Fluorescent Dye-Doped Silica Nanoparticles with Polyclonal Antibodies for the Rapid Detection of *Salmonella* spp.

Nut Songvorawit[1], Pravate Tuitemwong[2], Krisada Tuchinda[1] and Kooranee Tuitemwong[1*]

[1]Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[2]Risk and Decision Analysis Lab, Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand

**Corresponding author. E-mail: fscikrt@ku.ac.th*

ABSTRACT

This study investigated a novel detection method of Salmonella spp. based on immunofluorescence microscopy using fluorescent dye-doped silica nanoparticles (FDS-NPs). Tetraethyl orthosilicate (TEOS) was used as a precursor. The FDS-NPs produced with microemulsion combined with sol-gel techniques were spherical in shape and 40-70 nm in diameter. The observation of the particles under fluorescence microscope showed high intensity orange color luminescent (Rubpy dye) with high photostability. Surface modifications for bioconjugation with polyclonal antibodies were performed by two methods, amine and carboxyl group modifications. The FDS-NPs were coated with polyclonal antibodies (purified IgGs) against Salmonella spp. Results of bacterial detection by FDS-NPs indicated that nanoparticles with amine modified surface could attach onto the cells of the test organisms, Salmonella weltevreden and S. enteritidis, producing a distinctively bright color under fluorescence microscope. However, the nanoparticles with carboxyl group modification alone did not bind well with the test organisms. The FDS-NPs developed here show a high potential for use in the rapid detection of Salmonella and other bio-molecules.

Keywords: *Salmonella*, Immunofluorescence microscopy, Fluorescent dye-doped silica nanoparticles, Photostability, Bioconjugation

INTRODUCTION

Rapid detection and identification of pathogens are of great importance for medical studies, therapeutic applications, the food industry and biological analyses. Traditional microbiological methods of detecting and enumerating bacteria usually require several days to yield reliable results. In many cases, consumers have already used the products before the analyses are completed. Alternative and rapid assays based on different microbiological methods are constantly being developed (Vanne et al., 1996). The effective testing of bacteria requires methods of analysis that meet a number of challenging criteria. Time and sensitivity of

analysis are the most important limitations related to the usefulness of microbiological testing. The detection method must provide rapid results and be highly sensitive, since the presence of even a single pathogenic organism in the body or food may be an infectious dose (Ivnitski et al., 1999).

Fluorescent labeling probes are among the most popular detection methods. However, traditional fluorophore, such as fluorescein isothiocyanate (FITC), are not photostable, in addition to having relatively low fluorescence intensity (Lian et al., 2004). Fluorescent dye-doped silica nanoparticles (FDS-NPs) have been developed by Santra et al., (2001). The advantages of these particles are the high intensity of the fluorescent signal, high photostability, high solubility, non-toxicity and efficient conjugation with various biomolecules because the silica surface is simple to modify. In this experiment, we synthesized and characterized FDS-NPs for use in rapid detection of *Salmonella* spp.

MATERIALS AND METHODS

Bacteria

The Department of Microbiology, Faculty of Science, Kasetsart University (Bangkok, Thailand) supplied the *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella weltevreden* and *Escherichia coli.* All test bacteria were cultured on nutrient agar (NA) at 37°C for 24 h before preparing the cell suspension in 0.1 M PBS (pH 7.4).

Synthesis of FDS-NPs

The method was modified from that described by Lian et al. (2004). Briefly, cyclohexane (15 ml), *n*-hexanol (3.6 ml), Triton X-100 (3.54 ml), Rubpy dye solution (20 mM, 0.96 ml), TEOS (0.2 ml) and NH_4OH (0.18 ml) were mixed together in a 50 ml flask for 24 h. After the reaction was completed, FDS-NPs were precipitated with acetone. The product was characterized by transmission electron microscope (TEM; Jeol, model JEM-1010, Japan) for morphology. Luminescence properties and photostability were also studied by fluorescence microscope (Olympus, model Cover-Q18, Japan), spectro-fluorometer (Cary Eclipse, Australia) and UV-visible spectrophotometer (Hitachi, model U-188, Japan).

