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ARTICLE TYPE

Quantification of DNA through a fluorescence biosensor based on Click Chemistry

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A simple, sensitive and selective method for fluorescence determination of DNA using CuS particles based on click chemistry has been reported. Biotin modified capture DNA had been modified on Streptavidin Magnespheres Paramagnetic Particles (PMPs) and hybridized with the target-DNA (hepatitis B virus DNA as example), then target-DNA bound was hybridized with DNA-CuS particles and formed a sandwich like structure. The CuS particles on the sandwich structures can be destroyed by acid to form Cu(II), and Cu(II) can be reduced to Cu(I) by sodium ascorbate, which in turn catalyzes the reaction between a weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form a fluorescent 1,2,3-triazole compound. By this means, the target DNA concentration can be determined by the change of the fluorescence intensity of the system. It is found that the fluorescence increase factor has a direct linear relationship with the logarithm of target DNA concentrations in the range of 0.1 to 100 nM, and the detection limit was 0.04 nM (S/N=3). The proposed sensor not only allows high sensitivity and good reproducibility, but also has a good selectivity to single nucleotide mismatches.

Introduction

The development of detection methods for various analytes related to health and environment is important in analytical chemistry. DNA detection has attracted increasing attentions because of its important roles in pathogen analysis, genetic disorder diagnosis and forensic tests.[1,2] Traditional methods for DNA detection usually based on DNA microarrays and polymerase chain reaction (PCR).[3] These methods own the characters of high sensitivity and efficiency, but sophisticated instruments and well trained operators are required. Some simple methods, such as fluorescence,[4] nanomaterial-based amplification,[5] colorimetric,[6] electrochemistry and so on,[7] have been successfully developed and applied for DNA detection. These methods presented the characters of high sensitivity, simple equipment and easy operation.

Click chemistry possesses many significant advantages, such as excellent selectivity, high purity, high efficiency and mild reaction conditions.[8] Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) is one of the most mature click reactions, in which azide can react rapidly with terminal alkyne to form 1,2,3-triazole species under mild conditions in the presence of Cu(I) catalyst, which has been applied in diverse areas, such as surface modification, dendrimer design and drug discovery.[9] Early report showed that nonfluorescent 3-azidocoumarins can react with terminal alkynes to yield strong fluorescent 1,2,3-triazole products through CuAAC reaction.[10] Many sensitive biosensor had been developed based on this reaction for different targets, such as histidine,[11] copper in serum sample and pesticide residues.[12-13] But to the best of our knowledge, no report about DNA detection based on this reaction had been reported till now.

In this study, by introducing DNA-modified CuS particle to a DNA sandwich assay, a highly sensitive and selective fluorescence sensor for DNA (hepatitis B virus (HBV) DNA fragment has been chosen as an example) detection has been proposed. This sensor has combined the advantages of high selectivity of CuAAC reaction and high sensitivity of fluorescence detection method. The specificity and the reproducibility of the proposed sensor had been checked also.

Experimental

Chemicals

Sodium ascorbate, propargyl alcohol, imidazole and other reagents were obtained from Alfa Aesar China Co. Ltd. (Tianjin). The synthesis of 3-azido-7-hydroxycoumarin has been described elsewhere.[14] Streptavidin Magnespheres Paramagnetic Particles (PMPs) was purchased from Promega Corporation (Madison, USA). The other chemicals were bought from Shanghai Chemical Reagent Company (Shanghai, China) and used directly without further purification. 1-Ethyl-3-(3-dimethylammoniapropyl)-carbodiimide (EDC) and bovine serum albumin (BSA) were bought from Shanghai Sangon Biotech. Co. Ltd. (Shanghai, China), and the following oligonucleotides DNA (left to right: 5' to 3') were synthesized by Shanghai Sangon Biotech. Co. Ltd. (Shanghai, China):

5'-biotion modified capture DNA for HBV (Capt-DNA):

