Synthesis of DNA-Cu/Ag NCs and Its Application in Pyrophosphate and Phosphatase Detection

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Abstract

We synthesized a fluorescent sensor for detection of pyrophosphate (PPI) and alkaline phosphatase (ALP) based on copper/silver nanoclusters (DNA-Cu/Ag NCs) with single-stranded DNA as template. The whole synthesis process was only 30 min. When PPI was added to the reaction liquid for the preparation of DNA-Cu/Ag NCs, the fluorescence of DNA-Cu/Ag NCs would be quenched due to the strong complexation between PPI and Cu$^{2+}$. As ALP could catalyze the hydrolysis of PPI to Pi, the complexation between Cu$^{2+}$ and PPI would be destroyed and the fluorescence intensity would increase. Based on this principle, we developed a new method for detecting PPI and ALP with a detection limit of 136.7 nmol/L and 0.01 U/mL, respectively. Finally, by detecting the PPI in human serum, the diagnostic ability and practical application of the method were verified.

Keywords Bimetal nanoclusters, DNA-templated, pyrophosphate, alkaline phosphatase, biosample detection, spectrophotometry.
Introduction

Recently, bimetallic nanoclusters attract more and more attention. The bimetallic metal can be obtained by reducing in turn or reducing the two metal ions in the presence of stabilizers. Due to the synergistic effect of physical and chemical properties, bimetallic nanoclusters have better dispersion and strong fluorescence emission than single metal nanoclusters. In addition, bimetallic nanoclusters have potential applications in changing the electronic structure, enhancing the fluorescence quantum yield and changing the wavelength of fluorescence emission. Meanwhile, Cu/Ag NCs has been widely concerned and studied because of its low cost, simple operation, high quantum yield and good stability. Dodecyl mercaptan, glutathione, D(-)-Penicillamine, and DNA have been used as stabilizers to synthesize Cu/Ag NCs. Chang etc synthesized a copper/silver alloy nanocluster (DNA-Cu/Ag NCs) with DNA as template in 2010 at the first. The copper and silver alloy nanoclusters (DNA-Cu/Ag NCs) were synthesized with DNA as a template. The fluorescence quantum yield was about 4.5 times higher than the Ag NCs synthesized by the same DNA template, and the time required for the synthesis was greatly shortened (from 120 h to 1.5 h). DNA-Cu/Ag NCs has been used to detect Cu$^{2+}$, acetylcholinesterase inhibitors, adenosine, H$_2$S, single strand DNA binding protein (SSB), etc. Phosphate, especially the product of ATP (P$_2$O$_7^{4-}$, PPi), plays an important role in biological metabolism, such as energy conduction, skeletal structure, protein regulation, cell signal transduction and identification of organelles. The state of PPi in living cells has important effects on certain diseases, such as calcinosis and two calcium pyrophosphate crystal deposition. At present, a variety of methods have been used to detect PPi, such as colorimetric method, fluorescence method (organic dye, quantum dot, metal nanoparticle, metal ion complex), surface enhanced Raman scattering, electrochemical method, etc. However, these methods encounter some challenges including the need for modification, precision instruments and organic reaction equipment. ALP is one of the most commonly used enzyme markers based on immunological and aptamer analysis, genetic analysis, histochemical staining, and related affinity methods for monitoring proteins, nucleic acids, drugs, enzymes, and other analytes. ALP is also present in human serum and is used as an indicator of hepatobiliary and bone disease in routine clinical analysis. ALP is mainly used for dephosphorylation of nucleic acids in molecular biology. Therefore, the development of low-cost, simple operation, fast detection of quantitative detection of PPi and ALP is of great significance. For example, Ma and coworkers presented a new copper-mediated on-off switch for the detection of ALP and PPi based on DNA/AgNCs. In this paper, we present a novel bimetallic fluorescence sensor with strong fluorescent emission based on coordination between Cu$^{2+}$ and PPi and ALP catalytic hydrolysis of PPi. We synthesized DNA-Cu/Ag NCs using DNA as a template, and the entire synthesis process was only 30 minutes. The sensor has high sensitivity and high selectivity. When PPi was added to the DNA-Cu/Ag NCs preparation system, the strong complexation between PPi and Cu$^{2+}$ inhibited the DNA-Cu/Ag NCs formation, and resulted in the fluorescence quench of the obtained
DNA-Cu/Ag NCs. The degree of fluorescence attenuation was related to the concentration of PPi. At the same time, in the presence of ALP, PPi was hydrolyzed to orthophosphate (Pi) and the complexation between Cu²⁺ and PPi was destroyed. This facilitated the DNA-Cu/Ag NCs generation and the fluorescence intensity increased (Fig. 1).

