Preparation of Fluorescent Nanoparticles Based on Natural Silica for Bioimaging

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大腸菌の検出など、生物医学分野のさまざまな検査に用いられる蛍光ナノ粒子。よく用いられるのはシリカ（SiNPs）だが、その精製過程で有毒な廃棄物が出る。そこで本稿では天然シリカを用い、より環境にやさしい精製方法を提案する。

Abstract

Fluorescent nanoparticles are used in a plethora of biomedical applications including bioimaging and tracing of molecules. Hence, these nanoparticles may aid in the development of new disease diagnostics and treatments. One nanostructure of particular importance is silica (SiNPs), due to the range of favorable material properties such as high surface area, ability to bind biomolecules covalently to the surface, biocompatibility, biodegradability and non-toxicity. The current methods for the preparation of SiNPs are not environmentally friendly considering the toxicity of the generated waste. Geothermal silica may overcome these problems as a natural source of silica and offers a more environmentally friendly pathway. Geothermal silica already possess the main intrinsic nano/mesoparticles properties and functional groups such as silica-dioxide on the surface of which allows the capturing of fluorophores inside the nanoparticles both physically and chemically.

This work covers the preparation of SiNPs from natural/geothermal silica, followed by modification with fluorophore, forming the fluorescent SiNPs (FSNP). Characterization of the FSNP include transmission electron microscopy (TEM), Fourier Transform Infra-Red (FT-IR) spectroscopy, and fluorescence spectroscopy. The FSNP was further applied for the detection and bioimaging of bacteria *Escherechia coli* (*E.coli*), an important pathogen indicator, which were monitored by fluorescence spectroscopy and UV visible light.

Keywords fluorescence silica nanoparticles, bioimaging, silica geothermal, biosensing

Introduction

Escherichia coli (*E. coli*) is one of the fecal indicator organisms indicating the potential presence of pathogens in water environment, food products and agriculture products [1]. Monitoring this pathogen is crucial to prevent water related diseases, which is one of the major causes of mortality in the world, especially in the developing countries. According to WHO, diarrheal diseases cause approximately 1.8 million death per year [2]. Therefore, proper sanitation and improved quality of water supplies are important to maintain the public health.

New techniques have been developed for the detection and differentiation of bacteria, including *E. coli*. The plate assay using trioelylglycerol and the fluorescent dye Rhodamine B has been well known to detect bacterial lipases. Upon UV irradiation, orange fluorescent halos around the bacterial colonies are present due to substrate hydrolysis [3]. The so-called pink assay, in which the Rhodamine B showed a high level of pink fluorescence, was also used to detect the presence (or absence) of *E. coli* protein [4]. The utilization of fluorogenic substances for the detection of bacteria has indeed increased the performance of

the detection system to identify specific enzyme and protein activities. However, due to its importance as pathogen indicator in the environment, the detection system for *E. coli* still requires improved accuracy and sensitivity along with rapid sensing times.

In the emerging of nanotechnology, designing and modeling of new materials at the atomic scale are feasible, thus opening up new opportunities for numerous important applications. Nanoparticles offer unique electrical, optic and magnetic properties that are very different compared to its bulk properties [5, 6]. Moreover, these nanostructures have been reported to significantly improve the spectral properties of fluorophores such as the quantum yield, photostability and fluorescence lifetime [7, 8]. One nanostructure of particular importance is silica (SiNPs), due to the range of favorable material properties such as high surface area, ability to bind biomolecules covalently to the surface, biocompatibility, biodegradability and non-toxicity [9, 10].

Silica-based fluorescent nanoparticles are used in a plethora of biomedical applications ranging from bioimaging, molecule tracings, biosensors to drug delivery systems [11, 12]. Over the past decade, such fluorescent nanoparticles have been intensively studied and often offered remarkable sensitivity in detecting fluorescence signals due to the improved signal-to-noise ratio [11, 13, 14]. In addition, silica-based fluorescent nanomaterials have been reported to increase the photostability and fluorescence lifetime of the incorporated organic fluorophores [12, 15].

Silica is abundantly available in nature, one of which is through the waste of geothermal power plants. However, silica scaling in these power plants causes the production capacity to decrease by 40%. Recovering the "amorphous" silica from geothermal installation with total amount of about 3000 ton/year will indeed provide benefits to the power plants, as these geothermal silica can be utilized as building blocks for silica based nanomaterials [16]. The current method for the preparation of SiNPs often applies silica precursors or co-precursors which are expensive and requires the use of organic solvents and strong acids during the preparation process. In addition, this preparation process is not environmentally friendly considering the toxicity of the generated waste. Geothermal silica may overcome these problems as it already possess the main intrinsic properties of silica nano/mesoparticles such as having large surface area and functional groups such as silica-dioxide on the surface.