Biotin-AAAAAAAAAAAAACCTTTAACCTAA

3'-amino group modified DNA for CuS particles conjugation (NH_2-DNA):

TCCTCCCCCAACTCCTCCCAAAAAAAAAAAAA-NH_2

HBV DNA sequence (Target-DNA):

TGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGT

Mismatch sequence of adenine in target DNA (A-mis DNA):

TGGGAGGAGTGGGGGGAGGAGATTAGGTAAAAGGT

Mismatch sequence of guanine in target DNA (G-mis DNA):

TGGGAGGAGTGGGGGGAGGAGATTAGGTGAAAGGT

Mismatch sequence of cytosine in target DNA (C-mis DNA):

TGGGAGGAGTGGGGGGAGGAGATTAGGTCAAAGGT

Target DNA was designed to be much longer than capt-DNA used for the detection. In order to maximize its potential to recognize the nucleotide's base at the mismatch site, the locations of the single mismatch site were designed around the middle of the binding arms with capt-DNA. NH_2-DNA was designed to hybridize with the rest of target DNA which hybridized with capt-DNA. And amino group modified DNA reacted with carboxyl groups modified CuS particles under catalysis of EDC to form CuS particles modified DNA.

Buffer solution used in this study: 0.25 M NaCl, 0.15 M sodium phosphate buffer solution, pH 7.3, 0.05% Tween-20.

Synthesis of CuS particles

CuS particles with carboxyl groups were prepared according to the published method.[15] Briefly, 3.0 μL of mercaptoacetic acid as the stabilizer was added to 25 mL of 0.4 M $Cu(NO_3)_2$ solution, and the pH of the mixture was adjusted to 9.0 with 0.5 M NaOH solution. After the mixture was bubbled with nitrogen for 30 min, 25 mL of 1.34×10^{-3} M Na_2S solution was added to the solution dropwise. The reaction was carried out for 24 hours under nitrogen protection, and a brown colloid appeared gradually. After reaction, CuS particles with carboxyl groups were formed.

Modification of NH_2-DNA on CuS particles

200 μL of 0.1 M imidazole solution (pH 6.8) was added into NH_2-DNA (1.24 μM). After gentle shaking for 30 min, 100 μL of 0.1 M EDC solution (cross-linking agent) and 2.0 mL of CuS colloid were added to the mixture and reacted at room temperature for 24 h. Under these conditions, condensation between amino groups and carboxy groups was performed to form NH_2-DNA tagged with CuS particles, and then it was separated from other reagents by centrifugation at a rotate speed of 10000 rpm for 30 min. The precipitate was washed for 3 times with water and then re-suspended in water. The solution of CuS particles modified with DNA (DNA-CuS particles) was stored at 4 °C for the hybridizations later.

Procedures for DNA quantification

A portion of 0.6 mL of 1 mg/mL PMPs was washed by buffer solution once and then dispersed in 0.6 mL of buffer solution. Capt-DNA was added into the solution to achieve a final concentration of 0.5 μM, and the mixture was mixed on a shaker for 30 min at room temperature. The specific combination of streptavidin with biotin contributed to the combination of PMPs with capt-DNA.[16] After that, PMPs with capt-DNA were separated from the mixture by a magnet, for the reason that PMPs with paramagnetic were able to be attracted by a magnet. And then, they were further washed by buffer solution once and then dispersed in 0.6 mL of buffer solution. Consequently, PMPs with capt-DNA (PMPs-DNA) was formed.

