanoparticles selectively recognized saccharides on S. aureus.

4. Conclusion

Bt/dpa-HCC/FSiNP and B/FSiNP were synthesized. Bt/dpa-HCC/FSiNP showed a stable dispersibility in water because of the positive charge by betaine on the surface of FSiNP. Cu-Bt/dpa-HCC/FSiNP formed aggregates with both S. aureus and E. coli. However, B/FSiNP formed aggregates with S. aureus selectively. This result was confirmed by observation of microscopy and measurement of fluorescent intensity. Bt/dpa-HCC/FSiNP could be used to
confirm the existence of bacteria in water and B/FSiNP could detect the species of bacteria; *S. aureus* and *E. coli*. These results demonstrated that silica nanoparticles could detect bacteria in water within 10 min, which was well-satisfied of the requirements of the rapid bacteria detection.

**Acknowledgements**

This work was financially supported by a Grant-in-Aid for Scientific Research (C) (Grant No. 15K05548) from Japan Society for the Promotion of Science (JSPS) and a Grant-in-Aid for Scientific Research (A) (Grant No. 26248038) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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