The purpose of this work is to develop and synthesize fluorescent silica nanoparticles (FSNP) based on geothermal silica in which the dimensions, and optical properties of the synthesized nanoparticles can be finely tuned and adjusted. The fluorescence nanoparticle is then applied as an optical biosensor to detect *E. coli*.

To the best of our knowledge, the preparation of fluorescent silica nanoparticles from geothermal waste, in particular for biomedical purposes, has not been reported elsewhere. Hence, this approach will give added value to the geothermal silica as advanced functional nanostructures. In addition, this work will not only give advantage of the usage of natural Indonesian resources, but the outcome will also serve as a basis for future development of natural silica as nanostructured materials in other more sophisticated biomedical applications.

Experimental

Materials

Geothermal sludge, as the source of silica, was collected from PLTP Geodipa Dieng, Central Java, Indonesia. Sodium hydroxide (NaOH) was purchased from Merck Chemicals. Hydrochloric acid (HCl) 37% was an analytical grade from Merck Chemicals. Rhodamine-6G was purchased from Sigma-Aldrich. All chemicals were used without further purification. Deionized water was used for all synthesis and sensing experiments.

Synthesis of Fluorescent Silica Nanoparticles

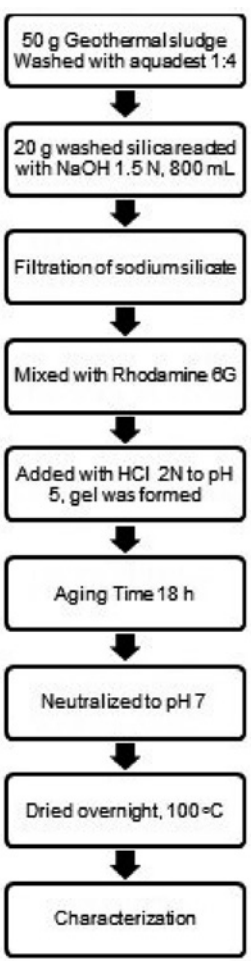
As shown in Scheme 1, a total of 20 g of washed silica powder was mixed with 800 ml of 1.5 N sodium hydroxide (NaOH) in a cup glass to form sodium silicate (Na_2SiO_3). The mixture was then stirred using a magnetic stirrer with heating constantly maintained at 90ºC within 60 minutes. Subsequently, the mixture was filtered through filter paper to separate the solution with solids. The sodium silicate solution was added with 0.05 g of Rhodamine-6G and stirred until homogeneous, then titrated with 2N HCl to form the gel and then allowed to stand for 18 hours. The gel formed was filtered with filter paper and washed with aquadest until pH 7. The neutralized gel was dried in an oven at 100 ºC, overnight. The resulting fluorescent solid samples were identified as the fluorescent silica nanoparticles (FSNP).

Characterization of Fluorescent Silica Nanoparticles (FSNP)

Transmission Electron Microscopy (TEM) images were obtained with TEM Tecnai G-20 S-Twin (FEI, USA) scanning TEM instrument (200 kV accelerating voltage) equipped with Tungsten cathode and an Eagle CCD camera. For TEM measurement, a suitable amount of ethanolic solution of FSNP was dropped onto a porous carbon film on a copper grid and then dried in vacuum.

Fourier Infrared Spectroscopy (FTIR) spectra were recorded on a FTIR Prestige-21 (Shimatzu, Japan), in transmittance mode, at 16 cm^{-1} resolution, over the range of 300-400 cm^{-1} with an accumulating average of 10 scans. The software used to generate the spectra was IR Solution (Shimatzu).

Emission spectrum of FSNP was recorded on Varioskan Flash (Thermo Scientific) at excitation wavelength of 553 nm.



Scheme 1. Flowchart of the preparation of FSNP from geothermal silica

E. coli Sensing Experiments

E. coli bacterial culture and preparation

E. coli InaCC-B5 bacterial culture was provided from the Research Centre for Chemistry-LIPI, Indonesia. Nutrient agar (NA) was prepared by dissolving nutrient agar powder in aquadest, continued with constant heating until dissolved completely. An amount of 4 ml of dissolved NA was sterilized in an autoclave for 15 min at 121 °C. The sterilized NA was allowed to settle and subsequently 1 one stock of *E. coli* bacteria was incubated for 24 h at 37 °C. The cultured E.coli was diluted with sterilized water for further sensing experiments.