PMPs-DNA solution (0.5 μM) and various concentrations of target-DNA were prepared in buffer solution. In order to ensure the formation of DNA double strand, the mixtures were heated to 37°C for 2.0 h. After that, the PMPs residue was washed by buffer solution containing 2.0 mg/mL BSA to remove unbound target-DNA and block nonspecific binding sites, then dispersed in 0.2 mL of buffer solution. DNA-CuS particles (0.465 μM) in buffer solution were added to the above DNA double strands solution, and the mixture was also heated to 37°C for 2.0 h. Therefore, DNA-CuS particles hybridized with target-DNA which had formed DNA double strands with PMPs-DNA previously to generate the sandwich like structure, then washed the sandwich like DNA residue again using buffer solution, and dispersed in 0.2 mL of buffer solution. Then HNO_3 (50 μM) was added to the above mixture to make CuS dissolved to produce Cu(II). 2.0 min later, propargyl alcohol (25 μM), 3-azido-7-hydroxycoumarin (25 μM) and sodium ascorbate (3 mM) were added into the above mixed solution. Cu(II) can react with sodium ascorbate to produce Cu(I). The reaction mixture was held for 3.0 h to make the CuAAC reaction between the azide and alkyne groups occurred efficiently in the presence of the Cu (I) catalyst at room temperature. Fluorescence spectra of the mixtures were recorded on a Varian Cary Eclipse at the excitation wavelength of 395 nm.

Results and Discussion

Design and characterization of the sensor

The principle of the fluorescent sensor was shown in Fig.1(A). The biotin modified capt-DNA with PMPs was hybridized to target-DNA, then target-DNA bound was hybridized to CuS particles modified DNA and formed the sandwich like structure. The sandwich like DNA can be separated easily by a magnet, which was re-dispersed into the buffer solution and the CuS particles in the sandwich like structure can be destroyed by acid to produce Cu(II). In the presence of sodium ascorbate, Cu(II) can be reduced to Cu(I) and which in turn initiates the CuAAC reaction between weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form the fluorescent 1,2,3-triazole compounds. Therefore, an obvious fluorescence enhancement can be identified. While a weaker fluorescence intensity can be observed in the absence of the target DNA because that the lack of the target-DNA hinders the formation of the DNA sandwich structure.

Simple experiments are performed to verify our principle. It is found that fluorescence intensity in the absence of the target DNA (curve a in Fig.1(B)) is much lower than that in the presence of target DNA (curve b in Fig.1(B)). The reason lies in that the lack of the target-DNA hindered the DNA sandwich hybridization, so the structure of DNA-CuS particles bound

sandwich can not be formed, consequently, there is no Cu(II) generated by acid, so Cu(I) can not exist in the reaction system, and the CuAAC reaction not occurred in the absence of Cu(I). Thus the reaction system shows weak fluorescence in the absence of the target DNA. On the contrary, if the target-DNA was added into the reaction system, a strong fluorescence signal can be observed. Moreover, a wavelength shift from 461 nm to 471 nm can be observed in Fig. 1(B). The reason lies in that the CuAAC reaction between the weak-fluorescent 3-azidocoumarin and propargyl alcohol occurs to yield the 1,2,3-triazole compound which showed a strong fluorescence at 471 nm in the presence of target DNA.

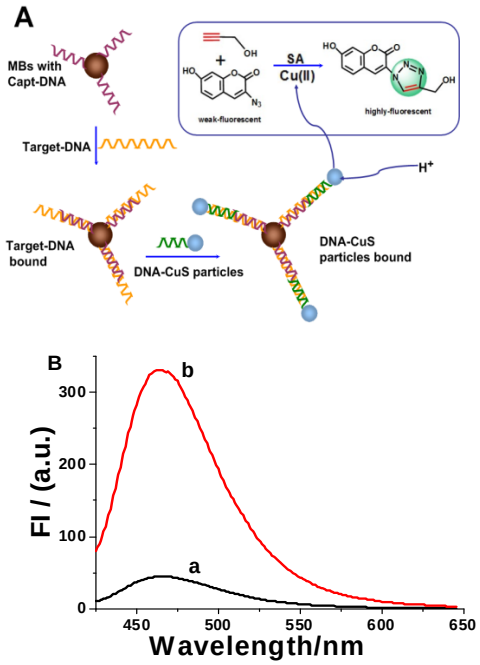

Fig. 1 (A) Schematic representation of the fluorescence sensor based on the CuAAC reaction. (B) The fluorescence of the system in the absence (a) and presence (b) of target-DNA. Excitation wavelength: 395nm, C_{azido}=2.5×10^{-5} M, C_{alkyne} =2.5×10^{-5} M, C_{SA}=3×10^{-3} M.

