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Highly selective detection of bacterial alarmone ppGpp with an off–on fluorescent probe of copper-mediated silver nanoclusters

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ABSTRACT

In this study, a facile strategy for highly selective and sensitive detection of bacterial alarmone, ppGpp, which is generated when bacteria face stress circumstances such as nutritional deprivation, has been established by developing an off–on fluorescent probe of Cu^{2+}-mediated silver nanoclusters (Ag NCs). This work not only achieves highly selective detection of ppGpp in a broad range concentration of 2–200 μM, but also improves our understanding of the specific recognitions among DNA–Ag NCs, Cu^{2+}, and ppGpp. The present strategy, together with other reports on the Ag NCs-related analytical methods, has also identified that Ag NCs functionalized with different molecules on their surfaces can be engineered fluorescent probes for a wide range of applications such as biosensing and bioimaging.

1. Introduction

Guanosine 3′-diphosphate-5′-di(tri)phosphate, ppGpp, is a bacterial alarmone that can generate a stringent response, which involves a survival mechanism for bacteria in the face of stress circumstances such as nutritional deprivation (Zhao and Huang, 2010). In this case, ppGpp will be swiftly generated on the bacterial ribosome by transferring a pyrophosphate from adenosine triphosphate (ATP) to the 3′–OH of guanosine diphosphate (GDP) or guanosine triphosphate (GTP; Ferullo and Lovett, 2008). Except for facilitating bacterial persistence under stress conditions, ppGpp also affects many biological functions of microorganisms such as gene expression, antibiotics production, virulence, symbiosis, and colony morphology (Jain et al., 2006). Owing to the significant roles of ppGpp in bacterial physiology, many studies have been dedicated to the establishment of analytical methods for the detection of ppGpp (Fischer et al., 1982; Takahashi et al., 2004; Saito et al., 2006; Rhee et al., 2008; Zhao and Huang, 2011).

The methods on the basis of high-performance liquid chromatography (HPLC) have usually been employed for the detection of ppGpp (Fischer et al., 1982; Takahashi et al., 2004; Saito et al., 2006). No matter what kind of signal (*e.g.*, by UV–vis absorption or by the isotope in thin layer chromatography) was used in the HPLC-related methods for the detection, all of these protocols could not achieve high sensitivity. To this end, Hong et al. reported the first fluorescent chemosensor for the quantitative assay of ppGpp, in which a complex of pyrene and bis(Zn^{2+}–dipicolylamine) was used for recognizing ppGpp and reporting the signal simultaneously (Rhee et al., 2008). A few years ago, we also developed a fluorescent probe, a complex of Cu^{2+} and 2,6-bis(2-benzimidazolyl)pyridine, for the selective detection of pyrophosphate and ppGpp (Zhao and Huang, 2011). However, the synthesis of organic ligands for the recognition of ppGpp usually needs to be conducted with complicated processes and under rigorous conditions. Moreover, ideas and actual results often come into conflict because most of the designed organic ligands cannot selectively distinguish ppGpp from other phosphate-containing anions such as nucleotides. Therefore, there is an urgent requirement to develop simple, rapid, reliable, and highly selective and sensitive sensors for the ppGpp.

Fluorescent metal nanoclusters (NCs) are a class of fluorophores that consist of several to hundreds of metal atoms and exhibit molecule-like properties. They are promising sensing materials with attractive features, including brightness, tunable emission, photostability, subnanometer size, biocompatibility, and easy preparation (Xu and Suslick, 2010). Herein, based on the strong fluorescent quenching effect of Cu^{2+} on DNA–Ag NCs as well as the specific recognition between Cu^{2+} and ppGpp, we report a “turn-on” fluorescence method for the detection of ppGpp.

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This work not only achieves highly selective detetion of ppGpp in the range of 2–200 μM, but also improves our understanding of the specific recognitions among DNA–Ag NCs, Cu^{2+}, and ppGpp.

