LANGMUIR

Interface Components: Nanoparticles, Colloids, Emulsions, Surfactants, Proteins, Polymers

Lighting Up Fluorescent Silver Clusters via Target-Catalyzed Hairpin Assembly for Amplified Biosensing

Min Pan, Meijuan Liang, Junlin Sun, Xiaoqing Liu, and Fuan Wang

Langmuir, **Just Accepted Manuscript** • DOI: 10.1021/acs.langmuir.8b01576 • Publication Date (Web): 25 Jul 2018

Lighting Up Fluorescent Silver Clusters via Target-Catalyzed Hairpin Assembly for Amplified Biosensing

Min Pan,[‡] Meijuan Liang,[‡] Junlin Sun, Xiaoqing Liu and Fuan Wang**

Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, Hubei, 430072, P. R. China

KEYWORDS: catalytic hairpin assembly; silver nanoclusters; lighting up; amplification; label-free; enzyme-free

ABSTRACT: Isothermal enzyme-free nucleic acid circuits have been developed for carrying out diverse functions ranging from dictate biocomputing to amplified biosensing. Catalytic hairpin assembly (CHA), the catalyzed cross-opening of two hairpin substrates by an initiator, attracts increasing attentions for its facile design and high amplification capacity. The complex labelling and frequent photobleaching of conventional fluorescent CHA biosensor still remains a challenge that needs to be solved. Herein, we constructed a new label-free and enzyme-free isothermal CHA lighting up AgNCs strategy for amplified nucleic acid assay by integrating the interfacially and spatially sensitive feature of DNA-templated fluorescent silver nanoclusters (DNA-AgNCs) and the high signal amplification capability of CHA circuit. In this strategy, one poly-guanine-grafted hairpin and the other AgNCs-capturing hairpin were engineered as assembly constitutes, which were kinetically impeded from cross-hybridizations without target. However, in the presence of target, the CHA-catalysed assembly of two functional hairpins was successively progressed and concomitantly accompanied by an efficient accommodation of AgNCs to the poly-guanine-elongated dsDNA product, leading to highly efficient AgNCs-lighting up and to the generation of an amplified fluorescence signal. As a simple mix-and-detect strategy, the isothermal enzyme-free CHA-mediated lighting up AgNCs (CHA-AgNCs) system provided a facile visualization way for amplified detection of DNA with a detection limit of 20 pM, which was comparable to or even better than some enzyme-involved amplification methods. The homogeneous CHA-AgNCs system can be utilized as a general sensing platform and be easily adapted for analyzing other biologically important analytes, e.g., microRNA (miRNA), by introducing the sensing module consisting of an auxiliary hairpin through an easy-to-integrate procedure. By taking advantage of the signal amplification features of CHA and the robust AgNCs-lighting up procedure, we anticipate that the CHA-lighting up AgNCs system can

provide an important tool for biomedicine and bioimaging applications and thus should hold great promise in clinical diagnose and treatment fields.

INTRODUCTION

Sequence-specific analysis of nucleic acids is of vital importance in clinical diagnosis and gene therapy.[1-3] Fluorescence assay enables a robust, sensitive and selective analysis of target, thus attracting more attentions in various research fields.[4-6] However, the cumbersome labelling and frequent photobleaching of conventional fluorophore probes represent a main challenge that needs to be solved before their extensive applications could be envisaged. Thus, the development of a robust and versatile fluorescent probe is more appealing for assembling DNA biosensors.

Recently, DNA-templated silver nanoclusters (DNA-AgNCs), a noble fluorescent nanomaterial with molecular-like properties, have received substantial interests due to their intrinsic merits of high biocompatibility, excellent photostability, tunable luminescence and sub-manometer size.[7-10] As a new signal transducer, the use of DNA-AgNCs not only avoid the covalent connection of DNA with signal indicators (organic dyes or quantum dots), but also make their integration into amplified biosensing easier *via* their corresponding DNA templates. Notably, Werner firstly reported on the fluorescent switchable DNA-AgNCs that were modulated by their nearby intrinsic guanine bases.[11-13] These AgNCs can be "lightened up" with 500-fold enhanced fluorescence once it is placed in close proximity with guanine-rich sequences (AgNCs enhancer). Two possible mechanisms are proposed to explain such an interesting phenomenon. One is the secondary structure of G-rich sequences (e.g., G-quadraplex) that favor the formation of red-emitting DNA-AgNCs. And the other is a possible electron transfer from guanine bases to DNA-AgNCs that trigger on their fluorescence. These features paved the way

on sensing nucleic acids through lighting up DNA-AgNCs by guanine-rich sequences. However, conventional AgNCs-lighting up strategy is usually based on a well-established stoichiometry procedure[12] and is usually suffered with insufficient sensitivity. It is interpretable since the target signaling occurs in an equivalent reaction ratio and usually requires delicately balanced affinities of interacting biomolecules in competitive assays. To overcome these drawbacks, different enzyme-involved isothermal amplification strategies, such as rolling cycle amplification (RCA)[14-17] has thus been developed for diverse sensing applications. Unfortunately, these enzyme-involved schemes might have the limitations of environmentally sensitive and poor repeatability. Moreover, AgNCs is easily susceptible to the surrounding protein enzymes and the accompanying enzymatic reaction conditions. Therefore, further efforts are needed in the development of new enzyme-free isothermally amplified AgNCs-based sensing platforms.