Optimization of the DNA sensor

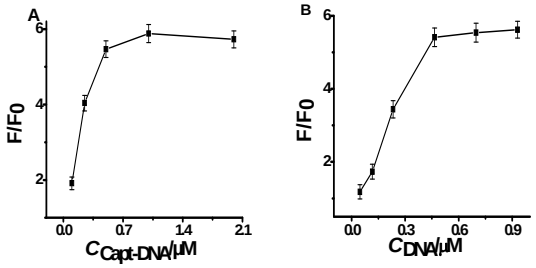

Fig. 2 (A) The relationship between the fluorescence increase factor and the Capt-DNA concentration. (B) The relationship between the fluorescence increase factor and the concentration of DNA which had been used to modified on CuS particles.

In order to perform the best performance of the system, some experimental conditions which affected the fluorescence enhancement had been studied. Firstly, the relationship between the fluorescence increase factor (defined as F/F_0, F and F_0 are defined as the fluorescent intensity of the sensor with and without target-DNA, respectively) and Capt-DNA concentration was studied (Fig. 2(A)). It was found that fluorescence increase factor increased with the enhancement of Capt-DNA concentration firstly and then reached a plateau when the Capt-DNA concentration was over 0.50 μM. So 0.50 μM of Capt-DNA had been chosen in the following study.

The effects of concentrations of DNA modified on CuS particles were investigated also (Fig. 2(B)). The fluorescence increase factor of the system increased with the extension of DNA concentrations in the range of 0.046 to 0.93 μM and then reached saturation at over 0.46 μM. Therefore, 0.46 μM of DNA was selected as the optimal concentration for the later experiments.

Quantification of DNA

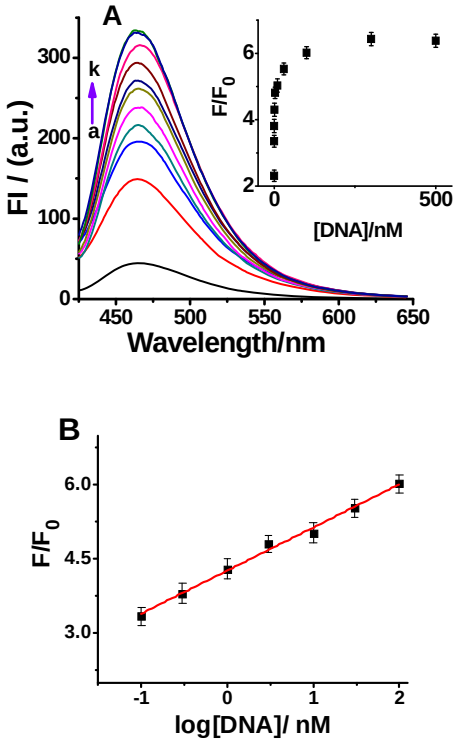

Fig. 3 (A) The fluorescence spectra at different concentrations of target DNA, from a to k: 0 nM, 0.03 nM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM and 500 nM; inset: the relationship between the values of F/F_0 and different DNA concentrations. (B) Target DNA concentration-dependent change in the fluorescence increase factor. The insert shows the calibration curve between the fluorescence increase factor and logarithm of target DNA concentrations

To study the feasibility of this method for the quantitative detection of target DNA, various concentrations of target DNA have been added and the fluorescence of the mixed solutions is monitored. Fig.3(A) shows the fluorescence spectroscopy at different DNA concentrations. It is found that the fluorescence intensity increases with the extension of the target DNA concentration in the range of 0.03 to 300 nM, and then reaches a plateau at over 300 nM. The reason may lie in that higher target