2. Experimental

2.1. Chemicals and materials

Ag NCs were synthesized using DNA as the templates (Guo et al., 2009; Richards et al., 2008; Zhang and Huang, 2011; Sharma et al., 2010). The DNA sequences used in this work are listed as follows. **T7**: 5′-GGC AGG TTG GGG TGA CTA AAA ACC CTT AAT CCC C-3′ (34 mer); **T1**: 5′-CCC CCC CCC CCC-3′ (12 mer); **T2**: 5′-AAT TCC CCC CCC CCC CAA TT-3′ (20 mer); **T3**: 5′-CCT CCT TCC TCC-3′ (12 mer); **T4**: 5′-CCC TTA ATC CCC-3′ (12 mer); **T5**: 5′-CCA CAA CCA CAA CAC ACA-3′ (18 mer); **T6**: 5′-AGT CAC CCC AAC CTG CCC TAC CAC GGA CT-3′ (29 mer). All of the above oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Sodium pyrophosphate (PPi) was purchased from Beibei Chemical Plant (Chongqing, China). Adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate disodium salt (ADP), adenosine 5′-monophosphate disodium salt hydrate (AMP), cytidine 5′-triphosphate disodium salt hydrate (CTP), uridine 5′-triphosphate trisodium salt hydrate (UTP), guanosine 5′-triphosphate disodium salt hydrate (GTP), guanosine 5′-diphosphate sodium salt (GDP), and guanosine 5′-monophosphate disodium salt hydrate (GMP) were all obtained from Sigma (USA). Guanosine 3′-diphosphate-5′-diphosphate (ppGpp) was commercially purchased from Trilink (USA). $Cu(NO_3)_2$, $MnCl_2$, $ZnCl_2$, $FeCl_3$, $CrCl_3$, $CdCl_2$, $AlCl_3$, $PdCl_2$, $Eu(NO_3)_3$, $Tb(NO_3)_3$, $HgCl_2$, $CoCl_2$, Na_2HPO_4, NaH_2PO_4 and Na_3PO_4 were all commercially available and used without further purification. Phosphate buffer (PB, 0.2 M) was used to control the acidity of the reaction solution. Mili-Q purified water (18.2 MΩ · cm) was used throughout the experiments.

2.2. Instrumentation

Fluorescence spectra were measured with an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). A Jacso J-810 circular dichroism (CD) spectropolarimeter (Tokyo, Japan) was used to measure the conformations of the molecules. The fluorescence lifetimes were recorded with a FL-TCSPC fluorescence spectrophotometer (Horiba Jobin-Yvon Inc, France). Zeta-potential was measured using a Zatasizer Nano-ZS90 System (Malvern Inc.).

2.3. Synthesis of DNA–Ag NCs

3 μM of DNA (HPLC purified) and 18 μM of $AgNO_3$ (Sigma-Aldrich) were firstly mixed in PB buffer (20 mM, pH 6.6) for 5 min, and then kept away from light at room temperature. After 20 min, 18 μM of freshly prepared $NaBH_4$ (Sigma-Aldrich) was quickly injected, thoroughly mixed, and then kept in dark without further disturbance at 4 °C overnight.

2.4. Procedure for fluorescence detection of ppGpp

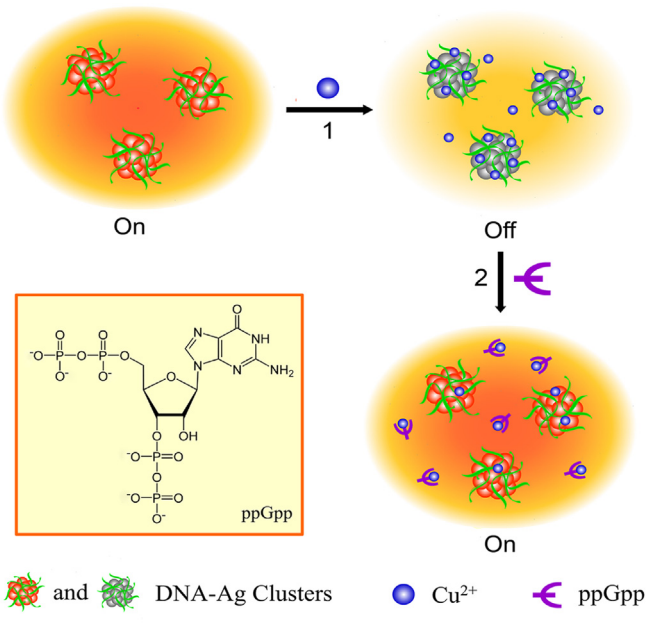
In a standard precedure, a certain concentration (as indicated in the figure caption) of ppGpp was added to the mixed solution of 300 nM DNA-Ag NCs and 20 mM PB buffer (pH 7.4). After 15 min, 800 nM Cu^{2+} was added at room temperature, and then the mixture was measured by the F-2500 fluorescence spectrophotometer with an excitation wavelength of 585 nm.