Catalytic hairpin assembly (CHA)[18] and hybridization chain reaction (HCR)[19] are two characteristic enzyme-free nucleic acid circuits with efficient signal amplifications. CHA is an inherently modular and scalable circuit, requiring only the design of base-pairing of strands. As a versatile isothermal amplification strategy, the target catalyses the assembly of two hairpins into dsDNA structures for amplified detection of nucleic acids,[20-22] proteins,[23-25] and small molecules.[26-27] Nevertheless, these CHA-mediated fluorescent assays usually require fluorophore-labels which are accessible to photobleaching and external interferers, making them costly and inconvenient biosensing systems. There is thereby an urgent demand and also a significant challenge to rationally design the label-free CHA-mediated sensing platform.

Herein, we constructed an isothermal non-enzymatic and label-free fluorescent sensing platform based on interfacially engineering DNA-templated silver clusters (AgNCs) and CHA-mediated lighting up of fluorescent AgNCs. It is expected that a proper integration of

autonomous CHA amplifier and robust AgNCs lighting up strategy could be implemented for more reliable and efficient biosensing. The AgNCs enhancer (G-rich) sequence and the AgNCs accommodation sequence were respectively grafted onto two different hairpin structures. The analyte initiated the CHA reaction that autonomously and successively brought the fluorescence enhancer and AgNCs into close proximity, leading to the fluorescence enhancement that could be visualized easily by a simple apparatus. In particularly, by incorporating an auxiliary sensing module, the integrated CHA-AgNCs system could be further adapted as a general sensing strategy for highly sensitive and selective detection of microRNA (miRNA-21) through a simple "plug-and-play" method. Given the attractive analytical characteristics of autonomous CHA-amplified lighting up of AgNCs, such as facile design, rapid operation, high sensitivity and selectivity, the unprecedented strategy holds great promise for biological and biomedical researches.

EXPERIMENTAL SECTION

Materials. DNA and RNA oligonucleotides were synthesized and HPLC-purified by Sangon Biotechnology (Shanghai, China) and Takara Biotechnology (Dalian, China), respectively. And these sequences were listed in Table S1. Stock solutions of DNA or RNA were prepared with phosphate buffer (RNase-free PB, 20 mM, pH 7.0) and stored at -20 °C. All other chemicals of analytical grade were purchased from Sigma-Aldrich (MO, USA) unless otherwise indicated. Ultrapure water used throughout the study was obtained by using a Milli-Q apparatus (Millipore, Bedford, MA).

Preparation of DNA-templated AgNCs. DNA-templated AgNCs with low fluorescence were prepared following the reported protocol with minor modifications.[11] Briefly, 15μL of the DNA

template oligonucleotides (100 μM) was mixed with 73 μL PB, followed by adding 4.5 μL of freshly prepared $AgNO_3$ (2 mM). After 15 min, 4.5 μL of freshly prepared $NaBH_4$ (2 mM) was introduced and incubated in dark at 25 °C for 18 h before use. The obtained DNA-AgNCs were then directly utilized without further purification or washing steps in the subsequent experiments. The absorption spectra of CHA-generated AgNCs were acquired by using a UV 2600 spectrometer (Shimadzu) with the wavelength ranging from 420 to 720 nm.

CHA-mediated lighting up AgNCs for amplified sensing. All of the assays were performed in reaction buffer (10 mM PB, 200 mM $NaNO_3$, 5 mM $Mg(NO_3)_2$, pH 7.0). Each hairpin probe (10 μM) was heated to 95 °C in reaction buffer and then slowly cooled down to room temperature for at least 2 h before use.

For detecting DNA analyte (I) by means of CHA-lighting up AgNCs, DNA analyte was introduced into the mixture of two hairpin probes (1 μM each) to initiate the self-assembly process at 25 °C for 2 h unless specified. Then the as-prepared AgNCs (2 μM) were introduced into the CHA mixture to get the fluorescence readout signal.

For amplified detection of microRNA target, miR-21 was added into the mixtures of a "helper" hairpin probe (H_3, 50 nM) and CHA hairpin probes (1 μM each) to initiate the self-assembly process at room temperature for 2 h unless specified. The AgNCs-involved transduction of microRNA is consistent with that of DNA assay. Unless specifically indicated, all of the control experiments were performed without changing the concentration of DNA probes except subtracting or replacing some of the oligonucleotides from these mixtures.

Fluorescence assay and visualization. All fluorescence measurements were performed by using a Cary Eclipse spectrometer (Varian Inc) at room temperature. The emission spectra of

AgNCs were acquired by exciting these samples at 580 nm, and the corresponding fluorescence emission spectra were collected from 600 to 780 nm. The kinetically monitoring the fluorescence changes were recorded at a fixed emission wavelength of 630 nm with an excitation wavelength of 580 nm.

