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Research Article

Antibody-Conjugated Rubpy Dye-Doped Silica Nanoparticles as Signal Amplification for Microscopic Detection of *Vibrio cholerae* O1

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This study demonstrated the potential application of antibody-conjugated Rubpy dye-doped silica nanoparticles for immunofluorescence microscopic detection of *Vibrio cholerae* O1. The particle synthesis of 20X of the original ratio was accomplished yielding spherical nanoparticles with an average size of 45 ± 3 nm. The nanoparticles were carboxyl functionalized and then conjugated with either monoclonal antibody or polyclonal antibody against *V. cholerae* O1. The antibody-conjugated nanoparticles were tested with two target bacteria and three challenge strains. The result showed that monoclonal antibody-conjugated Rubpy dye-doped silica nanoparticles could be effectively used as signal amplification to detect *V. cholerae* O1 under a fluorescence microscope. Their extremely strong fluorescence signal also enables the detection of a single cell bacterium.

1. Introduction

The standard or conventional methods for microbial detection are the cultural methods which primarily rely on the preenrichment and specific enrichment steps to enumerate the organisms that may occur in low numbers in the sample, followed by the isolation of the bacteria in solid media and a final confirmation by biochemical identification and/or serological tests. These methods are considered time consuming and labor intensive. In many cases, rapid methods to identify the pathogens with high accurately and sensitivity are very crucial. For example, in clinical diagnostics and veterinary, early detection of trace amount of the infectious pathogens can prevent epidemics and loss of lives [1]. In food industry, as contamination can occur in many steps throughout the whole process, the fast detection of the target microorganisms provides better control over the manufacturing process, offers prompt administration as preventive measures, and allows for earlier release of the product. Rapid microbiology methods have long been practiced including PCR [2–4], ELISA [5, 6], immunosensor [7], biosensors [8, 9], and immunochromatographic test strips [10–12].

Recently, fluorescent dye-doped silica nanoparticles (FDSNPs) have been developed and applied for the detection of undesirable matter such as bacteria and toxin [13–17]. These fluorescent nanoparticles are of high interest in ultrasensitive bioassays due to many reasons. Firstly, each particle encapsulates thousands of fluorescent dye molecules in a silica matrix providing stronger fluorescence intensity and superior photostability than single dye itself [18, 19]. Secondly, the silica surface can be easily modified with different types of functional groups such as hydroxyl, amino, and carboxyl groups which can be used to incorporate with a variety of biomolecules as needed [19–21]. Moreover, because

of the transparency of silica to visible light, the use of silica NPs is interesting in bioassays with optical detection [22].

Most applications of FDSNPs as signal reporter for bacteria or toxin determination in a sample requires specific equipments such as plate reader fluorometer [13], confocal microscope [16], and flow cytometer [23] to detect or monitor the fluorescence signals. Compared to other instruments, a fluorescence microscope is relatively cheaper. Besides, the technique for sample preparation for microscopic detection was common and easier to perform than other optical measurement.

Although a method of labeling organisms with conventional fluorescent dyes and examining with a specially designed fluorescent microscope has long been practiced for the detection and identification of microorganisms, we expected that using FDSNPs to label the target bacteria in this study would offer more advantage because they provide stronger signal intensity and higher photostability.

In the present work, we observed the result of mass synthesis of FDSNPs and used them as signal amplification for microscopic detection of target bacteria. Since the target bacteria were bound to signal reporter via antigen-antibody reaction, *Vibrio cholerae* O1 was chosen as model analyte due to the available antibodies in the experiments. This bacterium is a causative agent of cholera which is a food and waterborne gastroenteric infection and remains a significant threat to public health in the developing countries [24]. A rapid method which can screen and detect this infection is still required.

2. Methodology

2.1. Materials. Tetraethylorthosilicate (TEOS), Tris(2,2′-bipyridyl) dichlororuthenium (II) hexahydrate (Rubpy dye), ammonium hydroxide (NH_4OH), trimethoxysilyl-propyl-diethylene triamine (DETA), succinic anhydride, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Z-morpholinoethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Merck. Cyclohexane, n-hexanol, N, N-dimethylformamide (DMF), Triton X-100, and all other chemicals of analytical reagent grade were obtained from either Fisher Scientific or a local company. Polyclonal rabbit anti-*V. cholerae* O1 antibody and monoclonal antibody specific to *Vibrio cholerae* O1 were obtained from the National Institute of Health, Department of Medical Science, Thailand.