**Experimental**

**Reagents and Chemicals**

Oligonucleotide and alkaline phosphatase (ALP) were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) and were ULTRAPAGE (polyacrylamide gel electrophoresis) purified by manufacturer. Sodium borohydride (NaBH₄) and silver nitrate (AgNO₃) were purchased from Sigma-Aldrich. Copper sulphate (CuSO₄) and 3-Morpholinopropanesulfonic acid (MOPS) were purchased from Aladdin Industrial Corporation. Sodium pyrophosphate (PPi) was purchased from Alfa Aesar. All other reagents were of analytical reagent grade and used as received.

**Apparatus**

Fluorescence measurements were carried out with a FluoroMax-4 spectrophotometer (HORIBA Scientific). UV–Vis absorption spectra were recorded using a Shimadzu UV-2100 spectrophotometer. pH measurements were performed by using a PHS-3C pH meter (Shanghai Leici Chuangyi Apparatus & Instrument Co. Ltd. Shanghai, China). Transmission electron micrograph (TEM) images were recorded using a JEOL-2010 electron microscope (JEOL Ltd, Tokyo, Japan) operating at an accelerating voltage of 200 kV and using Energy Dispersive Spectrometer matched with the transmission electron microscope, the EDS spectrum of the nano-cluster elements is obtained according to the X-ray characteristic wavelength of each element.

**Synthesis of water-soluble DNA-Cu/Ag NCs**

The mixture of single-stranded DNA, AgNO₃ and Cu(NO₃)₂ was incubated in an ice bath for 15 min. Then, NaBH₄ solution was added, and the resultant mixture was kept at 35 °C water bath for 30 min. Different molar ratios of DNA:CuSO₄:AgNO₃ were prepared to select the optimum preparation conditions.

**Fluorescence detection of PPi**

Fluorescence detection was performed at room temperature. First, we studied the quenching effect of Cu²⁺ concentrations on the fluorescence intensity of DNA-Cu/Ag NCs by adding different concentrations of Cu²⁺ into DNA-Cu/Ag NCs to determine the appropriate concentration. The PPi aqueous solutions with different concentrations were freshly prepared before use. Specifically, 6.0 μL DNA (100 μM), 12.0 μL Ag⁺ (0.3 mmol/L), 10.0 μL Cu²⁺ (0.18 mmol/L) and 10.0 μL PPi aqueous solution with different concentrations were put into 250 μL MOPS buffer (pH 7.0). The reaction was carried out in an ice bath for 15 min. Then, the mixture was reduced by the addition of NaBH₄. The solution was thoroughly mixed and was kept in the dark at 35°C for 30
The fluorescence measurements were carried out. In order to investigate the selectivity of this assay, influence of the other anion ions on the fluorescence of the DNA-Cu/Ag NCs system was detected. All the fluorescence measurements were carried out under the same conditions throughout the experiment: the slit widths of excitation spectrum and emission spectrum were 5 nm and 5 nm, respectively.

**Human serum sample preparation**

Fresh human serum samples were obtained from three healthy adult volunteers at a local hospital and stored frozen until the assay. In order to convert disulfides to free thiols, a reducing reagent Tris(2-carboxyethyl)phosphine (TCEP) (35 mM) was added into the serum sample (TCEP:serum sample = 1:10). After incubation at 40 °C for 10 min, the same amount of precipitation agent acetonitriile (acetonitriile:serum sample = 1:1) was added into the sample to eliminate the interference of proteins, followed by centrifugation at 3000 rpm for 20 min. The supernatant which contained biothiols in serum was used for further analysis. All the experiments were performed in compliance with the relevant laws and institutional guidelines, and approved by the relevant institutional committees (Ethics Committee of China Pharmaceutical University). Informed consent was obtained for all experiments involving human subjects.