E. coli sensing with FSNP

A total of 20 µl of FSNP and 180 µl of *E. coli* suspension were added into a microplate. The microplate was covered in aluminium foil to avoid exposure of light and the mixture was allowed to react for 4 h. A control positive was prepared by adding 20 µl of FSNP and 180 µl of sterilized water in a microplate, and treated the same way as previous. The fluorescence intensity of both samples were measured using Fluorescence Spectral Scanning Multimode Reader (Thermo Scientific Varioskan Flash) with excitation wavelength of 553 nm and emission wavelength of 580 nm.

Bioimaging test

E. coli was cultured on NA in a microplate following the same procedure as previous. A total of 2 ml of FSNP was added on the *E. coli* culture and the mixture was exposed under UV irradiation.

Results and Discussion

Fabrication and Characterization of the FSNP

The luminescent nanoparticles investigated in this study was derived from amorphous geothermal silica. The silica nanoparticles were fabricated using the common approach of sol-gel process using silica obtained from the side product of geothermal plant [17] as precursor, which until now has not been reported elsewhere. Rhodamine-6G was added to the silica nanoparticle as fluorophore producing FSNP. The FSNP obtained from this process had an irregular structure with a particle diameter about 10-20 nm, which was measured by means of

TEM (see Figure 1).

The surface chemistry of the FSNP samples were characterized by Fourier Transform Infrared Spectroscopy (FTIR) (Figure 2). Figure 2 presents the FTIR spectra of the raw geothermal silica (black trace) and FSNP (blue trace). The raw geothermal silica has a broad band at around 1000 – 1300 cm^{-1} with the peak at 1070 cm^{-1} which was assigned to Si-O-Si asymmetric stretching vibrations. A small band at 940 cm^{-1} and 800 cm^{-1} were attributed to $Si\text{-}O^-$ stretching vibrations or silanol group due to alkali silica glasses and Si-O-Si symmetric stretching vibration.

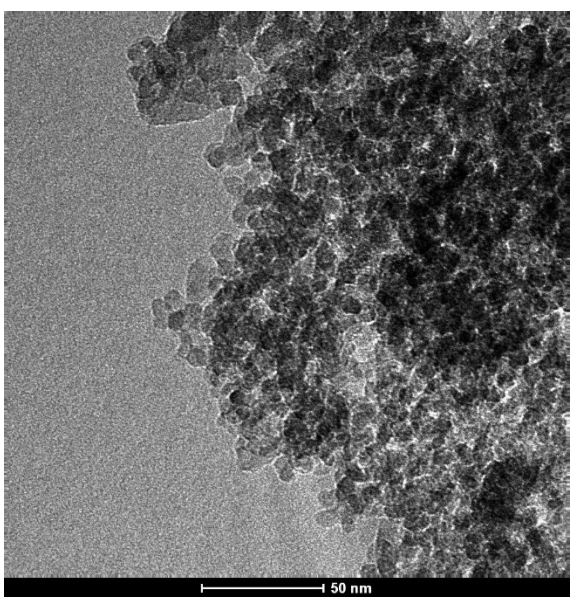


Fig. 1. Representative TEM images showing the nanostructures of FSNP

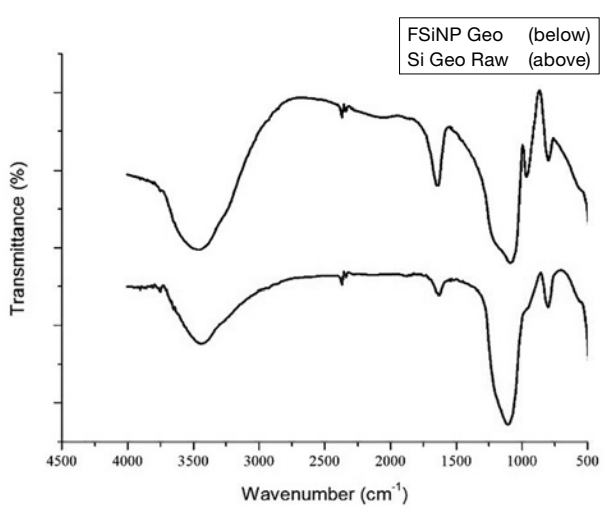


Fig. 2. FTIR spectra of silica geothermal as the precursor (above) and after modification into Si nanoparticles with Rhodamine-6G, FSNP (below).