DNA concentration will cause higher CuS particles modified DNA to couple with the PMPs, resulting in that more Cu(II) can be reduced to Cu(I) by sodium ascorbate, which in turn initiates the CuAAC reaction between weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form more 1,2,3-triazole compounds, leading to the fluorescence intensity increase. However, the CuAAC reaction may have been complete when the target DNA concentration is over 300 nM, and the amount of 1,2,3-triazole compounds has no further increase, resulting in the fluorescence intensity has no obvious change.

Fig. 3(B) showed the relationship between the fluorescence increase factor and the target DNA concentrations. It is found that the fluorescence increase factor increases with the extension of target DNA concentration. And there is a good linear relationship between the fluorescence increase factor (Y) and the logarithm of target DNA concentration (X) in the range of 0.1 to 100 nM (Figure 3(B) inset). The equation is:

$$Y=4.27+0.87\log X \qquad R^2= 0.9927$$

The detection limit (LOD) is estimated to be 0.04 nM according to the definition of $3\sigma_b$/slope, where σ_b is defined as the standard deviation of the blank samples, slope is obtained from the calibration curve. The low LOD of the proposed method may lie in that CuS particles are released and functioned as a catalyst for the CuAAC reaction, which amplifies the variation of fluorescence responses at different target DNA concentrations. The LOD is better than the previously reported fluorescence methods, such as fluorescence turn-on detection (0.41 nM) and fluorescence resonance energy transfer amplification (0.077 nM).[17-18]

Reproducibility and selectivity

In order to investigate the reproducibility of the proposed sensor, five parallel prepared sensors were used to detect target DNA (10 nM). The relative standard deviation (RSD) is calculated to be 4.1%, this indicates that the proposed method has good reproducibility. The prepared sandwich like structure was stored in a refrigerator for six weeks at 4°C and then was used to test the fluorescence increase factor, the results show that there has no significant change compared with the freshly made one, which suggests that the prepared sandwich structure has good stability in a long time.

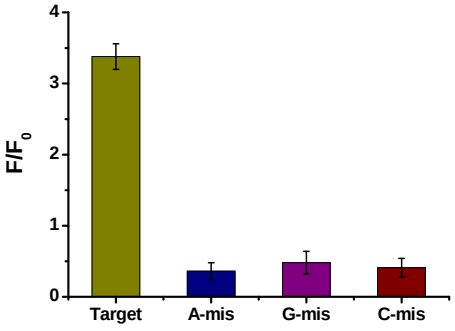

Fig. 4 Selectivity of the sensor towards target-DNA. The concentration of DNA is 0.1 nM. C_{azido}=2.5×10^{-5} M, C_{alkyne} =2.5×10^{-5} M, C_{SA}=3×10^{-3} M.

The selectivity of the present sensor was investigated by using NH_2-DNA labeled CuS particles to hybridize with the same concentration of target DNA or different single nucleotide mismatch target DNAs. As shown in Fig.4, the single nucleotide mismatch causes little changes of the fluorescence increase factor. It is because that the single nucleotide DNA may cause inefficient hybridization, but they could not stop hybridization thoroughly, and hybridization is enhanced with cooling.[19] So a few of them still hybridized with capt-DNA, and DNA with CuS particles to form the sandwich like structure at room temperature, finally induces the CuAAC reaction between 3-azido-7-hydroxycoumarin and propargyl alcohol to form a small amount of fluorescent 1,2,3-triazole compounds. This means the mismatch DNA sequences causes no obvious interference to target DNA detection, indicates that the proposed sensor has excellent selectivity for target DNA.