Samples for visualizing were reacted in 1.5 mL Eppendorf tube. After 2 h of incubation, the samples were used for recording fluorescent spectra firstly and then transferred into the quartz cuvette for visualizing. For visualizing these AgNCs samples through fluorescence imaging, the lighting-up photo of the red-emitting AgNCs was carried out by incubating with different reaction system under visible and UV light (λ=365 nm). The AgNCs samples were hold in quartz cuvette and placed in a commercial gel imager. The image was acquired by using a digital camera and presented here without any contrast/color adjustment.

Gel electrophoresis analysis. For gel electrophoresis assay, 20 μL of DNA analyte (20 nM) was incubated with CHA mixtures (H_1 and H_2, 500 nM each) in reaction buffer (10 mM PB, 200 mM $NaNO_3$, 5 mM $Mg(NO_3)_2$, pH 7.0) for 2 h at room temperature that was similar to the fluorescent DNA assay. Then each of the samples was mixed with loading buffer and transferred into the notches of the freshly prepared 9% native polyacrylamide gel. Subsequently, electrophoresis was performed at a constant voltage of 100 V in 1×TBE buffer (89 mM Tris, 89 mM boric acid, 2.0 mM EDTA, pH 8.3) for 1 h. The gel was then stained with GelRed™ and imaged by FluorChem FC3 (Protein Simple, USA) under UV (λ=365 nm) irradiation.

Atomic force microscopy (AFM) characterization. DNA samples were prepared in reaction buffer (10 mM PB, 200 mM $NaNO_3$, 5 mM $Mg(NO_3)_2$, pH 7.0) for AFM characterizations. The hairpins H_1 and H_2 (500 nM each) and DNA-AgNCs (1 μM) were incubated with 20 nM DNA analyte for 2 h at room temperature. Prior to their use, all hairpin reactants (10 μM) were heated

to 95 °C for 5 min and cooled down to 25 °C for at least 2 h to form highly stable hairpin structures. Then, a freshly cleaved mica was modified (by dipping into 5 mM $MgCl_2$ for 5 min) to bear positive charges on its surface to facilitate DNA sample loading and AFM imaging. The generated CHA-AgNCs complex was diluted and deposited on freshly prepared mica for 15 min to allow its adsorption, followed by its rinsing with ultrapure water and drying under a stream of nitrogen. The prepared sample was scanned in tapping mode by Multimode 8 Atomic Force Microscope with a Nano Scope V controller (Bruker Inc.). The silicon tips used for AFM analysis were SCANASYST-AIR (tip radius: ~2 nm; resonance frequency: ~70 kHz; spring constant: ~0.4 N/m; length: 115 μm; width: 25 μm).

RESULTS AND DISCUSSION

Construction of the CHA-mediated lighting up AgNCs system. As shown in Figure 1A, the system consists of two CHA-involved hairpins (H_1 and H_2) and one DNA-templated AgNCs probe (DNA-AgNCs, P). H_1 includes the sequence a-b that is complementary to the analyte I. The loop region c of H_1 is complementary to the stem domain c* of H_2 while its stem region b* assists the formation of a thermally metastable hairpin structure. H_1 also includes an elongated G-rich domain d that functions as fluorescence enhancer in the subsequent AgNCs-lighting up mediated by CHA process. H_2 includes a partially complementary sequence b*-c* of H_1 that is elongated with caging sequence c and AgNCs capture sequence e* at its 5'- and 3'-end, respectively. The AgNCs probe P consists of a template sequence of AgNCs to accommodate low fluorescent AgNCs, and a capture sequence e to selectively immobilize fluorescence enhancer G-rich sequence. The dark AgNCs could be remarkably enhanced in fluorescence upon their connection with G-rich sequences.[25] The design of each DNA sequence is theoretically

optimized[28] and is listed in Table S1. In the absence of analyte (I), the AgNCs probe (P) connects with H_2 to form P-H_2 complex through e/e* duplex. However, these two hairpins are kinetically impeded, due to the firmly assembled hairpin structures, leading to no fluorescence enhancement. I opened H_1 via a toehold-mediated strand displacement mechanism, leading to the formation of sequence b* of H_1 and an intermediated I-H_1 hybrid. The newly exposed sticky docks to the toehold region b of H_2, and then opens H_2 *via* branch-migration, resulting in the generation of H_1-H_2 DNA duplex and the concomitant regeneration of analyte I for successively triggering subsequent CHA process. Thus, the CHA-catalysed toehold-mediated strand displacement triggers the successive hybridizations between hairpins H_1 and H_2 for continuously generating H_1-H_2 duplex product. The resulting DNA assembly, upon capturing AgNCs through e/e* hybridization, brings the AgNCs and G-rich enhancer sequence into close proximity to export high fluorescence readout of AgNCs. Therefore, by combination of autonomous CHA reaction and label-free AgNCs, the facile fluorescence amplification strategy can be successfully implemented for sensing application purpose. Figure 1B shows the time-dependent fluorescence changes of AgNCs upon incubating the CHA-AgNCs mixture with and without DNA analyte I. In the absence of I, the fluorescence intensity kept nearly unchanged (curve a of Figure 1B), indicating that the mixture is stable enough without any obvious signal leakage (spontaneous CHA reaction). However, when the CHA-AgNCs mixture was incubated with initiator I, a dramatically increased fluorescence was observed and it reached a plateau after ~2 h, curve b of Figure 1B. Figure 1C shows the corresponding fluorescence spectra of the CHA-AgNCs system. Clearly, the CHA-mediated regeneration of the analyte leads to a successive lightening up of AgNCs and the amplified fluorescence detection of the analyte I. The CHA-AgNCs system could be visualized by using digital camera under visible light and UV light (Figure 1C inset). No