The band at 1070 cm^{-1} and at 800 cm^{-1} are common bands appear to all silicates with tetrahedrally coordinated silicon. The band at 1650 cm^{-1} was assigned to bending vibration of H-OH bonds, while the broad band around 3400 – 3650 cm^{-1} correspond to stretching vibration of –OH bonds [18, 19].

After modification with Rhodamine-6G forming the FSNP, the FTIR spectra (below) showing some changes in intensity for some of the adsorption bands but there is no additional band confirming the fluorescent agent (Rhodamine-6G) was not chemically bound to the silica geothermal nanoparticles but only physically entrapped in the pores of the particles.

The emission spectrum of FSNP was recorded at excitation wavelength of 553 nm, which is the excitation wavelength of the dye Rhodamine-6G. As shown in Figure 3, the maximum emission peak was observed at 580 nm. This wavelength will further be used for to observe the detection performance of FSNP towards *E. coli*.

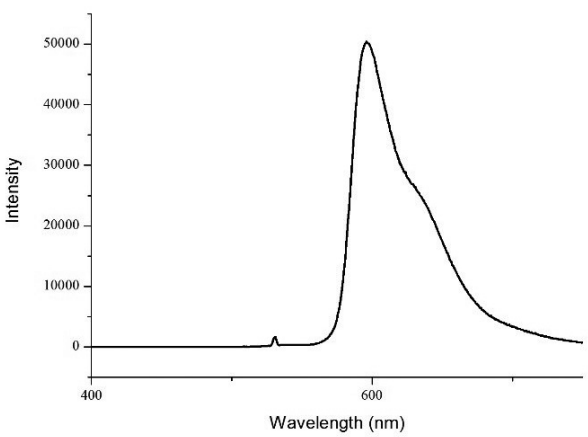


Fig. 3. Emission spectrum of FSNP at excitation wavelength 553 nm

Sensing Experiments of the FSNP for the Detection of E. coli

The FSNP was then used to detect *E. coli* in the culture media. The presence of *E. coli* protein will quench the fluorescence emission of Rhodamine-6G as the biorecognition compound. Therefore during sensing, the fluorescence intensity of the FSNP was measured using fluorescence spectroscopy before and after added to the culture media containing *E. coli*. The concentration of the FSNP was varied from $1x10^{-1}$ mg/mL down to $1.95x10^{-4}$ mg/mL. During sensing, the fluorescence emission

signals were decreased after the addition of FSNP to the culture media containing *E. coli* in each different FSNP concentration tested, indicating the quenching of fluorescence emission due to the presence of *E. coli*. The loss of the fluorescence intensity signal in different concentration of the FSNP is summarized in Table 1.

Table 1. The fluorescence intensity loss after FSNP added to the culture media containing *E. coli* for each FSNP concentration tested in the range of $1x10^{-1}$ mg/mL to $1.95x10^{-4}$ mg/mL.

FSNP concentration (mg/mL)	Fluorescence intensity loss (%)
1×10^{-1}	26.11
5×10^{-2}	30.64
2.5×10^{-2}	47.87
1.25×10^{-2}	18.44
6.25×10^{-3}	27.37
3.12×10^{-3}	5.02
1.56×10^{-3}	57.02
7.81×10^{-4}	59.76
3.9×10^{-4}	74.51
1.95×10^{-4}	74.70

From Table 1, it can be seen that the percentage of fluorescence signal loss increases gradually with the decrease concentration of the FSNP until the FSNP concentration of $6.25x10^{-3}$ mg/mL. Lower than that concentration, the percentage of fluorescence signal loss was decreased (at the concentration of $3.12x10^{-3}$ mg/mL) and then increased significantly at the concentration lower than $3.12x10^{-3}$ mg/mL. It suggests the lowest working concentration of the FSNP for *E. coli* detection was $6.25x10^{-3}$ mg/mL.

The next experiment was to run the control experiment using Rhodamine-6G without any silica nanoparticle as negative control to investigate whether the fluorescence signal loss was due to the presence of *E. coli* or not. The concentration of Rhodamine-6G used in this experiment was lower than the concentration of FSNP used in the previous experiments (Table 1). This Rhodamine-6G concentration was equal to the concentration of Rhodamine-6G used to modify the geothermal silica nanoparticle thus the fluorescence signals emitted from the control (Rhodamine-6G only) and the FSNP are comparable. In this experiment, the fluorescence was emitted at 627 nm as the Rhodamine-6G emission, which is higher than the emission signal Rhodamine-6G embedded in the geothermal silica nanoparticles.