Preparation of purified IgGs

Polyclonal antibodies against *Salmonella* were prepared by immunizing a rabbit with O antigen of *Salmonella anatum* and *Salmonella enteritidis*. The antiserum was obtained from the rabbit after 1-week immunization and was further purified by protein precipitation with ammonium sulfate. The precipitate was dissolved in binding buffer (20 mM PBS, pH 7.0) and filtrated with 0.22 μm pore size membrane before it was subjected into the protein G-sepharose affinity column to obtain only IgGs. The purified IgGs were dialyzed, lyophilized and stored at -20°C until use.

Fabrication of FDS-NPs

The surface of the particles was modified by attachment of carboxyl and amine groups as described by Amnatrungtrakool et al., (2009). Then, the FDS-NPs were washed several times and dispersed in 0.1 M PBS (pH 7.4) containing 1 mg/ml purified IgG against *Salmonella* spp., followed by shaking for 4 h. The product was dispersed in quenching solution. Finally, it was transferred into storage buffer and kept at 4°C until use.

Detection of *Salmonella*

The antibodies coated FDS-NPs were dispersed in 0.1M PBS buffer and then mixed with cell suspension of *Salmonella* (10^4 cfu/ml) at 1:4 volume ratio, incubated at 37°C for 30 min, centrifuged and the supernatant was discarded. The pellet was smeared on a glass slide and observed under a scanning electron microscope (SEM; Hitachi, model S-510, Japan)

RESULTS AND DISCUSSION

Synthesis of FDS-NPs

FDS-NPs were successfully synthesized by modified sol-gel reaction via water using an oil microemulsion method. After 24 h reaction, the mixture changed from clear orange to turbid, indicating the formation of NPs. The resulting dried NPs weighed about 40 mg. The TEM images (Figure 1) showed the FDS-NPs were quite uniform, with a spherical shape ranging from 40-70 nm in diameter. In the synthesis of FDS-NPs, TEOS, as a precursor, reacts with water to form a polymer of SiO_2 or silica by sol-gel process (Santra et al., 2001; Amnatrungtrakool et al., 2009).

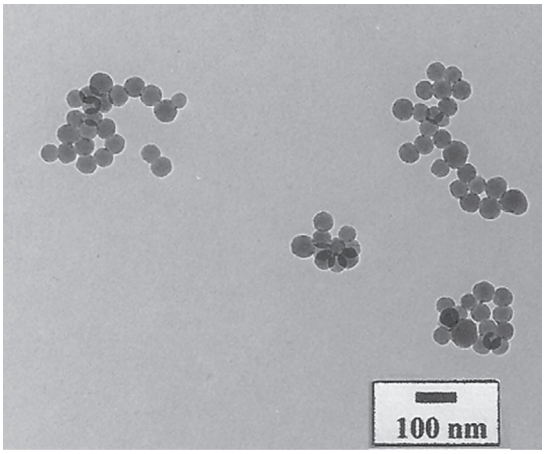

Figure 1. SEM image of FDS-NPs at 48,000x.

A polymerization reaction is initiated by NH_4OH. In water-in-oil microemulsion, the aqueous phase is dispersed as microdroplets surrounded by a monolayer of surfactant molecules (Triton X-100 and *n*-hexanol) in the continuous hydrocarbon phase (cyclohexane). The aqueous cores of microemulsion systems can serve as compartmentalized media for chemical reactions, as a microreactor for the synthesis of NPs (Zhao et al., 2004). In addition, the size of the NPs can be manipulated as needed, by changing the water-to-surfactant molar ratio (Abarkan et al., 2006). While this reaction is proceeding, many Rubpy dye molecules, which are dissolved in the water phase, are trapped inside a silica matrix, giving these particles luminescence properties. However, in this experiment, FDS-NPs formed with aggregation, rather than monodispersion, due to either low speed agitation or insufficient time to disperse the NPs by ultrasonicator.

Luminescence and photostability

This study chose high hydrophilicity Rubpy dye as an inorganic fluorescent dye since it can be dissolved in water and is thus suitable for FDS-NPs synthesis using water-in-oil microemulsion. The study of luminescence properties by spectrofluorometer showed that pure Rubpy had an emission at 606 nm when excited at the 455 nm excitation band maxima in aqueous solution. The excitation and emission spectra remained the same for the pure Rubpy and FDS-NPs in the aqueous solution (Figures 2 and 3).