visual color change could be observed for CHA-AgNCs system with and without target DNA under visible light. Meanwhile, a bright red-emitting fluorescence readout could be acquired for CHA-AgNCs system with target DNA while the non-triggered CHA-AgNCs system shows scarcely no visible fluorescence signal, implying that the present CHA-AgNCs system could be utilized as a powerful visualization-based sensing platform through a simple apparatus. Accordingly, a fixed time-interval of 2 h was chosen as the most appropriate reaction time for acquiring fluorescence spectra in the subsequent experiments. This ensures the complete CHA reaction and an efficient AgNCs coupling with its enhancer. Control experiments show that H_1 or/and H_2 alone has no (enhancement) effect on the fluorescence of AgNCs (Figure S1), indicating that the fluorescence enhancement of robust AgNCs indeed originates from the CHA-mediated hybridizations between H_1 and H_2 monomers.

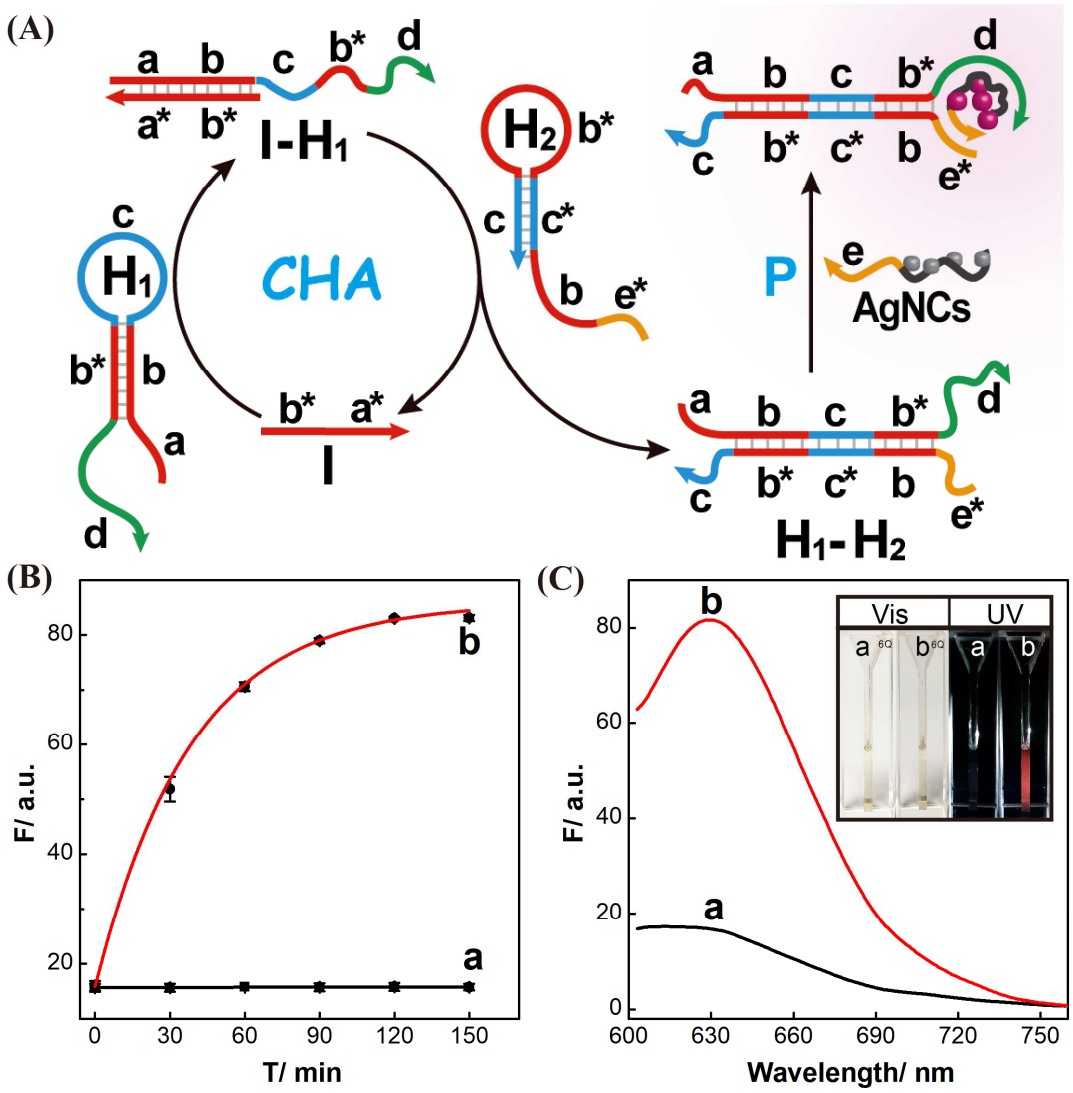

Figure 1. (A) Scheme of CHA-mediated lighting up of dark DNA-AgNCs for amplified DNA assay. (B) Time-dependent fluorescence changes and (C) fluorescence spectra of the CHA-AgNCs system without (a) and with (b) DNA analyte (100 nM), respectively. Inset: photograph of the lighting-up CHA-AgNCs system shown in (C) under visible and UV light (λ=365 nm). The CHA-AgNCs system was carried out in reaction buffer for a fixed time interval of 2 h. Error bars were derived from n = 5 experiments.

