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Effect of ammonium citrate concentration variation on the carbon dots colistin modification for *Escherichia coli* detection

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Abstract

World Health Organization (WHO) mentioned that diarrhea is one of the main causes of under-five mortality in developing countries due to *Escherichia coli* (*E. coli*) bacteria. Synthesis of colistin-conjugated-carbon dots using ammonium citrate as a precursor for *E. coli* detection has been carried out to study its application for detection in water samples from urban population wells. Synthesis of carbon dots (CDs) was carrried out using conventional pyrolisis method. Ammonium citrate with mass variation of 10; 15; 20; and 25 mg were added to 2.5 g colistin and heated at 180 °C for 1 h. Brown residue was yielded; more over the functional groups and morphology were characterized by FT-IR and TEM. The brown residue is further diluted by distilled water, centrifuged at 8000 rpm for 10 min. The absorbances were analyzed using spectrophotometer UV-Vis. Carbon dots colistin with different concentration of ammonium citrate variations were analyzed using spectrophotometer fluorescence at 310-450 nm and the highest intensity would be used for *E. coli* detection. From the experimental part, it was found that CDs colistin has spherical morphology with diameter around 3-10 nm. The highest intensity was achieved at excitation wavelength of 360 nm from CDs colistin with 20 mg ammonium citrate. In the detection process of three household urban wells water in Yogyakarta-Indonesia, it was concluded that one sample was found to contain *E. coli* whose concentration was higher than the permitted limit of 235 cfu per 100 mL set by Ministry of Health-Republic of Indonesia.

Keywords: *Escherichia coli, carbon dots, ammonium citrate, colistin, fluorescence.*

1. Introduction

Development of water pathogenic bacteria analysis methods are highly important due to water quality monitoring necessity since diarrhea constantly becoming major problem in many countries [1-3]. *Escherichia coli* is one of the top pathogenic bacteria associated foodborne illnesses such as diarrhea with 1.9 1.9 million children under 5 dies from diarrhea every year according to WHO (World Health Organization) and UNICEF [1]. In other hand, these bacteria could decompose organic substances in food into inorganic substances, that is CO_2, H_2O, energy, and mineral [4]. Carbon dots (CDs) is nanomaterial that obtained from single-walled nanotube through electrophoresis process with about 10 nm size. Previous research stated that CDs possibly to synthesized by top-down and bottom-up methods [5]. For top-down method, larger carbon structures to be broke into CDs such as electrochemical oxidation, laser ablation and arc-discharge technique. While CDs is formed from raw or precursor material by means combustion/thermal/hydrothermal, supported synthesis, microwave, and chemical dissolution methods. Colistin, which popular as polymyxin E, is a polycationic peptide antibiotic with two hydrophilic and lipophilic groups. Colistin could interact with the lipopolysaccharide molecules in the outer membrane of the bacteria [6]. Previous report has reported a synthesize amikacin modified fluorescent carbon dots and then utilized as material sensor for Gram-negative *Escherichia coli* as pathogenic bacteria [7]. CDs usage as material detection for bacteria was also studied as a carrier for biological study because some advantages of CDs such as their chemical stability, the surface can be easily functioned or modified, and the range of their wide emission/excitation spectrum [8]. CDs can also be used as a marker of bacterial cells that exhibit a high affinity for cell membranes. In addition, modified CDs was successfully utilized for *E. coli* detection by variation of colistin sulphate concentration in the synthesis process [9]. In this research, we tried to synthesize colistin-conjugated CDs to evaluate the effect of ammonium citrate concentrations in CDs modification by observing its fluorescence spectra and how their performances in detecting *E. coli* in water samples.

2. Experimental Section

2.1. Synthesis of CDs

CDs were synthesized through bottom-up approach by pyrolysis method [9-11]. And then, spectrophotometer UV-Vis instrumentation was used to measure absorbance to get the highest peak at certain wavelength.

2.2. CDs synthesis with variation concentration of ammonium citrate

Ammonium citrates concentration variation of 10; 15; 20 and 25 mg and colistin sulphate at 2.5 g was pounded in pestle, heated for 60 min at 180 °C. The resulting brown solids were characterized by FT-IR, SEM and TEM. The solids were then dissolved in 10 mL distilled water and the procedure was continued by centrifugation at 8500 RPM for 10 min. The measurement of absorbance was conducted using UV-VIS spectrophotometer.