Observation under florescence microscope showed that FDS-NPs exhibited high intensity, orange-color luminescence (Figure 4) when excited with the blue light of a fluorescence microscope. The FDS-NPs were excited with a 100W mercury lamp for 60 min (Figure 5). After excitation for 60 min, the FDS-NPs showed only a small decrease (2%) in emission intensity. The excellent photostability was due to the large number of dye molecules incorporated inside a very

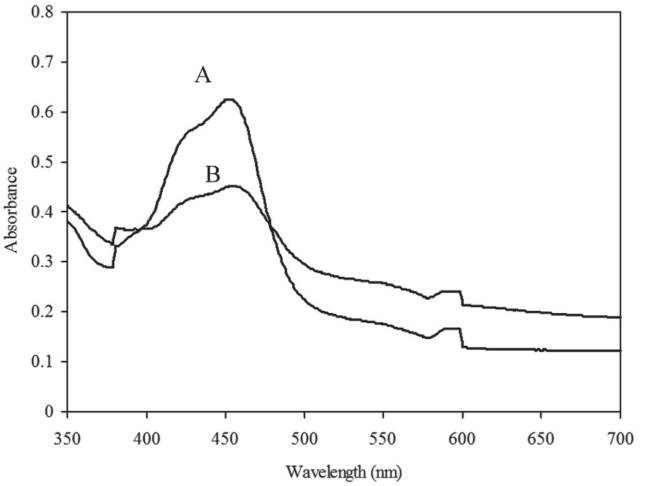

Figure 2. Absorption spectra of (A) FDS-NPs and (B) pure Rubpy dye.

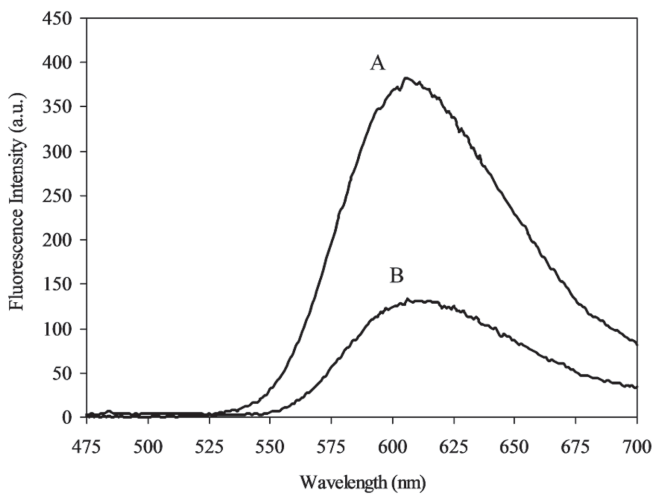

Figure 3. Emission spectra upon excitation at 455 nm of (A) FDS-NPs and (B) pure Rubpy dye.

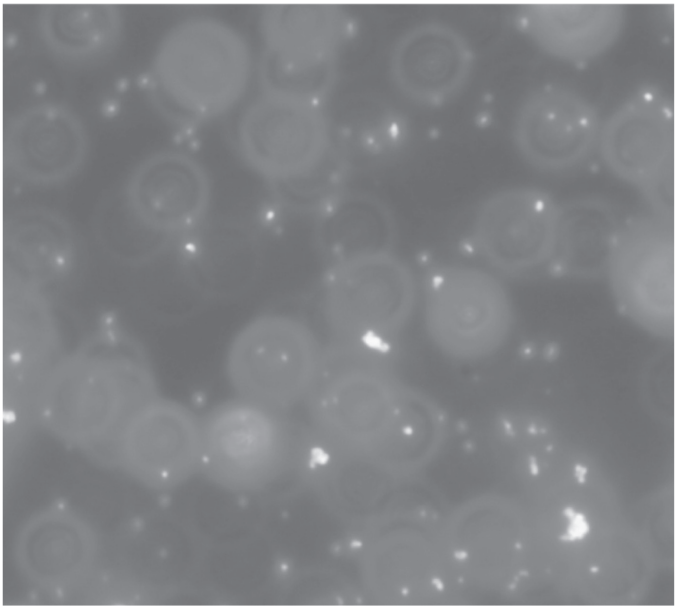

Figure 4. Fluorescence image of FDS-NPs excited with blue light of fluorescence microscope (1000x).