Conclusions

In summary, a novel fluorescence sensor for DNA has been proposed based on the CuAAC reaction. The Cu(II) comes from target-dependent binding of DNA-CuS particles, and can be reduced to Cu(I) by ascorbate, which in turn induces the CuAAC reaction between weak-fluorescent 3-azido-7-hydroxycoumarin and propargyl alcohol to form a fluorescent 1,2,3-triazole compound. It is found that the quantification of DNA is relevant to the fluorescent increase factors. In addition, the proposed sensor shows high sensitivity and good selectivity, even in the presence of single nucleotide mismatches. Moreover, this method may be helpful to expand the utility of click chemistry in fluorescence detection for bioassays.

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Notes and references

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1 Y. Huang, Y. Zhang, X. Xu, J. Jiang, G. Shen and R. Yu, *J. Am. Chem. Soc.*, 2009, **131**, 2478-2480.

2 W. Xu, X. Xue, T. Li, H. Zeng and X. Liu, *Angew. Chem. Int. Ed.*, 2009, **48**, 6849-6852.

3 G. M. Makrigiorgos, S. Chakrabarti, Y. Zhang, M. Kaur and B. D. Price, *Nat. Biotechnol.*,2002, **20**, 936-939.

4 S. Zhu, Z. Liu, W. Zhang, S. Han, L. Hu and G. Xu, *Chem. Commun.*, 2001, **47**, 6099-6101.

5 J. M. Nam, S. I. Stoeva and C. A. Mirkin, *J. Am. Chem. Soc.*, 2004, **26**, 5932-5933.

6 H. Zhou, J. Liu, J. Xu and H. Chen, *Chem. Commun.*, 2001, **47**, 8358-8360.

7 T. G. Drummond, M. G. Hill and J. K. Barton, *Nat. Biotechnol.*, 2003, **21**, 1192-1199.

8 H. C. Kolb, M. G. Finn and K.B. Sharpless, *Angew. Chem. Int. Ed.*, 2001, **40**, 2004-2021.

9 Y. Zhou, S. X. Wang, K. Zhang and X. Y. Jiang, *Angew. Chem. Int. Ed.*, 2008, **47**, 7454-7456.

10 R. F. H. Viguier and A. N. Hulme, *J. Am. Chem. Soc.*,2006, **128**, 11370-11371.

11 S. Y. Qiu, X. H. Li, W. M. Xiong, L. D. Xie, L. H. Guo and B. Qiu, *Biosens. Bioelectron.*, 2013, **41**, 403-408.

12 C. M. Wang, L. J. Lu, W. M. Ye, O. Zheng, B. Qiu, Z. Y. Lin, L. H. Guo and G. N.Chen, *Analyst.*,2014,**139**,656-659.

13 L. J. Lu, L. L. Yang, H. J. Cai, L. Zhang, Z.Y. Lin, L. H. Guo, B. Qiu and G. N. Chen, *Food. Chem.*,2014, **162**,242-246.

14 J. P. Collman, N.K. Devaraj and C. E. D. Chidsey, *Langmuir.*, 2004, **20**, 1051-1053.

15 T. N. Milica, I. C. Mirjana, V. Veana and I. M. Olga, *J. Phys. Chem.*, 1990, **94**, 6390-6396.

16 P.C. Weber, D. H. Ohlendorf, J. J. Wendoloski and F.R. Salemme, *Science.*, 1989, **243**, 85-88.

17 M. Wang, D. Q. Zhang, G. X. Zhang, Y. L. Tang, S. Wang and D. B. Zhu, *Anal Chem.*, 2008, **80**, 6443-6448.

18 L. G. Xu, Y. Y. Zhu, W. Ma, H. Kuang, L.Q. Liu, L. B. Wang and C. L. Xu, *J. Phys. Chem. C.*, 2011, **115**, 16315-16321.

19 C. H. Wohlgamuth, M. A. McWilliams and J. D. Slinker, *Anal. Chem.*, 2013, **85**, 1462-1467.