2.3. Spectrofluorometric assay

The fluorescence emission of varied CDs was recorded between 310-450 nm using spectrofluorophotometer. *E. coli* detection was then performed based on excitation wavelength that produced the highest intensity.

2.4. Fluorescence detection of E. coli

E. coli concentration of 1×10^8 cfu mL^{-1} was diluted with PBS at pH 7.4 to concentrations of 10^4; 7.5×10^3; 2.5×10^3 and 10^2 cfu mL^{-1}. Each concentration of *E. coli* in 1 mL was added to falcon bottle and followed by the introduction of 0.1

mL of CDs colistin. Vortex was then conducted for 5 min and continued by gentle shaking with orbital shaker at 200 RPM for 60 min at room temperature. Separation of the mixture was done by centrifugation at 8000 RPM for 10 min. The fluorescence spectra were analyzed using spectrofluorophotometer at excitation wavelength of 360 nm.

3. Results and Discussion

3.1. IR study

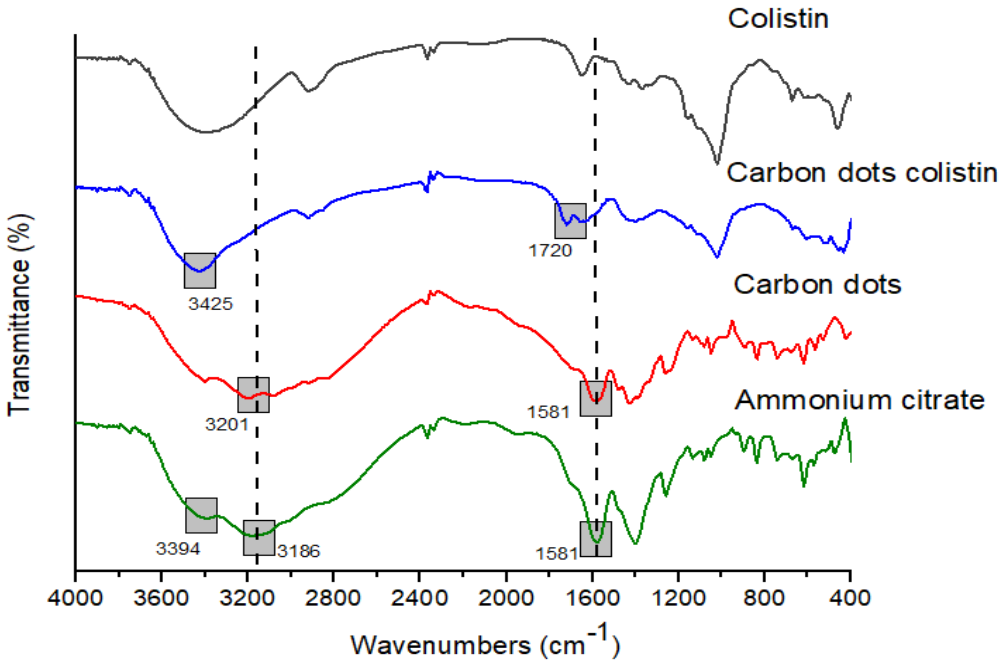

Figure 1. FT-IR spectra of material for biosensor.

Figure 1 shows FT-IR spectra of precursors and CDs colistin. Based on CDs spectrum, broad band at 3201 cm^{-1} indicated stretching vibration of hydroxyl functional group while at 3402 cm^{-1} denoted N-H stretching vibration of amine. Other prominent vibrational bands at 1581 and 1381 cm^{-1} signified the stretching vibration of C-H [12]. Small bands at ca. 1000 to 1200 cm^{-1} can be associated to bending vibration of C-O and therefore the resulting CDs was corresponded with ammonium citrate precursor. Furthermore, the presence of small bands at around 2800 to 3400 cm^{-1} due to heating process eliminating -OH functional group. Thus, stretching vibration of C-H would be more observable following the pyrolysis process. According to spectrum of CDs colistin, there was a wavenumber shift from 1581 cm^{-1} of CDs to 1720 cm^{-1} as a sign of the attachment of colistin to CDs. This result in agreement with Sk and Chattopadhyay which stated that the appearance of wavenumber at 1720 cm^{-1} does not indicate the existence of carbonyl functional group, however, it signifies the formation of amide bond [13]. Based on the previous explanation, it can be concluded that CDs colistin was successfully synthesized. Based on Figure 1, CDs and CDs colistin had oxygen-containing functional groups that generated hydrophilic properties and were suitable for biosystem application.