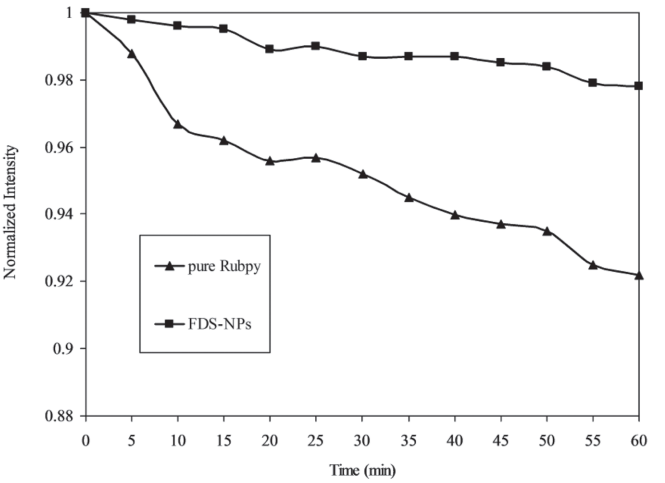

Figure 5. Comparison of photostability of FDS-NPs and Rubpy dye. Liquid solutions were exposed to 100W blue light of mercury lamp.

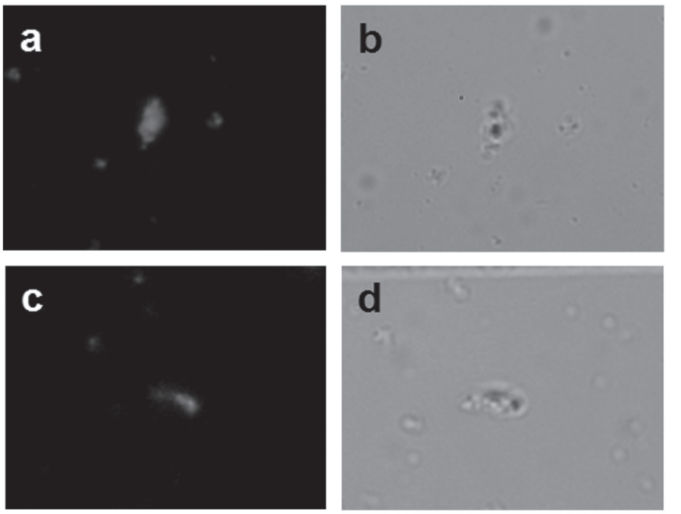

Figure 6. Images of bacterial cells (1000x) after being incubated with FDS-NPs. (a) and (c) are fluorescent images, (b) and (d) are phase contrast images for *S. weltevreden* and *S. enteritidis*, respectively.

small volume silica particle (Yan et al., 2007). The FDS-NPs trapped the dye in the silica matrix, providing protective effects for Rubpy dye. Through this technique, the photobleaching and photodegradation that often affect conventional dyes can be minimized (Amnatrungtrakool et al., 2009).

Attachment of FDS-NPs on *Salmonella* cells

In order to estimate the detection capability of the FDS-NPs, purified IgG against *Salmonella* spp. was tested for specificity against the target bacteria by slide agglutination test. Results indicated that the polyclonal antibody was specific to *S. weltevreden* and *S. enteritidis*, but did not react to *S. typhimurium* and *E. coli.* FDS-NPs with amine-modified surface could detect *S.* Weltevreden and *S.* Enteritidis. Bacterial cells, which were attached by FDS-NPs, exhibited a bright orange spot distinct from the dark background when observed under a fluorescence microscope (Figure 6). In contrast, *S. typhimurium* and *E. coli* were not detected. The results corresponded well to the slide agglutination test, indicating that the efficiency of FDS-NPs depend on a coated antibody on the surface of the particles. However, FDS-NPs with carboxyl group modified surface could not detect any test bacteria, suggesting that there were no antibodies on the surface, which might be due to failed modification.

The time needed for detecting *Salmonella* spp. with FDS-NPs was less than 2 h. The attachment of FDS-NPs on bacterial cells was also confirmed by SEM imaging (Figure 7), which showed bacterial cells attached with a large number of NPs on the surface.