3. Results and discussion

3.1. On–off–on fluorescent sensor for detection of ppGpp

As illustrated by Scheme 1, DNA–Ag NCs with strong fluorescence emission were firstly synthesized using DNA as a template. In the presence of Cu^{2+}, the fluorescence of Ag NCs could be greatly quenched (turn-off) *via* electron or energy transfer due to the specific interaction of Cu^{2+} and DNA. Cu^{2+} is usually reported to attach chiefly to the phosphate of DNA, and can also interact with the nucleobases (Zhang and Ye, 2011). Moreover, Cu^{2+} is also a well-known efficient fluorescence quencher due to its paramagnetic properties, and has been widely used in sensing (Zhang and Ye, 2011; Shang and Dong, 2008; Liu et al., 2011). Combined together, Cu^{2+} is used as the mediator to tune the fluorescence of DNA–Ag NCs in the present work. On the other hand, the rich electrons in the phosphate groups of ppGpp allow it to show strong coordination affinity with Cu^{2+}. In this case, part of Cu^{2+} could be easily disassociated from Ag NCs–Cu^{2+} complex and coordinated with ppGpp when a specific amount of ppGpp was introduced. As a result, relatively strong fluorescence of Ag NCs could be observed again (turn-on).

Previous reports have demonstrated that Ag^+ can selectively coordinate with cytosine (C base) and form stable C–Ag^+–C complexes (Gwinn et al., 2008; Lan et al., 2011). On the basis of this interaction, Ag NCs are able to be synthesized using DNA as a template. It has also been demonstrated that the DNA templates with different sequences have distinct binding capabilities toward Ag^+ ions, which can determine the size of the formed Ag NCs and thus their fluorescence quantum yield (QY) as well as the emission wavelength. (Gwinn et al., 2008; Lan et al., 2011). To this end, we designed a series of single-stranded DNA (ssDNA) with different proportions of C base and different lengths to synthesize the DNA–Ag NCs in the present work (Table. S1, T1–T7; Guo et al., 2009; Richards et al., 2008; Zhang and Huang, 2011; Sharma et al., 2010). The result shows that Ag NCs capped with different DNA sequences indeed have distinct fluorescence properties and display different responses to Cu^{2+} (Figs. S1 and S2), indicating that the fluorescence response



Scheme 1. Schematic illustration of the off–on fluorescent strategy for ppGpp detection based on the probe of Cu^{2+}-mediated DNA–Ag NCs.