3.2. Characterization of CDs using TEM

Figure 2 shows that CDs were spherical in shape without agglomeration. Based on the size distribution, diameter of synthesized CDs was between 3 and 10 nm with average of 7 nm. This result was in accordance with previous research that CDs have dispersed spherical morphology and size less than 10 nm [14]. This result is also confirmed by

Zhang and his coworkers that CDs are nanoparticles having small dimension between 2-10 nm, monodisperse, and near-spherical-shaped [15].

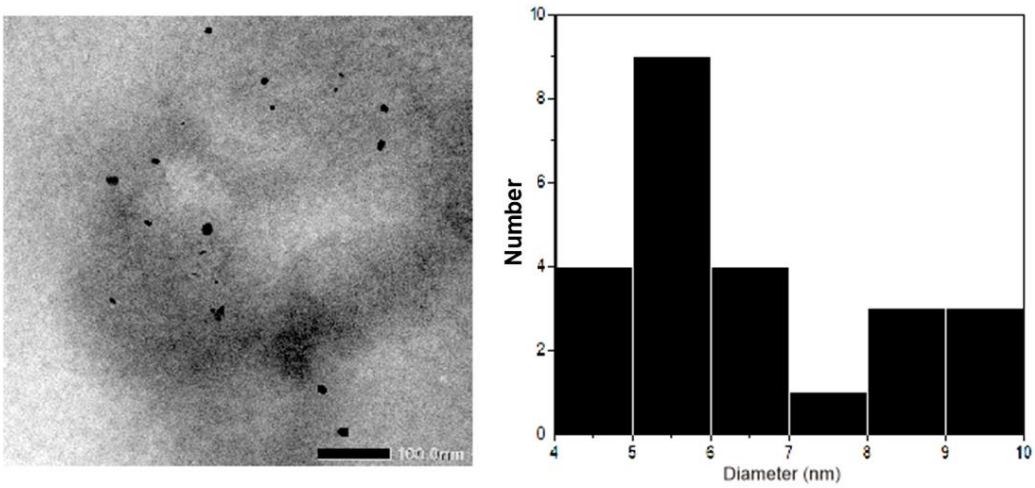

Figure 2. TEM image and size distribution of CDs.

3.3. Optical characterization of CDs and CDs colistin

The absorbances of CDs were observed on a range of UV wavelength (260-360 nm) that is related to the transition of n-π^* from C=O or another functional group resulted from modification or passivation process. Transition of n-π^* needed lower energy than the other electronic transitions and therefore the absorbance peak of CDs was 334 nm near the range of visible wavelength as shown in Figure 3. This absorbance was resulted from electronic transition of C=O of CDs and generated yellow color of the solution under visible light [16]. Based on Figure 3, red shift was observed as the wavelength changed from 334 nm of CDs to 337 nm of CDs colistin. This phenomenon was also reported by Chandra and his coworkers and an indication of the formation of amide bond [17]. The absorption spectra of CDs colistin exhibited pointless peak due to the existence of colistin. Bigger amount of colistin assured that entire CDs attached to colistin and consequently the absorbance of C=O was sealed by new amide functional groups that was covalently formed or -NH_2 functional groups of colistin.