Feasibility of the CHA lighting up AgNCs system. Primary results have demonstrated that AgNCs can be lightened up by G-rich sequence through an indispensable integration of G-rich

enhancer sequence and dark AgNCs (Figure S2 and S3). This dramatically amplified fluorescence signals could be easily visualized and discriminated under UV light. An observable fluorescence of the red-emitting AgNCs was emerged for the amplified CHA-AgNCs system (with an amplification efficiency of N) while the fluorescence of the non-amplified stoichiometric AgNCs-based system (with an amplification efficiency of 1) is too weak to be observable, demonstrating the significant signal amplification features of the present CHA-AgNCs sensing strategy (Figure S4). The compatibly designed CHA circuit was further demonstrated by native polyacrylamide gel electrophoresis (PAGE) experiments, Figure 2A. Although H_1 (lane 1) and H_2 (lane 2) was encoded with complementary sequences, the H_1+H_2 mixture showed no change of their corresponding bands (lane 4), indicating that H_1 and H_2 were stable in the form of hairpins and the false cross interaction between H_1 and H_2 was effectively blocked without analyte. A distinct new band (lane 3) appeared and corresponded to I-H_1 complex when H_1 was incubated with analyte I, demonstrating that I-H_1 hybridization occupied in the early stage of CHA reaction. However, after introducing analyte I into the H_1+H_2 mixture, a new band corresponding to H_1-H_2 duplex appeared with slower electrophoretic mobility (lane 5), while the bands of H_1 and H_2 monomers became weakened and even vanished. This confirms the successful implementation of CHA circuit and the efficient formation of stable H_1-H_2 duplex with higher molecular weight. More importantly, the formation of H_1-H_2 duplex through CHA strategy is manifested as the mobility of CHA product is the same with that of the annealing product of H_1+H_2 mixture (lane 6). Gel electrophoresis shows a good agreement with that of fluorescence experiment in Figure 1B and 1C. In addition, the obtained CHA-AgNCs sample was characterized by AFM that showed highly ordered stripes over the entire area (Figure S5). In particular, the pitch of the stripes is measured to be ~2 nm, which corresponds to a typical height

of AgNCs.[23] Besides, the absorption spectrum of generated fluorescent AgNCs was also investigated (**Figure S6)**. The assembled CHA-AgNCs show an obvious absorption ranging from 500 nm to 600 nm (λ_{max} = 580 nm). Based on the aforementioned results, it is clear that the fluorescence activation is specially tuned by the interaction between AgNCs and G-rich sequences of CHA product. The AgNCs-lighting up strategy is attributed to two possible mechanisms. The secondary structure of G-rich sequences favors the assembly of stable DNA-AgNCs, and guanine base-donated electron transfer to silver nanoclusters that mediates the formation of red-emitting AgNCs.

CHA lighting up AgNCs for Amplified DNA assay. Based on the previous convincing results, it is evident the current CHA-AgNCs system as shown in Figure 1A can be adapt for the amplified detection of target DNA (Figure 2B). A substantial enhancement of fluorescence intensity was observed as the concentration of DNA analyte increased, consistent with the enhanced formation of H_1-H_2 duplex as a result of the CHA reaction, which then facilitates the subsequent integration of G-rich enhancer sequence with AgNCs and the generation of bright red-emitting AgNCs. The performance of traditional stoichiometric AgNCs-lighting up system (as non-amplified control) was also examined for analysing the same target of varied concentrations under the same incubation environment. A dramatically lower signal response was observed for non-amplified AgNCs system (Figure S4), which was reasonable since the amplification capacity of the CHA strategy (N) was much higher than that of the traditional stoichiometric AgNCs (1) system. By plotting the fluorescence intensity of AgNCs (λ=630 nm) with the concentration of DNA target, the respective derived calibration curves were collected for CHA-AgNCs and non-amplified AgNCs systems (Figure 2C). A linear correlationship between the fluorescence intensity and DNA concentration was acquired to be 0.5-15 nM and 1-

25 nM for the amplified CHA-AgNCs system and traditional non-amplified AgNCs system, respectively (Figure 2D). The detection limit of the CHA-AgNCs system corresponded to 20 pM according to the 3σ/S calculation method, which is much lower than that of non-amplified AgNCs system (450 pM). The stability of the DNA-AgNCs is of vital importance for acquiring a reliable sensing performance. The fluorescence stability of the CHA-generated DNA-AgNCs was thus explored with different incubation time and was shown in **Figures S7**. A negligible decreasement of the present fluorescent DNA-AgNCs was revealed after 4 h of light illumination, suggesting the reliable and robust features of our CHA-AgNCs-amplified sensing strategy. Noted that the present system is a more general sensing strategy since there is no specific G-rich sequence that needs to be considered even the signal amplification efficiency is different. It is reasonable since these two system are based on different working principles even the lighting up AgNCs-based transduction is the similar. The enhancer sequence is incubated with the AgNCs probe of our system without analyte while the G-rich enhancer sequence was utilized as analyst (at least partially) to lighten up fluorescent AgNCs.