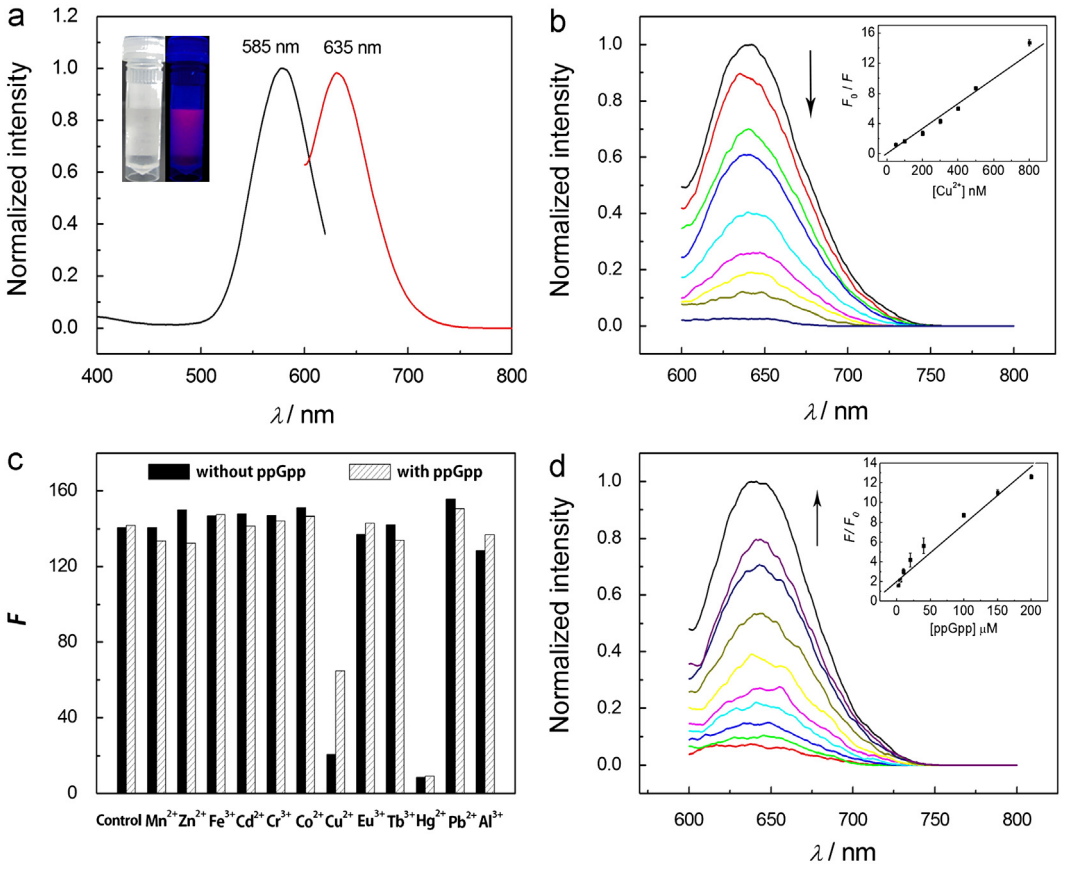


Fig. 1. The performances of the on–off–on fluorescent sensor for the detection of ppGpp: (a) fluorescent excitation and emission spectra of T7-Ag NCs, together with a photograph (inset) of the Ag NCs solution under white and UV light (λ_{ex}: 365 nm), respectively; (b) fluorescence quenching of the T7-Ag NCs with the addition of different amounts of Cu^{2+} (from top to bottom: 0, 50, 100, 200, 300, 400, 500, 800, and 1000 nM). The inset shows the values of F_0/F as a function of the concentration of the introduced Cu^{2+}; and (c) response of different metal ions to the T7-Ag NCs in the absence and presence of ppGpp. The concentrations of DNA–Ag NCs, ppGpp, metal ions and PB buffer are 300 nM, 20 μM, 500 nM and 20 mM, respectively; (d) fluorescence restoration with the addition of increased concentrations of ppGpp (from bottom to the top: 0, 2, 5, 10, 20, 40, 100, 150, 200 μM) into the Ag NCs–Cu^{2+} complex. The inset shows the values of F/F_0 as a function of the concentration of the ppGpp.

pattern of Ag NCs to a specific analyte is highly dependent on the nature of the DNA template (Huang et al., 2011).

Compared with the Ag NCs prepared by other DNA templates, the T7-Ag NCs display much higher QY and stronger capability of binding with Cu^{2+}. The as-obtained T7-Ag NCs are highly dispersed in aqueous solution and show a pink-red color under a UV lamp (λ_{ex}: 365 nm). The maximal excitation and emission peaks are located at 585 and 635 nm, respectively (Fig. 1a). When a specific amount of Cu^{2+} was introduced, the fluorescence of T7-Ag NCs could be quenched immediately. The fluorescence intensity gradually decreased with the increase of different concentrations of Cu^{2+} in the range of 50–800 nM (Fig. 1b). This fluorescence quenching could be attributed to the interaction between Cu^{2+} and the surfaces of the DNA-Ag NCs (Zhang and Ye, 2011; Shang and Dong, 2008; Liu et al., 2011). Specifically, Cu^{2+} could easily attach to the surfaces of T7-Ag NCs by the coordination with the DNA, and thereafter effectively quench their fluorescence *via* electron or energy transfer. Moreover, it was found that the degrees of fluorescence quenching were almost identical under different pH values (pH 5.8–8.0; Fig. S3), demonstrating that the quenching of fluorescence by Cu^{2+} could not be affected by the pH fluctuation of the system.