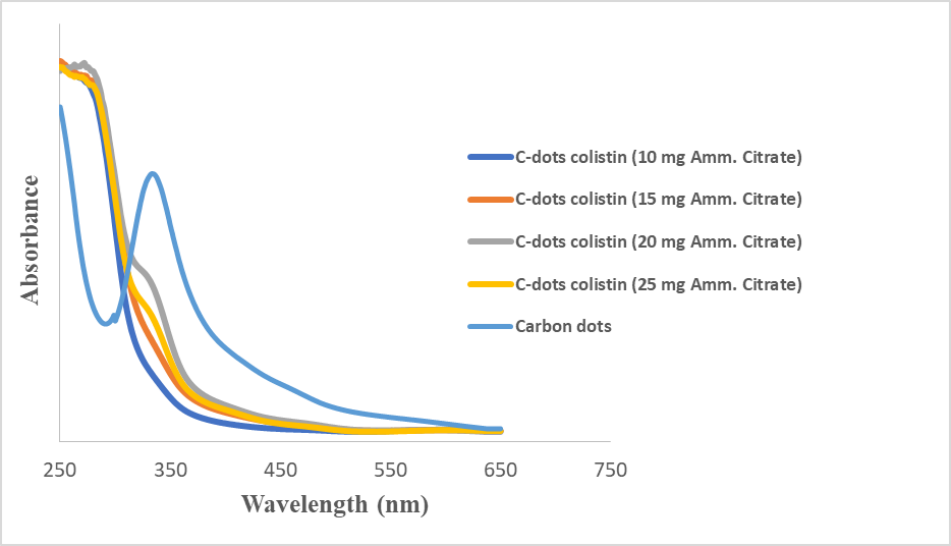

Figure 3. UV-Vis spectra of CDs and CDs colistin with variation of ammonium citrate concentrations.

Figure 3 showed that CDs colistin (20 mg ammonium citrate) had optimum absorbance at 337 nm while variation of 10 mg induced weak absorbance and resulted no peak. This weak absorbance was resulted from insignificant number of CDs colistin which was due to small amount of ammonium citrate and abundance of colistin. Moreover, C=O functional group contributing to the absorbance was sealed by -NH_2 functional groups of colistin sulfate and consequently negligible absorbance was produced. All of CDs colistin variations showed similar characteristic specifically shoulder peaks in their spectra at ca. 300 nm. The shoulder peak was produced from the n-π* transition from carbonyl or other functional groups [18] and be the indication of the attachment of colistin to CDs. In other words, modification can affect the absorbance of CDs in range of UV-Vis wavelength depending on added materials.

Besides their various structure determined by the precursors, CDs have similarity in optical properties, related to absorbance and fluorescence. CDs generally exhibit dominant optical properties at UV region (230-320 nm), with constant end curve until visible wavelength. The existence of other functional groups in CDs determines the absorbance and fluorescence in UV-vis region. Furthermore, the differences in absorption and fluorescence spectra signify the composition and structure of diverse hybridization [19].

3.4. Fluorescence spectra of CDs colistin

Fluorescence properties of all varied CDs colistin at specified excitation wavelength ranging from 310 until 450 nm as shown in Figure 4. The highest intensities of all varied CDs colistin were observed at excitation wavelength of 360 nm. Based on Figure 4, variation of 20 mg ammonium citrate showed the greatest intensity and was suitable to detect *E. coli*. Due to its intense intensity, detection of *E. coli* will be more effective. The differences in amount of ammonium citrate affect the fluorescence emission which represent the variance in composition and hybridization structure of CDs as well as size of emission trap [20].