2.2. Synthesis and Characterization of Fluorescent Dye-Doped Silica Nanoparticles. Fluorescent dye-doped silica nanoparticles (FDSNPs) in this study were prepared using a water-in-oil microemulsion technique. The Rubpy dye was chosen as fluorescence dye encapsulated inside the silica matrix because it emits bright orange fluorescence which is attractive to the naked eye. This would be suitable for further application as signaling marker for microscopic detection. In this study, the synthesis was at 20 times of the original ratio as described by Lian et al. [25] with a slight modification to investigate the effect of mass production on the shape and size of the NPs.

Briefly, 150 mL cyclohexane, 35.4 mL Triton X-100 and 36 mL n-hexanol as oil phase, surfactant, and cosurfactant, respectively, were mixed together followed by 9.6 mL Rubpy dye solution (20 mM in deionized water) as water phase to form water-in-oil microemulsion. After that, 2 mL TEOS was added as silica precursor and 1.2 mL NH_4OH was added to initiate the polymerization. The mixture was mixed together in a flask wrapped in aluminum foil to prevent photobleaching for 24 h. The precipitation was obtained by adding acetone and collected by centrifugation. The FDSNPs were washed with ethanol and deionized water several times to remove surfactant and unabsorbed fluorescent dye from the particles' surface. The particles were air dried and weighed for yield.

To characterize shape and size of the nanoparticles, samples of FDSNPs in aqueous solution were imaged under a transmission electron microscope (TEM) (TECNAI 20 TWIN, USA). Approximately 100 particles on the pictures derived from TEM were randomly measured for diameter using program ImageJ.

For their optical properties, the FDSNPs in aqueous solution were analyzed for emission spectra and excitation spectra using a spectrofluorometer (Hitachi Model F2500, Japan).

2.3. Surface Modification and Antibody Conjugation. Before conjugating with antibody specific for the target microorganism, the surfaces of FDSNPs were needed to be chemically modified. In this study, the carboxyl groups were formed onto the nanoparticle surfaces using the method as described by Zhao et al. [13] with some modification. Firstly, FDSNPs were continuously stirred in 1% DETA in 1 mM acetic acid for 30 min at room temperature. After centrifugation and thoroughly washing for several times, the sample was mixed with 10% succinic anhydride in DMF solution under N_2 gas for 6 h with continuous stirring. The carboxyl-functionalized fluorescent silica nanoparticles were washed several times with deionized water before the next step.

To conjugate antibodies onto the particle surfaces, the particles reacted with 1% EDC and 1% NHS in MES buffer (0.1 M, pH 5.5) for 30 min with continuous shaking. After that the particles were washed with deionized water and resuspended in PBS (0.01 M, pH 7.4). Then, the suspension was mixed with purified antibody and allowed to react at room temperature for 4 hours with gentle shaking. The antibody-conjugated fluorescent dye-doped silica nanoparticles (Ab-FDSNPs) was collected, washed, and blocked with 1% BSA for 30 min. The Ab-FDSNPs were washed and kept in PBS containing 0.1% BSA and 0.01% NaN_3 at 4°C until use.

In this work, two antibodies specific to *Vibrio cholerae* O1 (monoclonal antibody and polyclonal antibody) were studied. To confirm the achievement of the antibody conjugation step, the *V. cholerae* O1 incubated with Ab-FDSNPs was imaged by a scanning electron microscope (SEM) (HITACHI SEM S-2500, Japan).