**Results and discussion**

**Characterization of DNA-Cu/Ag NCs**

In the present study, DNA-Cu/Ag NCs synthesized by AgNO₃ serves as a silver ion source, Cu(NO₃)₂ as a copper ion source and NaBH₄ as a reducing agent in the presence of cytosine and thymine rich ssDNA with the sequence 5’-CCCTTAATCCCC-3’³². Poly T was found to be a specific template for CuNPs formation due to binding interactions between thymine and Cu²⁺ ions.³³ Poly C was found to produce brightly fluorescent DNA-Ag nanoclusters.³⁴ Based on these works, we used the ssDNA consisting of poly C, poly T, and A as the loop to synthesize fluorescent DNA-Cu/Ag NCs. During the preparation experiment of DNA-Cu/Ag NCs, the concentrations of Cu²⁺ and Ag⁺ were the key factors. We studied the effect of concentrations of Cu²⁺ and Ag⁺ on the fluorescence intensity of the prepared DNA-Cu/Ag NCs. We found that the fluorescence intensity of DNA-Cu/Ag NCs was the strongest when the molar ratio of DNA:CuSO₄:AgNO₃ in the reaction mixture was 1:3:7. In the following experiments, this preparation condition was used throughout.

In order to confirm the formation of DNA-Cu/Ag NCs, the DNA-Cu/Ag NCs obtained from the preparation was characterized by TEM and the element analysis was carried out by a matching spectrometer. It can be seen from Fig. 2(a) that the dispersion of DNA-Cu/Ag NCs is good and the particle size is relatively uniform. From the element analysis (Fig.2(b)), it can be observed that DNA-Cu/Ag NCs mainly contains elements such as C, O, Na, S, Ag and Cu. We speculate that the Ag and Cu elements are derived from synthetic raw materials and are the main constituent elements of the nanometer, while the C, O, Na and S elements are derived from buffers, reducing agents and templates DNA. At the same time, the particle size of the randomly selected nanoscale clusters in the field of vision is calculated and the particle size distribution in
Fig. 2(c) is obtained. The results show that the particle size of DNA-Cu/Ag NCs is evenly distributed, and the average particle size is 1.87 ± 0.28 nm ($n = 35$).

To further explore the optical properties of DNA-Cu/Ag NCs, UV absorption spectra and fluorescence spectra of the DNA-Cu/Ag NCs were recorded. As shown in Fig. 3, there is one absorption peak at 470 nm in the spectra of the DNA-Cu/Ag NCs conjugates. The maximum excitation wavelength of the obtained DNA-Cu/Ag NCs is almost the same as that of the corresponding UV absorption peak, and the corresponding fluorescence peak is 560 nm.

**Fluorescence quenching of PPI by Cu$^{2+}$**

Because of the strong complexation of PPI and Cu$^{2+}$, the fluorescence intensity of the prepared DNA-Cu/Ag NCs decreases. Cu$^{2+}$-PPI complex competed with the interaction of Cu$^{2+}$ and DNA template and thus inhibited the nanocluster generation, which led to the decrease of fluorescence. The effect of Cu$^{2+}$ concentration on the fluorescence intensity of DNA-Cu/Ag NCs was investigated. With 470 nm as the excitation wavelength, the fluorescence emission spectra and the fluorescence emission intensity at 560 nm were recorded respectively. The fluorescence intensity change rate ($I_0 - I$)/$I_0$ before and after the addition of PPI was calculated. As shown in Fig. 4(a), with the increase of Cu$^{2+}$ concentration from 4.0 µM to 10.0 µM, the fluorescence intensity of the obtained clusters increases. By calculating the fluorescence intensity change rate ($I_0 - I$)/$I_0$ (Fig. 4(b)), it can be observed that the fluorescence intensity change of the detection system was the largest when the Cu$^{2+}$ concentration was 6.0 µM. So 6.0 µM was chosen as the best Cu$^{2+}$ concentration in the experiment.