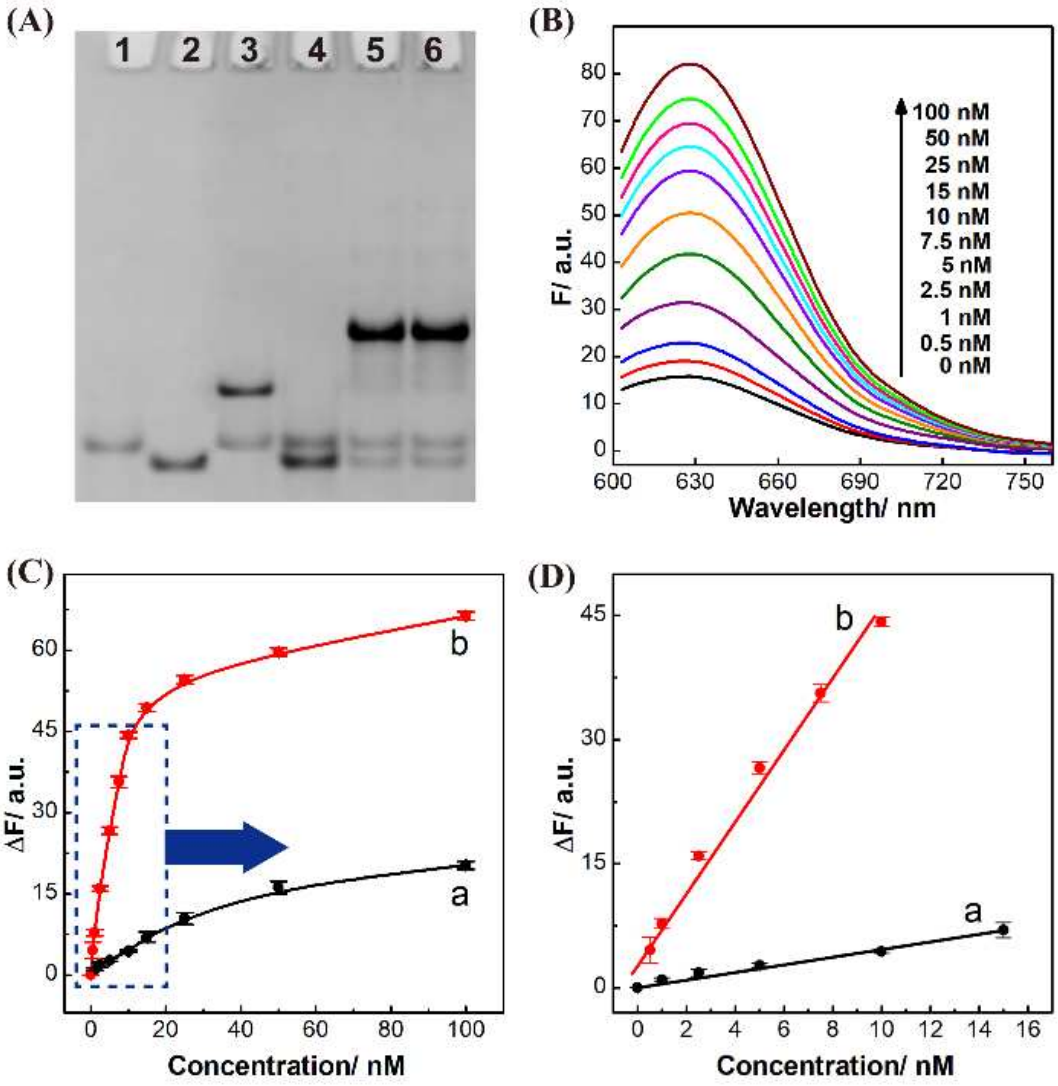

Figure 2. Validation of the CHA-AgNCs system for amplified DNA detection through the proposed CHA strategy. (A) Native gel electrophoresis characterization of the CHA-AgNCs system. Lane 1: H_1; lane 2: H_2; lane 3: I+H_1; lane 4: H_1+H_2; lane 5: H_1+H_2+I; lane 6: H_1+H_2 (slow annealing). (B) Fluorescence spectra of the CHA-AgNCs sensing platform in the presence of different concentrations of DNA analyte I. Resulting full range calibration curve (C) and the corresponding expanded linear calibration curve (D) of the non-amplified system (a) and the amplified CHA-AgNCs system (b). The respective AgNCs-based systems were carried out in reaction buffer for a fixed time-interval of 2 h. Error bars were derived from n = 5 experiments.