2.4. Microscopic Detection of Vibrio cholerae O1 with Antibody-Conjugated Fluorescent Dye-Doped Silica Nanoparticles. *V. cholerae* O1 serotypes Inaba and Ogawa were used as target bacteria while three enteric bacteria (*Campylobacter jejuni*,

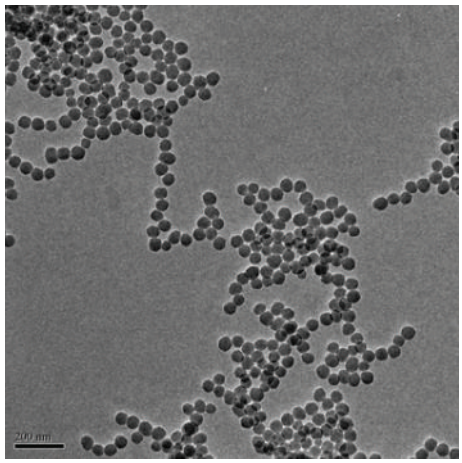


Figure 1: Transmission electron microscope images of FDSNPs at 11,500x magnification.

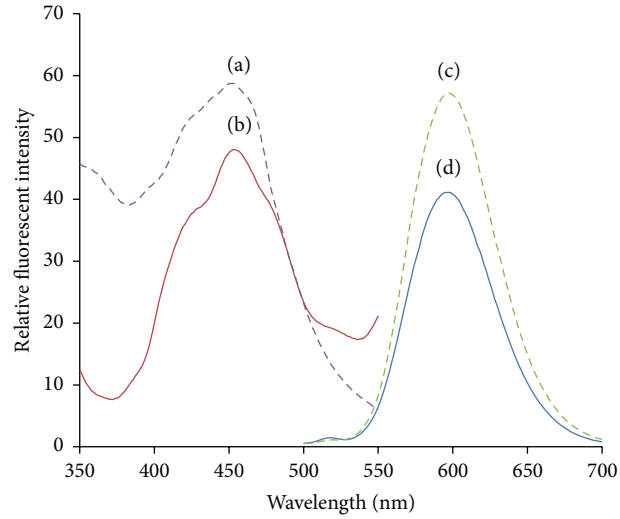


Figure 2: Fluorescence excitation (left panel) and emission spectra (right panel) of the FDSNPs ((a) and (c)) and Rubpy dye ((b) and (d)).

Escherichia coli, and *Salmonella* Typhimurium) were used as challenge microorganisms to study the specificity of the antibody-conjugated fluorescent dye-doped silica nanoparticles.

V. cholerae O1 was cultured in TSB with 1% NaCl at 37°C for 18 h. Other test bacteria were grown in TSB at the same condition. For each strain, the cell density of about 10^4 CFU/mL corresponding to McFarland standard was prepared. Then 1 mL of the cell suspension was incubated with 10 μL of 1 mg/mL Ab-FDSNPs for 5 min at room temperature. A loop of the sample was placed on a slide and covered with a coverslip. The sample was detected under a fluorescence microscope (Zeiss Primo Star iLED) at the excitation wavelength of 455 nm. The fluorescence images were recorded using a digital camera (Cannon EOS 550D).

3. Results and Discussion

3.1. Synthesis and Characterization of Fluorescent Dye-Doped Silica Nanoparticles. In this study, the silica nanoparticles were synthesized using a water-in-oil microemulsion method. The growth of silica nanoparticles takes place in water nanodroplets in the dispersing phase. The silica precursor (TEOS) undergoes hydrolysis and polycondensation reactions resulting in the formation of monodisperse spherical silica particles [22, 25]. The Rubpy dye which previously dissolved in water was encapsulated inside a silica network and consequently formed fluorescent dye-doped silica nanoparticles (FDSNPs). After centrifugal collecting, washing, and drying, the synthesis yielded small amount of fine reddish brown powder.

In our preliminary studies, the silica nanoparticles were synthesized using the same ratio as original ratio [25]. Ten batches were produced with an average yield/batch of 24 ± 5 mg. This amount was compared very well to the typical yield of about 20 mg/batch reported by Lian et al. [25]. The results from transmission electron microscope images show that FDSNPs have a spherical shape (Figure 1). However, the average diameters of the particles were moderately uniform within the same batch but slightly different among the batches (from 29 ± 5 nm to 43 ± 7 nm).