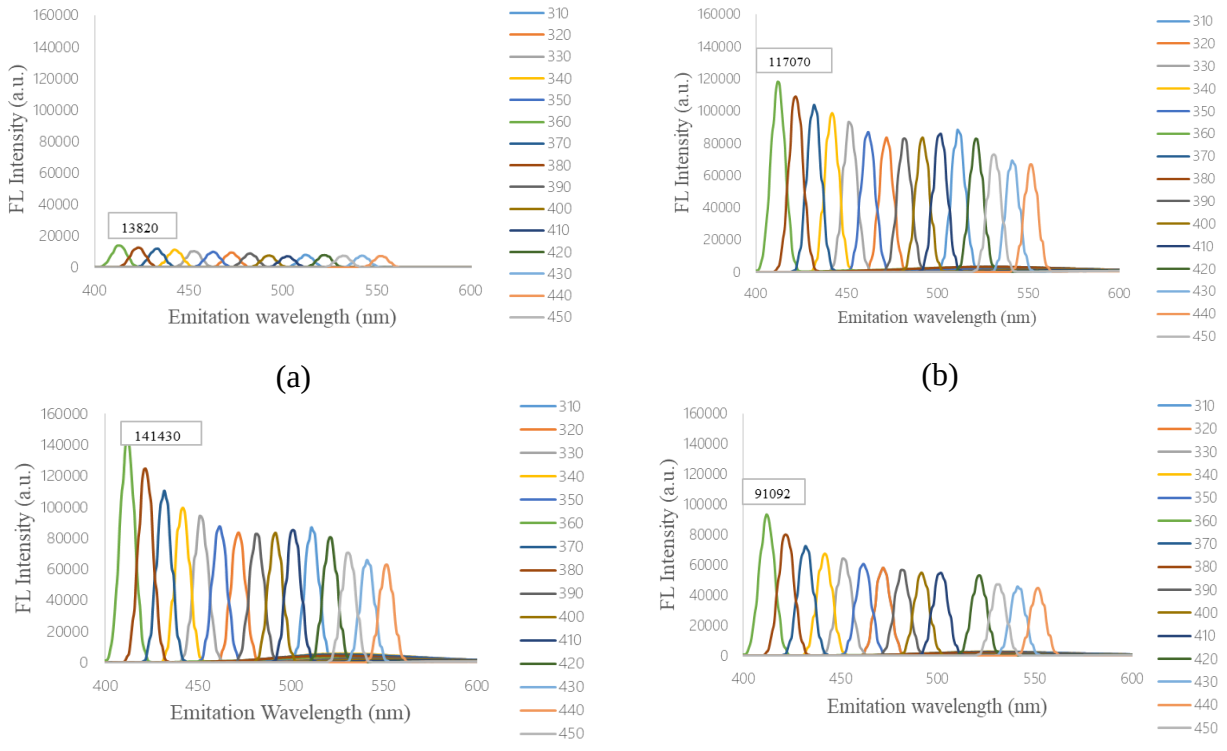

Figure 4. Spectra fluorescence for CDs with ammonium citrates of (a) 10 mg; (b) 15 mg; (c) 20 mg; and (d) 25 mg

CDs colistin with different amount of ammonium citrate modified the resulting fluorescence intensity. The least ammonium citrate generated the weakest intensity and the greatest one produced the highest intensity. This phenomenon is relevant with Chandra and his coworkers [17] that reported the incorporation of colistin on the surface of CDs reduces radiative recombination which the higher colistin concentration is, the lower produced fluorescence intensity becomes. Radiative recombination occurs when the sample receives energy from the light source, $hv > Eg$, to generate the pair of electron-hole. The reduction observed in fluorescence intensity of CDs colistin 25 mg ammonium citrate due to the presence of electron withdrawing groups from carbonyl. This functional groups hindered the radiative recombination of CDs colistin thus lowering the fluorescence intensity [21]. This indication was not detected in CDs colistin 10 mg ammonium citrate because of low concentration of ammonium citrate and negligible effect of electron withdrawing groups.

3.5. E. coli detection using CDs colistin

The detection of *E. coli* used CDs colistin 20 mg ammonium citrate due to its intense fluorescence intensity. The measurement of fluorescence intensity was conducted at excitation wavelength of 360 nm at which highest intensity was produced. CDs colistin was utilized for *E. coli* detection because the existence of colistin performed as both antibacterial agent and sensor of *E. coli*. CDs colistin interacted electrostatically with lipopolysaccharide (LPS) in outer membrane of *E. coli*. This kind of interaction initiated the replacement of divalent cations of phosphate groups in lipid membrane [22].

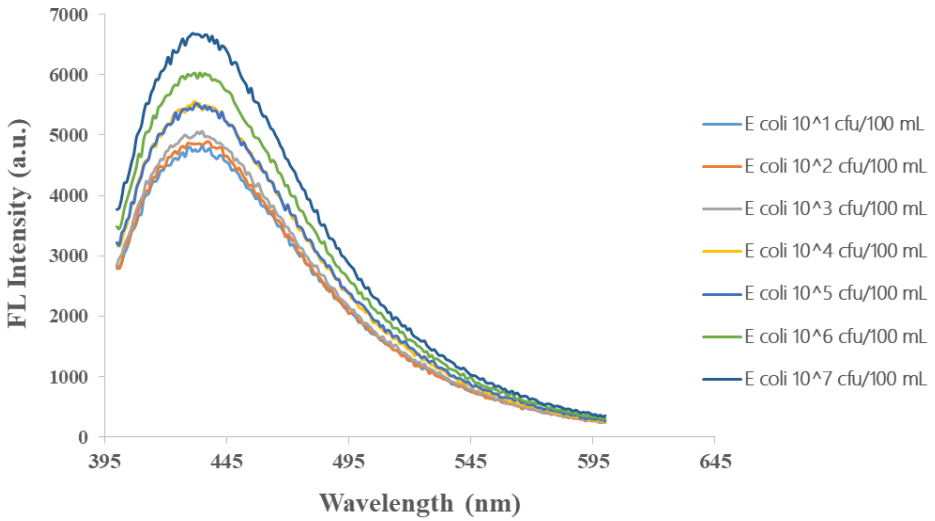

Figure 5. The results of fluorescence CDs colistin detection of *E. coli* using the optimum ammonium citrate.