Amplified microRNA assay based on CHA lighting up AgNCs. The present optimized CHA-AgNCs system represents a general sensing strategy, which can be easily adapted for analysing other nucleic acid targets, e.g.; microRNA (miRNA), by introducing an additional sensing module. As a group of endogenous noncoding RNA of ~20 nt, miRNA plays a key role in regulating the expression levels of intracellular mRNA and also shows great potential in cancer diagnosis and prognosis fields.[29-31] Here, microRNA-21 (miR-21) was chosen as the model target. Aberrant miR-21 is overexpressed in a large variety of human cancers and has been recognized as an important oncogenic biomarker. As shown in Figure 3A, an additional sensing module consisting of a "helper" hairpin H_3 was simply introduced into the CHA-AgNCs system to recognize miR-21, and upon opening, it triggered the CHA-mediated successive lighting up AgNCs and the efficient conversion of dark AgNCs into bright red-emitting AgNCs, giving rise to the turn-on amplified detection of miR-21 target. Figure S9 depicts the time-dependent fluorescence changes of the updated CHA-AgNCs sensing platform without and with miR-21 target. Scarcely no fluorescence change was observed for non-triggered CHA-AgNCs system, demonstrating the high stability of the updated CHA-AgNCs-involved sensing platform, curve a of Figure S9. A remarkably intensified fluorescence was observed for miR-21-triggered CHA-AgNCs system and it reached to a saturation plateau after ca. 80 min, curve b of Figure S9. Thus, a fixed time interval of 80 min was chosen to acquire the fluorescence spectra subsequently. Figure 3B depicts the fluorescence spectra of the sensing module-integrated CHA-AgNCs system upon analysing different concentrations of miR-21 analyte. From the derived calibration curve (Figure 3C), the updated CHA-AgNCs system enabled the amplified detection of miR-21 with a detection limit corresponding to 38 pM (based on $3\sigma/S$ method) in a linear range of 0.75-15 nM, which is comparable to and even better than most of the enzyme-free AgNCs-based

sensing platforms (Table S2). Thus, by integrating a sensing module (foreign helper DNA), the CHA-mediated successively lighting up AgNCs scheme can be used as a general signal amplification module without further significant alteration of the present CHA-AgNCs system, and is specifically suitable for sensitively analysing trace amount of analyte through a simple "plug-and-play" approach.

Selective performance of CHA lighting up AgNCs. The updated CHA-AgNCs system shown in Figure 3A is not only sensitive, but also selective. To evaluate the specificity of the present miRNA detection system, the miR-21-targeting CHA-AgNCs platform was challenged with a series of interfering nucleic acids: β-actin mRNA, son DNA and let-7a miRNA, Figure S10 and Figure 3D. One may realize that the fluorescence of AgNCs generated by the different interfering nucleic acids is very close to the background signal of the system (without analyte). And only miR-21 could generate remarkable fluorescence change through the proposed CHA-AgNCs sensing strategy. Surprisingly, these signal differences could even be visualized and discriminated under UV light (Figure S10 inset). Obviously, only miR-21 can trigger our updated CHA-AgNCs system and generate bright red-emitting AgNCs. This comparison clearly demonstrates the high selectivity of our CHA-AgNCs system that can offer potential applications for discriminating target miRNA from other biological interference sequences in complex clinical samples. The present CHA-AgNCs could be furtherly extended to a more complicated biological environments, such as *in vivo* living cells, once the issue of biothiol interference could be solved in an appropriate way.