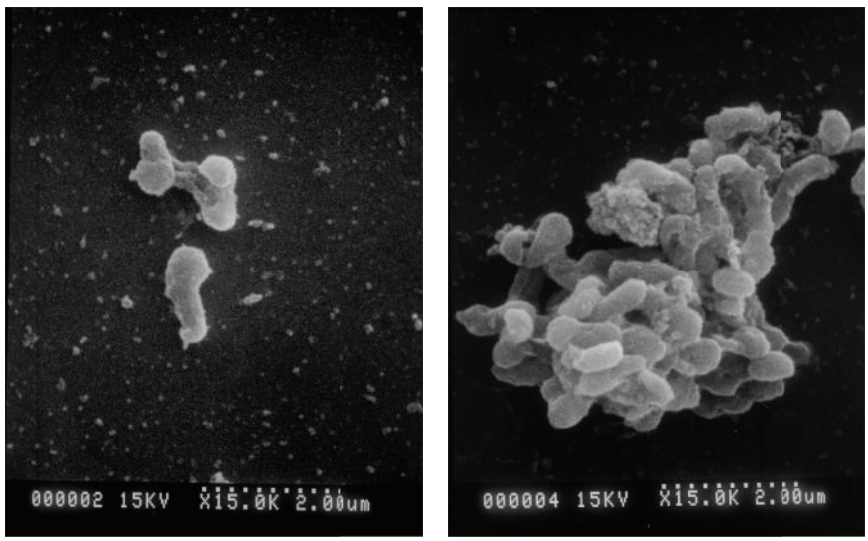
Table 1: Average particle size of fluorescent dye-doped silica nanoparticles in other studies synthesized using the same chemical ratio.

Researchers	Average size (nm)
Zhao et al. [13]	60 ± 4
Hun and Zhang [14]	47 ± 5
Santra et al. [19]	63 ± 4
Lian et al. [25]	70

As compared to particle sizes reported by other researchers who used the same chemical ratio of FDSNP synthesis in Table 1, our nanoparticles were relatively smaller than those of the three studies but close to those of Hun and Zhang [14].

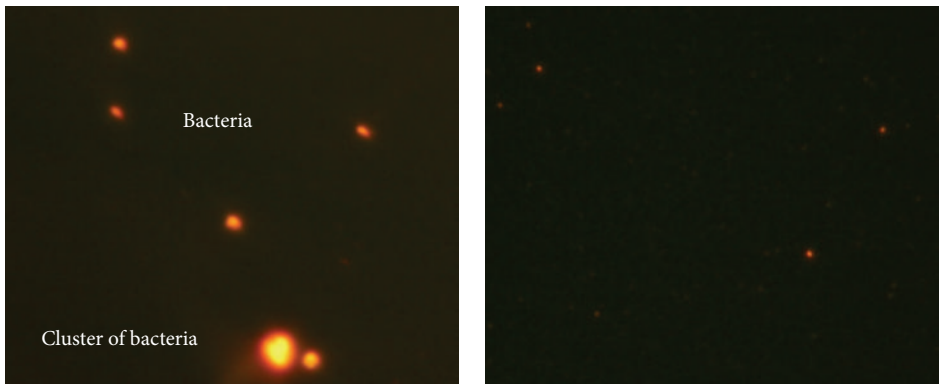
The reason for the size variation might be contributed to the differences in synthesis techniques and equipments such as time interval between microemulsion preparation and addition of silica precursor and ammonium hydroxide, mixing speed, and size of magnetic stirrer including size of flask or container. To some extent, these factors had an effect on the size of water droplets in the microemulsion. Since the silane hydrolysis and the formation of silica particles are restricted in the water phase, the diameter of the prepared particles is also affected by the size of the water cores in the system [26]. Therefore, to eliminate particle size variation caused by different synthesis batch, the fluorescent nanoparticles were produced with a proportion of 20 times of the original ratio. It was found that the FDSNPs from this batch were spherical with an average size of 45 ± 3 nm. This was a proof that a synthesis at 20X ratio did not affect the shape and size of the FDSNPs.