The fluorescence intensities, before and after interaction with *E. coli*, did not show any particular trend with the percentage of fluorescence signal loss, as presented in Table 2. It confirms that the fluorescence signals emitted by Rhodamine-6G itself is not stable at very low concentrations of the dye. This result corroborates that the decreased fluorescence intensity in the previous experiments (Table 1) was due to the quenching of the fluorescence signal from Rhodamine-6G entrapped in the geothermal silica nanoparticles in the presence of *E-coli*. This result also shows that the FSNP has increased photostability compared to that of the Rhodamine-6G, at the same amount of concentration of the dye.

Table 2. The fluorescence intensity loss after Rhodamine-6G added to the culture media containing *E. coli* for Rhodamine-6G concentration tested in the range of $6.25x10^{-4}$ mg/mL to $1.22x10^{-6}$ mg/mL.

FSNP concentration (mg/mL)	Fluorescence intensity loss (%)
6.25×10^{-4}	64.15
3.13×10^{-4}	-68.82
1.56×10^{-4}	90.71
7.81×10^{-5}	74.53
3.91×10^{-5}	63.02
1.95×10^{-5}	36.16
9.77×10^{-6}	-7.18
4.88×10^{-6}	-84.24
2.44×10^{-6}	-220.58
1.22×10^{-6}	-284.12

Bioimaging of E.coli using FSNP

E. coli detection using the FSNP was also observed under UV light to see the fluorescence during sensing (Figure 4). In Figure 4, it can be seen that, the FSNP itself emitted an intense orange fluorescence (a) and once *E. coli* introduce to the petri dish, the colour was changed into light orange (b) indicating the fluorescence intensity was decreased due to the presence *E. coli*. It corroborates that *E. coli* protein has ability to quench the fluorescence emission of the FSNP. Orange fluorescent halos around the *E. coli* colonies were also observed, proving that FSNP may be applied for bioimaging purposes.

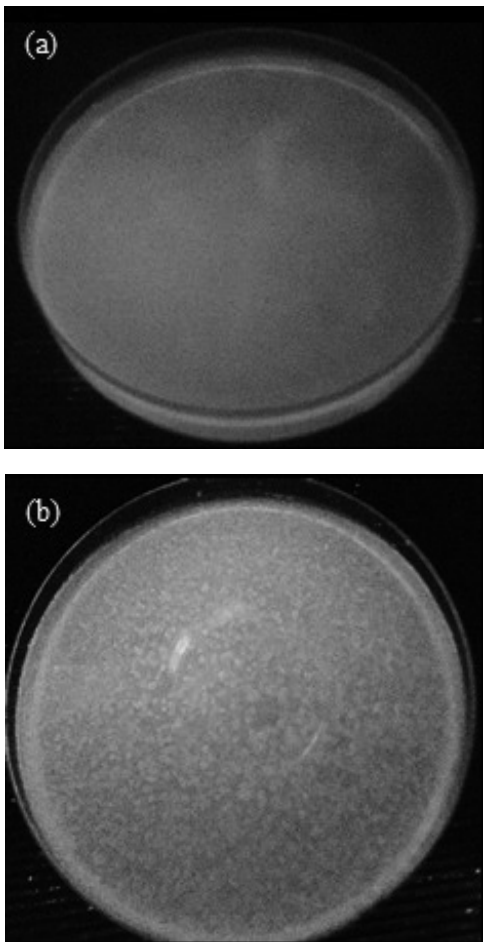


Fig.4. Fluorescence emission of the FSNP (a) and FSNP + *E.coli* (b)

Conclusion

This work presents for the first time the detection of *E. coli* using fluorescent silica nanoparticles (FSNP). The FSNP was derived from amorphous silica geothermal waste and modified with the dye Rhodamine-6G. The FSNP interacted with the protein of the *E. coli*, resulting in quenching or decrease of emission intensity of the FSNP. Furthermore, we observed the detection of *E. coli* under UV light, resulting in decrease of intensity compared to the FSNP in the absence of *E. coli*. Orange fluorescent halos around the *E. coli* colonies were also observed, proving that FSNP may be applied for bioimaging purposes. Continued studies are currently being conducted in our laboratory to optimize the properties of FSNP as well as its ability for bioimaging and detection systems.

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