The strong power of Cu^{2+} for quenching the fluorescence of DNA–Ag NCs could result in a low background signal, and thus improve the signal-to-background ratio of the "turn-on" fluorescence method for the detection of ppGpp. Compared with other metal ions, only Cu^{2+} could effectively quench the fluorescence of T7-Ag NCs and make it partially restore again when a specific amount of ppGpp was introduced (Fig. 1c). This result suggested that ppGpp could selectively and strongly bind with Cu^{2+}, and made Cu^{2+} to keep relatively far distances from the Ag NCs. As a result, the fluorescence of AgNCs could be recovered. Hg^{2+} was also able to greatly quench the fluorescence of T7-Ag NCs; however, the fluorescence could not be restored in the presence of ppGpp. This result demonstrated that only Cu^{2+} could be the most appropriate mediator for adjusting the fluorescence of T7-Ag NCs in an "on–off–on" mode for the detection of ppGpp.

Table 1
Fluorescence lifetimes (τ) of Ag NCs, Ag NCs–Cu^{2+}, and Ag NCs–Cu^{2+}–ppGpp.

Samples	τ (ns)	A (%)[a]
Ag NCs	3.14	100
Ag NCs–Cu^{2+}	3.11	100
Ag NCs–Cu^{2+}–ppGpp	3.13	100

[a] The spectral area was calculated from the fluorescence decay curve.

The average fluorescence lifetimes (as shown in Table 1) of Ag NCs and Ag NCs–Cu^{2+} complex were calculated by fitting the fluorescence decay curves (Fig. S4). The Stern–Volmer plot of F_0/F (F_0 and F are the fluorescence intensities in the absence and presence of Cu^{2+}, respectively) has good linearity and the Stern–Volmer constant (K_{sv}) derived from this plot is $6.6 \times 10^6\ M^{-1}$, indicating the high quenching efficiency of the excited state. In addition, the lifetime of Ag NCs almost did not change after they interacted with Cu^{2+} (3.14 ns vs. 3.11 ns), demonstrating that

the fluorescence quenching follows a static quenching mechanism (Guo et al., 2009).

As shown in Fig. 1d, the fluorescence intensity gradually increased with the addition of increased concentration of ppGpp into the Ag NCs–Cu^{2+} complex. Based on the specific interaction between Ag NCs–Cu^{2+} complex and ppGpp, a facile method can be developed for highly selective detection of ppGpp. The relative fluorescence intensity (F/F_0, F and F_0 are the fluorescence intensities in the presence and absence of ppGpp, respectively) is linearly proportional to the concentration of ppGpp in the range 2–200 μM (Fig. 1d, inset). The equation of $F/F_0=1.7+0.6c$ ($R^2=0.99$) can be used to describe the relationship between the fluorescence intensity and the concentration of ppGpp. The limit of detection (LOD) of this method is 0.75 μM (3σ). Compared with the previously reported methods (Fischer et al., 1982; Takahashi et al., 2004; Saito et al., 2006; Rhee et al., 2008; Zhao and Huang, 2011), the present strategy can achieve lower LOD together with broader detection range for the ppGpp (Table. S2). Moreover, this facile and rapid method can make the detection easy to be conducted and be completed within a few minutes.

3.2. Selectivity of the present detection method

Another advantage of this analytical method is the high selectivity for the detection of ppGpp. As shown in Fig. 2a, the fluorescence of Ag NCs–Cu^{2+} complex could not be recovered with the addition of other phosphate-containing anions except for ppGpp. In order to further understand the selectivity for ppGpp, a comparison study of the phosphate, pyrophosphate, and guanine-involved nucleotides containing different numbers of phosphate (*e.g.*, GMP, GDP, GTP) was also conducted using the standard procedure. The result shows that only ppGpp can result in the fluorescence restoration rather than other kinds of phosphate-containing anions (Fig. 2b). This result indicates that the specific recognition between ppGpp and Cu^{2+} is greatly dependent on the number of the phosphate in the nucleotides, making it an excellent method for selective detection of ppGpp. In this case, design and synthesis of complicated organic ligands for the recognition of ppGpp is not needed any more using the present protocol. To obtain the optimal experimental condition for the detection of ppGpp, the amount of Cu^{2+}, pH value of the system, and the order of regents added were further investigated. It seems that the concentration of Cu^{2+} of 800 nM and the pH value of 7.4 (close to physiological pH) were the optimal conditions (Fig. S5). In addition, the order for the addition of regents could also slightly affect the degree of fluorescence restoration (Fig. S6).