The excitation and the emission spectra of the FDSNPs were characterized and compared with those of the pure



(a) (b)

Figure 3: SEM images of (a) comma-shaped *V. cholerae* O1 coated with Ab-FDSNPs and (b) a cluster of *V. cholerae* O1 bound to Ab-FDSNPs.



(a) (b)

Figure 4: Fluorescence images of (a) *V. cholerae* O1 bound to Ab-FDSNPs and (b) Ab-FDSNPs without bacteria.

Rubpy dye. Both substances were prepared in aqueous solution for the spectrofluorometric measurement. The results show that pure Rubpy dye exhibited an emission at 595 nm when excited at 453 nm. The excitation spectrum of FDSNPs was not significantly different from that of the pure dye, but the maximum emission band of FDSNPs was slightly shifted to 598 nm (Figure 2). These findings indicated that the spectral characterization of the Rubpy dye did not change when it was surrounded by silica matrix.

3.2. Surface Modification and Antibody Conjugation. The achievement of surface modification antibody conjugation steps was confirmed via a SEM image of *V. cholerae* O1 bound to Ab-FDSNPs. Figure 3(a) shows that the antibody was successfully conjugated onto the nanoparticles as there were a number of tiny spherical particles of FDSNPs coating cell surface of each single bacterium like a breaded shrimp.

3.3. Microscopic Detection of Target Bacteria. In this experiment, when a sample of bacteria incubated with Ab-FDSNPs was being investigated under a fluorescence microscope, there were some orange rods or dots blinking here and there as the bacteria labeled with FDSNPs were still alive and actively moving directionless (Figure 4(a)). Occasionally, few bright orange chunks were found. These chunks resulted from clusters of bacteria bound to Ab-FDSNPs (Figure 3(b)).

On the contrary, when a sample of free nanoparticles without bacteria was investigated, it was very difficult to focus since the FDSNPs were too small. However, sometimes very tiny orange dots were found (Figure 4(b)). These visible bright dots were assumed to be some self-aggregation of Ab-FDSNPs for two reasons. First, they were smaller than a single bacterium coated with Ab-FDSNPs. Second, they either stayed still or were floating in the same direction whilst the fluid flew under the cover glass unlike the movement of live bacteria.