Based on Figure 5, *E. coli* concentration of 10^7 cfu per 100 mL gave the highest fluorescence intensity while the lowest one was produced by concentration of 10 cfu per 100 mL. In other words, the fluorescence response depended on the concentration of *E. coli*. The depletion of fluorescence intensity particularly in range of 10^4 to 10 cfu per 100 mL was not too obvious to be observed. This phenomenon can be caused by the interaction of entire CDs colistin to *E. coli*. Therefore, *E. coli* concentration of 10^4 cfu per 100 mL was the minimum concentration to be measured by CDs colistin 20 mg ammonium citrate. Another cause of this insignificant intensity reduction was the diverse sizes of *E. coli*. Size of *E. coli* ranges from 0.4-0.7 µm × 1.4 µm so that the surface area of *E. coli* is heterogeneous to be bind with CDs colistin. Hence, the fluorescence intensities between small concentration of *E. coli* are not very contrasting.

Detection of *E. coli* in real samples was utilized municipal well water samples. Three wells named well A, B, and C have same distance to specific septic tank which was 3-4 m. The basis to determine the quality of the samples is Ministry of Health of Republic of Indonesia regulation that stated the presence of *E. coli* in domestic water sample should not exceed 235 cfu per 100 mL. In addition, to get more precise measurement, *E. coli* concentrations of 10^2 cfu per 100 mL and 10^5 cfu per 100 mL are used as comparisons. Water samples from wells A and B resulted lower fluorescence intensities than *E. coli* concentration of 10^2 cfu per 100 mL as shown in Figure 6. Furthermore, these intensities were much lower than well C and can be an indicator that it has been polluted with *E. coli*. Although the three wells have relatively same distance to a septic tank, the groundwater flow direction is different. The move of groundwater flow is from the septic tank to well C while away from wells A and B.

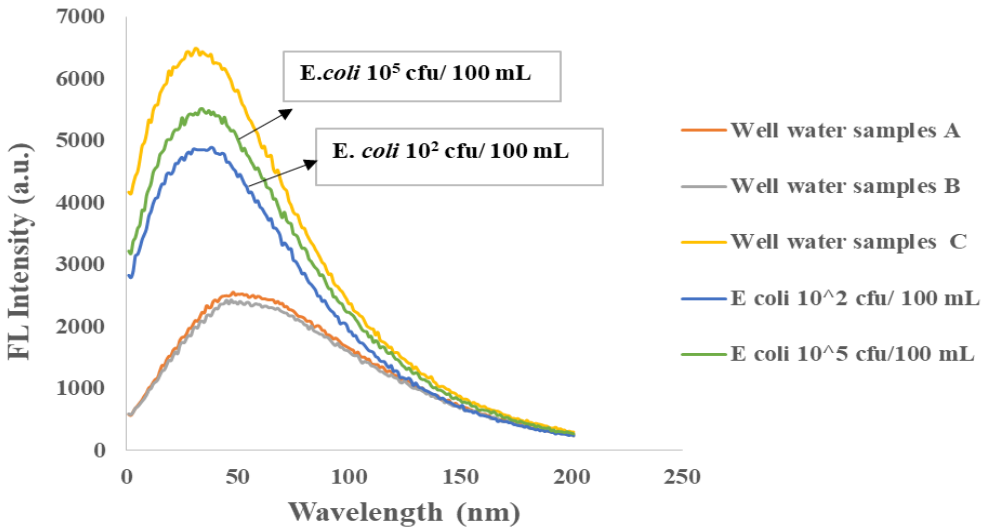

Figure 6. The detection of *E. coli* from municipal wells water samples

4. Conclusion

In conclusion, CDs colistin was successfully synthesized from ammonium citrate and colistin sulfate by conventional pyrolysis method. CDs colistin showed spherical morphology with average size of 7 nm. CDs colistin 20 mg ammonium citrate generated the highest fluorescence intensity and can be employed to effectively detect *E. coli* at the minimum concentration of 10^4 cfu per 100 mL.

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