3.3. Mechanism of the on–off–on fluorescence process

In order to elucidate the interaction between the fluorescent probe and ppGpp and thus the "on–off–on" fluorescence process, the measurements of zeta-potential and circular dichroism (CD) spectra were further conducted. As shown in Fig. 3a, the Ag NCs

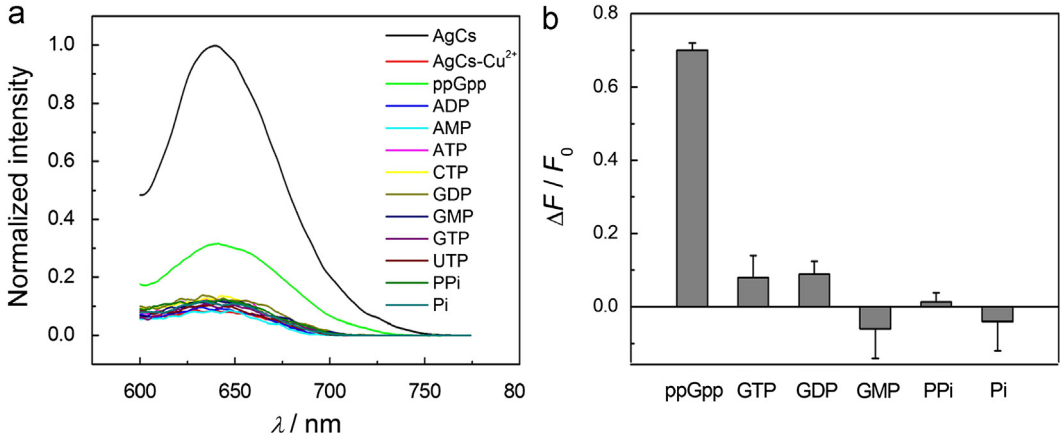


Fig. 2. Studies on the selectivity of the present detection method: (a) fluorescence spectra of Ag NCs–Cu^{2+} complex before and after the addition of different phosphate-containing anions; and (b) a comparison study shows the effect of different numbers of phosphate in the anions on the fluorescence restoration of Ag NCs–Cu^{2+} complex. The concentrations of DNA–Ag NCs, phosphate-containing anions, Cu^{2+}, and PB buffer are 300 nM, 20 μM, 800 nM, and 20 mM, respectively.