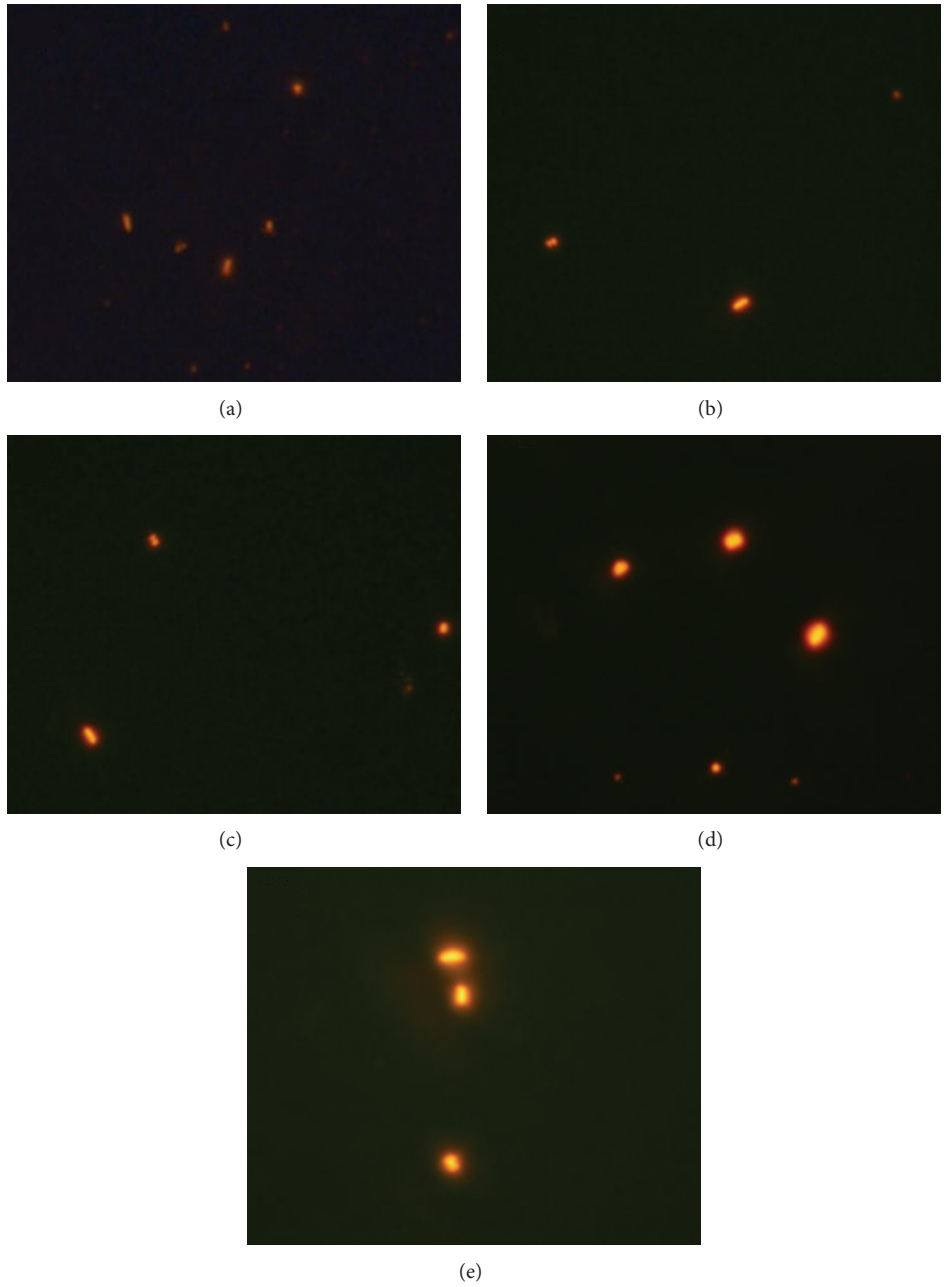


Figure 5: Fluorescence images of test bacteria incubated with PAb-FDSNPs (a) *Vibrio cholerae* O1 serotype Inaba; (b) *Vibrio cholerae* O1 serotype Ogawa; (c) *Campylobacter jejuni*; (d) *Escherichia coli*; (e) *Salmonella* Typhimurium.

Figures 5 and 6 show fluorescence images of test bacteria incubated with either PAb-FDSNPs or MAb-FDSNPs. It can be seen that PAb-FDSNPs could bind to all strains while MAb-FDSNPs bind to only *V. cholerae* O1. These findings indicated that the PAb against *V. cholerae* O1 in this experiment could cross react with other enteric bacteria which are nontarget bacteria. Normally, the polyclonal antibody can recognize several epitopes on the target protein while monoclonal antibody reacts only with single epitope. That is why the polyclonal antibody has a higher tendency for cross-reaction as compared to monoclonal antibody. Therefore, for the application of Ab-FDSNPs as signal amplification to detect *V. cholerae* O1 under a fluorescence microscope, MAb-FDSNPs developed in this study could be effectively employed while PAb-FDSNPs were not appropriate since cross reaction to nontarget bacteria is an undesired result.

Based on extremely strong fluorescence signal of these particles, a capability to detect a single bacterium can be achieved with these nanoprobes.

4. Conclusions

Fluorescent dye-doped silica nanoparticles have shown a potential application as strong intense signaling marker for