The pH of the buffer would affect the stability of DNA, the affinity of bases with metal ions, the presence of metal ions and the detected substances. Therefore, the effect of different pH buffer on DNA-Cu/Ag NCs system was investigated. Using MOPS as reaction buffer, the pH of solution was adjusted by varying the 1M NaOH volume. As shown in Fig. 5(a), after adding PPI, the pH value of the buffer has a significant effect on the fluorescence intensity of DNA-Cu/Ag NCs and the change rate of fluorescence intensity. First, with the increase of pH of buffer solution from 6.5 to 7.4, it can facilitate the formation of DNA-Cu/Ag NCs and the fluorescence intensity of DNA-Cu/Ag NCs has a trend of gradual increase. Then, the fluorescence intensity change rate ($I_0 - I$)/$I_0$ was calculated. As shown in figure 5b, the fluorescence intensity change rate of the detection system before and after the addition of PPI was the largest when the pH value is 7.0, so we chose the buffer solution of the pH value of 7.0.

**Selectivity of the DNA-Cu/Ag NCs for detecting PPI**

In order to confirm the specificity of the binding of PPI to Cu$^{2+}$, we examined the effect of other acid anion on the detection. When DNA-Cu/Ag NCs was synthesized, PPI, SO$_4^{2-}$, H$_2$PO$_4^-$, HPO$_4^{2-}$, CO$_3^{2-}$ and HCO$_3^-$ were added respectively (the final concentration was 10 umol/L). The same conditions were used to react and record the change rate of fluorescence intensity at the 560 nm emission peak under the excitation of 470 nm (Fig. 6(a)). It can be seen from the figure that the fluorescence intensity change rate of PPI is the most significant compared with the very weak response of other anions. Therefore, the DNA-Cu/Ag NCs prepared has a specific response to PPI, which lays a foundation for its application in practical biological samples.
Sensitive detection of PPI

To investigate the utility and sensitivity of this system as a quantitative fluorescence method for PPI detection, a series of PPI solute ions with concentrations from 0.5 μM to 5.0 μM were tested. As shown in Fig. 7(a), the fluorescence intensity of DNA-Cu/Ag NCs decreases with increasing PPI concentration. As shown in Fig. 7(b), the low concentration of PPI (0.5 μM – 5.0 μM) can cause a large degree of fluorescence quenching, and the fluorescence quenching rate has a good linear relationship with the concentration of PPI (Fig. 7(c)). When the concentration of PPI is greater than 5.0 μM, the frequency of the fluorescence quenching rate increases slowly. Among them, the linear regression equation between the concentration of PPI in the range of 0.5 μM – 5.0 μM and the change rate of fluorescence intensity is: \((I_0 - I)/I_0 = 0.08821C_{\text{PPI}} + 0.2312\) (\(R^2 = 0.9997\)), and the detection limit is 136.7 nmol/L according to the definition of detection limit = 3 \(\sigma_b\)/slope. The good linearity and low detection limit provide a necessary condition for the determination of PPI content in biological samples afterwards.

Sensitive detection of ALP

We also set a series of ALP solutions with concentrations from 0.02 to 0.20 U/mL to test this system. ALP was introduced into the solution of PPI in MOPS buffer (20 mM MOPS, pH 7.0) and subsequently incubated for 75 min at 37°C. Then, DNA, Cu\(^{2+}\), Ag\(^{+}\) were added into the mixture and incubated in an ice bath for 15 min. Finally, the mixture was reduced by the addition of NaBH\(_4\) at 37°C for 20 min. ALP catalyzed the hydrolysis of PPI to Pi which broke down the coordination between Cu\(^{2+}\) and PPI. The released Cu\(^{2+}\) promoted DNA-Cu/Ag NCs formation which exhibited a strong fluorescence. As shown in Fig. 8(a), the fluorescence intensity of DNA-Cu/Ag NCs increases with increasing ALP concentration. As shown in Fig. 8(b), the low concentration of ALP (0.02 to 0.10 U/mL) can cause a large degree of Fluorescence recovery, and the fluorescence recovery rate has a good linear relationship with the concentration of ALP (Fig. 8(c)). When the concentration of ALP is greater than 0.10 U/mL, the frequency of the fluorescence recovery rate increases slowly. Among them, the linear regression equation between the concentration of ALP in the range of 0.02 to 0.10 U/mL and the change rate of fluorescence intensity is \((I_0 - I)/I_0 = 161.0C_{\text{ALP}} - 2.481\) (\(R^2 = 0.997\)), and the detection limit is 0.01 U/mL.