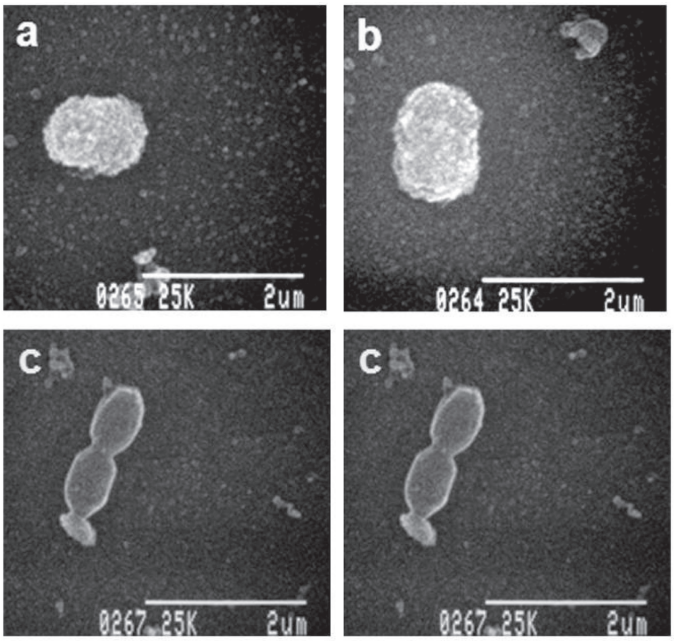

Figure 7. SEM images of bacterial cells after inoculated with FDS-NPs. (a) *S. weltevreden*, (b) *S. enteritidis*, (c) *S. typhimurium* and (d) *E. coli.*

Many previous studies (Santra et al., 2001; Lian et al., 2004; Zhao et al., 2004) have successfully used centrifugation to separate FDS-NPs binding bacteria from free NPs, because the weight of FDS-NPs binding bacteria are higher than that of free NPs or free bacterial cells. In this study, however, some free NPs remained in the pellet after centrifugation. The particles did not create a problem, however, because the light emitted from these particles is not as bright as that swarming around the bacterial cells. A false positive could happen only when FDS-NPs become bigger aggregates.

CONCLUSION

We have developed a new method for the detection of *Salmonella* spp. using FDS-NPs based on immunofluorescence microscopy. The technique provides for rapid detection (within 2 h) of *Salmonella* spp. The accuracy of the method depends on antibodies coated on a silica surface. In addition, this method shows promise in detecting low levels of *Salmonella* spp., due to its ability to amplify light signals in the detection step. This study did not attempt to compare the efficiency of this new technique with conventional methods.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding support from both the National Research Council of Thailand (NRCT) and the Faculty of Science, Kasetsart University.

REFERENCES

Abarkan, I., T. Doussineau, and M. Smaihi. 2006. Tailored macro/microstructural properties of colloidal silica nanoparticles via microemulsion preparation. Polyhedron 25: 1763-1770.

Amnatrungtrakool, S., P. Tuitemwong, and S. Angkaew. 2009. Preparation of bio-conjugated fluorescent nanoparticles for the detection of Salmonella spp., Proceedings of the 47th Kasetsart University Annual Conference. Kasetsart University, Bangkok. Thailand, Mar 17-20, 2009: 420-428.

Ivnitski, D., I. Abdel-Hamid, P. Atanasov, and E. Wilkins. 1999. Biosensors for detection of pathogenic bacteria. Biosens. Bioelectron. 14: 599–624.

Lian, W., S.A. Litherland, H. Badrane, W. Tan, D. Wu, H.V. Baker , P.A. Gulig, D.V. Lim and S. Jin. 2004. Ultrasensitive detection of biomolecules with fluorescent dye-doped nanoparticles. Anal. Bioanal. Chem. 334: 135-144.

Santra, S., P. Zhang, K. Wang, R. Tapec, and W. Tan. 2001. Conjugation of biomolecules with luminophore-doped silica nanoparticles for photostable biomarkers. Anal. Chem. 73: 498-4993.

Vanne, L., M. Karwoski, S. Karppinen, and A.M. Sjoberg. 1996. HACCP-based food quality control and rapid detection methods for microorganisms. Food Control 7: 236-276.

Yan, J., M.C. Estevez, J.E. Smith, K. Wang, X. He, L. Wang, and W. Tan. 2007. Dye-doped nanoparticles for bioananlysis. Nanotoday 2: 44-50.

Zhao, X., L.R. Hilliard, S.J. Mechery, Y. Wang, R.P. Bagwe, S. Jin, and W. Tan. 2004. A rapid bioassay for single bacterial cell quantitation using bioconjugated nanoparticles. Proceedings of the National Academy of Sciences of the United States of America 101: 15027-15032.

none