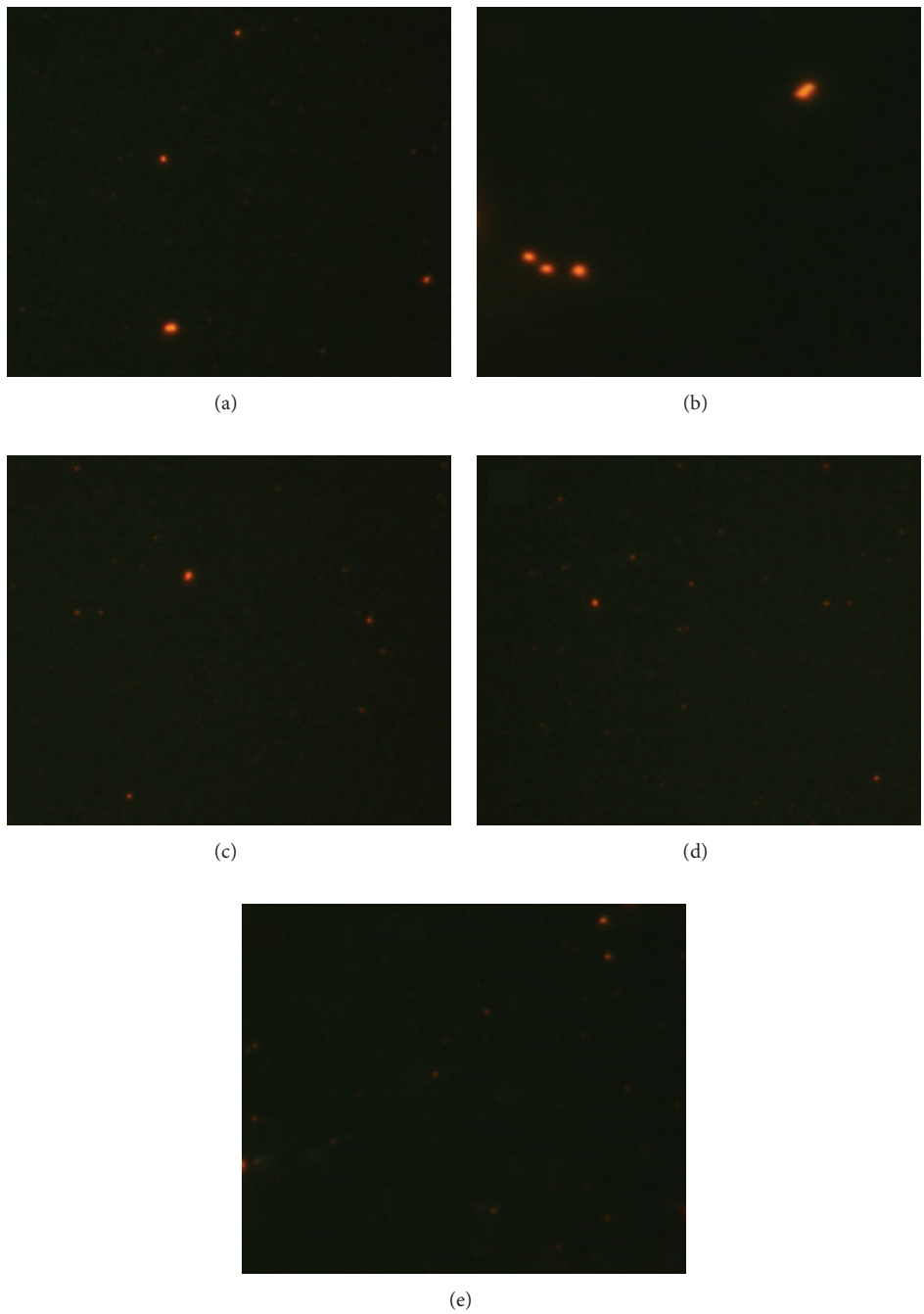


Figure 6: Fluorescence images of test bacteria incubated with MAb-FDSNPs (a) *Vibrio cholerae* O1 serotype Inaba; (b) *Vibrio cholerae* O1 serotype Ogawa; (c) *Campylobacter jejuni*; (d) *Escherichia coli*; (e) *Salmonella* Typhimurium.

ultrasensitive detection of target bacteria under a fluorescence microscope. However, the key component for the success is based on the selection of highly specific antibody to be conjugated onto the particle surface.

In this study, the synthesis of 20X ratio was accomplished. The carboxyl functionalization and antibody conjugation onto the nanoparticles were successful. The application of Rubpy dye-doped silica nanoparticles as signal amplification for microscopic detection proposes a reliable, easy, and rapid method for detection of *V. cholerae* O1 in a wide area such as food safety and clinical diagnosis.

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