Human serum analysis

To investigate the feasibility of the proposed method for practical applications, the detection of PPI in human serum samples was performed. The pretreated serum samples were diluted 100-fold and added different concentrations of ALP. The obtained ALP levels in three human serum samples were listed in Table 1. The recovery data ranging from 97.6% to 103.1% with a satisfactory RSD lower than 5.0 (Table 1), indicating convenient and promising analytical performance for detection of biothiols in serum samples.
Conclusions

In summary, by using single strand DNA as template, Cu (NO$_3$)$_2$ as a copper ion source, AgNO$_3$ as a silver ion source, NaBH$_4$ as a reductant, a simple and rapid synthesis of DNA-Cu/Ag alloy nanoclusters with strong fluorescence emission was developed. Then, the fluorescence quenching of DNA-Cu/Ag NCs caused by the strong complexation of Cu$^{2+}$ and PPi was used to obtain the linear relationship between the concentration of PPi and the change rate of the fluorescence intensity of DNA-Cu/Ag NCs, which provided a theoretical basis for the detection of the biological samples containing PPi. Meanwhile, because ALP catalyzed the hydrolysis of PPi to Pi, the complexation between Cu$^{2+}$ and PPi was destroyed and the fluorescence intensity increased. Based on this principle, we developed a new method for detecting PPi and ALP with a detection limit of 136.7 nmol/L and 0.01 U/mL, respectively.

Acknowledgements

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References

Table 1  Determination of P Pi in human serum samples

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Figure Captions

Fig. 1 Schematic illustration of the fluorescence sensing strategy for PPI and ALP detection based on DNA templated DNA-Cu/Ag NCs.

Fig. 2 (a) TEM image, (b) EDS image of the DNA-Cu/Ag NCs, and inset: (c) the size distribution histogram.

Fig. 3 UV-Vis and fluorescence spectra of the DNA-Cu/Ag NCs.

Fig. 4 Effects of the concentration of Cu$^{2+}$ on the fluorescence intensity of DNA-Cu/Ag NCs and the detection of PPI. Fluorescence emission spectra (a) of the reaction system containing different concentrations of Cu$^{2+}$ in the absence and presence of 5.0 μM PPI, and change rate of fluorescence intensity ($I_0 - I$)/$I_0$ responses to the different concentrations of Cu$^{2+}$(b).

Fig. 5 Effects of the pH value of MOPS buffer on the fluorescence intensity of DNA-Cu/Ag NCs and the detection of PPI. Fluorescence emission spectra (a) of the reaction system in buffer with different value in the absence and presence of 5.0 μM PPI, and change rate of fluorescence intensity ($I_0 - I$)/$I_0$ responses to the different pH values of MOPS buffer(b).

Fig. 6 The fluorescence intensity change ratio ($I_0 - I$)/$I_0$ values in response to PPI and other anions.

Fig. 7 Fluorescence emission spectra (a) of DNA-Cu/Ag NCs upon the addition of various concentrations of PPI (The concentration of PPI from top to bottom: 0, 0.5, 2.5, 5, 10, 25, 50, 75, 100 μM), and plot of fluorescence intensity change ratio ($I_0 - I$)/$I_0$ vs concentration of PPI (b), and inset: plot of linear region from 0.5 to 5.0 μM (c).

Fig. 8 Fluorescence emission spectra (a) of DNA-Cu/Ag NCs-PPI system upon the addition of various concentrations of ALP (The concentration of ALP from bottom to top: 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.15, 0.18 U), and plot of fluorescence intensity change ratio ($I_0 - I$)/$I_0$ vs concentration of ALP (b), and inset: plot of linear region from 0.02 to 0.1 U/mL (c).
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