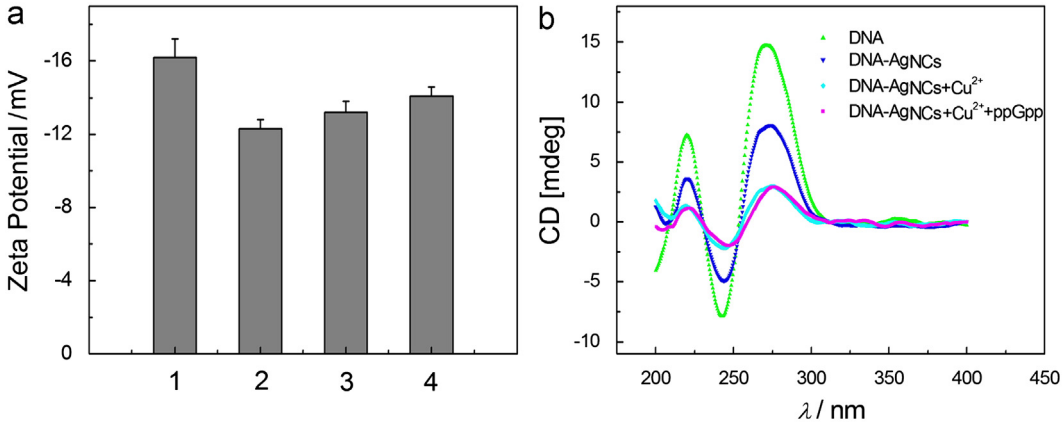


Fig. 3. Mechanistic studies on the on–off–on fluorescence process: (a) zeta-potential of (1) Ag NCs, (2) Cu^{2+}–Ag NCs complex, (3) the mixture of Cu^{2+}–Ag NCs and 10 μM of ppGpp, and (4) the mixture of Cu^{2+}–Ag NCs and 20 μM of ppGpp; and (b) CD spectra of DNA, DNA–Ag NCs, DNA–Ag NCs–Cu^{2+} and DNA–Ag NCs–Cu^{2+}–ppGpp.

show an average zeta-potential of −16.2 mV, indicating that a large number of DNA with negative charge were exposed on the surfaces of the Ag NCs. This value slightly increased to −12.3 mV with the addition of Cu^{2+}, owing to the coordination of Cu^{2+} with DNA on the surfaces of Ag NCs and thus neutralization of partial negative charges of the DNA. However, this value decreased again with the addition of ppGpp, and it further decreased with the increase of the concentration of ppGpp. This result confirmed that ppGpp could strongly bind with Cu^{2+} and make it relatively far away from the DNA–Ag NCs. As a result, the fluorescence quenching of the Cu^{2+} against the DNA–Ag NCs was weakened, and thus led to the fluorescence restoration.

CD spectra could also be a powerful means to confirm their interactions. As shown in Fig. 3b, the CD spectrum of pure ssDNA shows a positive Cotton effect at 271 nm and a negative Cotton effect at 242 nm, corresponding to the base stacking and helical conformation of DNA, respectively (Ota et al., 1998). After they had been used for the stabilization of Ag NCs, these two peaks red-shifted to 273 nm and 244 nm, correspondingly, together with the dramatic decrease of the peak intensities. It suggested that the DNA with reduced degree of base stacking and increased structural relaxation were obtained after they had been capped on the surfaces of Ag NCs (Zhang et al., 2012; Sang et al., 2010). Coordination of DNA-Ag NCs with Cu^{2+} resulted in further decrease of the peak intensities. With the addition of ppGpp into Ag NCs–Cu^{2+} complex, the peaks of Cotton effect further red-shifted to 277 nm and 249 nm, correspondingly. This result demonstrated that the strong interaction of Ag NCs–Cu^{2+} and ppGpp assuredly occurred, and thus caused the change of the microenvironment around Ag NCs–Cu^{2+} complex in the solution.

Taking together, we can conclude that Cu^{2+} is the most appropriate candidate to mediate the fluorescence of the Ag NCs for the detection of ppGpp. Specifically, Cu^{2+} can easily attach to the phosphates of DNA and greatly quench the fluorescence of DNA–Ag NCs *via* electron or energy transfer. It should be pointed out that the fluorescence response pattern of the Ag NCs to a specific species has a remarkable relationship with the nature of the DNA template used for the synthesis of the Ag NCs. When ppGpp was added to the Ag NCs–Cu^{2+} complex, part of the Cu^{2+} can be switched to bind with the phosphate groups of ppGpp and keep relatively far distance from the Ag NCs, resulting in the restoration of the fluorescence.

4. Conclusions

In summary, we have developed a Cu^{2+}-mediated "off–on" fluorescent probe with DNA–Ag NCs and successfully applied it in the detection of ppGpp. Compared with previous reports on the assay of ppGpp, our present protocol shows the advantages of being simple, rapid, and highly selective and sensitive, with relatively broad detection range, and avoiding the requirements of synthesis of complicated organic ligands for the recognition of ppGpp. This strategy, together with other reports on the Ag NCs-related analytical methods (Zhang et al., 2011, 2012; Shang and Dong, 2008; Liu et al., 2011; Guo et al., 2009; Huang et al., 2011; Han and Wang, 2011), has identified that Ag NCs functionalized with different molecules on their surfaces can be excellent fluorescent probes for a broad range of applications such as sensing and bioimaging.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.05.056.

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