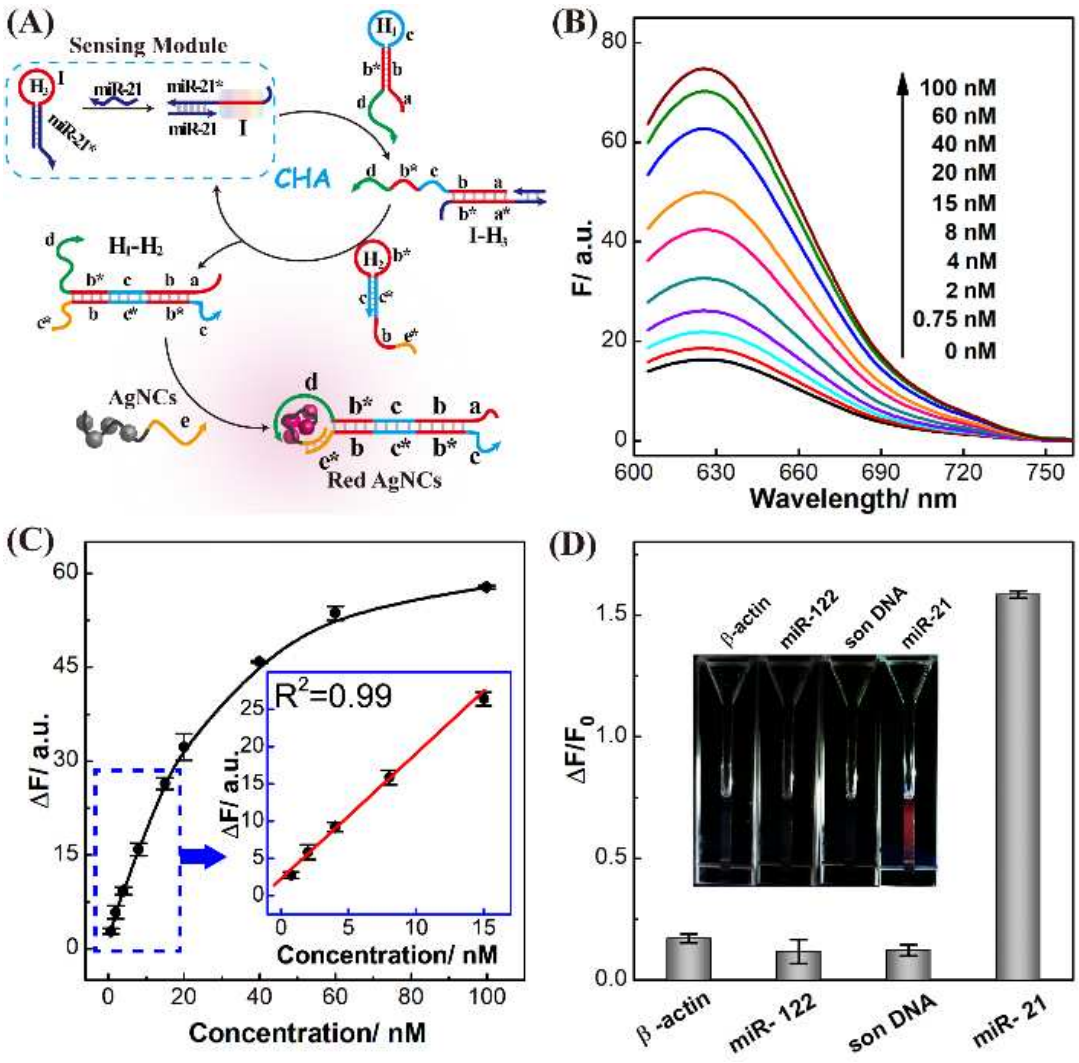

Figure 3. Amplified detection of miR-21 based on integrating an additional sensing module with the present CHA-AgNCs system. (A) Scheme of the general sensing platform by introducing a sensing module (consisting of H_3) into the well-established CHA-AgNCs system. (B) Fluorescence spectra of the updated CHA-AgNCs system upon analyzing different concentrations of miR-21 target. (C) Resulting calibration curve of miR-21-targeting CHA-AgNCs system. Inset: expanded linear calibration curve. (D) Selectivity of the present CHA-AgNCs system (miR-21 and interfering RNAs, 15 nM each), where F_0 represents the original fluorescence intensity of the system. The present miR-21-targeting system was incubated with

the corresponding analyte in reaction buffer for a fixed time interval of 80 min. Error bars were derived from n =5 experiments.

CONCLUSIONS

In summary, we have developed a new paradigm for isothermal enzyme-free and label-free amplified nucleic acid detection by combining the highly efficient signal-amplification capability of CHA reaction with the spatially sensitive fluorescent AgNCs. The analyte initiated the CHA reaction that autonomously and successively brought the fluorescence enhancer and AgNCs into close proximity, leading to the fluorescence enhancement that could be visualized easily by a simple apparatus. It is a simple amplification method that relies only on hybridization-involved strand-displacement reaction, without additional perishable enzymes, special instrumentations, and complicated thermal cycling procedures. The present CHA-lighting up AgNCs system is inherently modular and scalable, which enables us to utilize it as a versatile sensing platform for detecting other clinical important analyte, e.g., miRNA, through a convenient "plug-and-play" approach. The facile mix-and-detect operation of the present CHA-mediated visualization method could be carried out by a simple apparatus, opened up the possibility of an on-site fluorescence point-of-care testing (POCT) method and even smart diagnose devices through its integration with frequently used smartphones. The flexible and programmable nature of homogeneous CHA-lighting up AgNCs provides a versatile and robust toolbox for a wide range of fields, such as in vivo bioimaging, diagnosis and environmental monitoring, and even aptamer-related targets.

ASSOCIATED CONTENT

Supporting Information

DNA sequences, summary of Ag NCs-sensing platforms, demonstration of CHA-lighting up AgNCs, effect of CHA hairpin constitutes and mutant hairpin monomers on AgNCs, comparison of the non-amplified and amplified CHA-AgNCs systems, AFM and UV-vis characterizations, stabilities of the CHA-AgNCs system, traditional stoichiometric AgNCs-lighting up strategy, optimization and selectivity of CHA-AgNCs system. The Supporting Information is available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: xiaoqingliu@whu.edu.cn; fuanwang@whu.edu.cn.

Author Contributions

‡ M. Pan and M. Liang contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by National Basic Research Program of China (973 Program, 2015CB932601), Hubei Provincial Natural Science Foundation of China (2015CFB503), Jiangsu Provincial Natural Science Foundation of China (BK20161248, BK20160381), National Natural Science Foundation of China (21503151, 81602610) and 1000 Young Talent (to F.W. and X.L.).

REFERENCES

(1) Deng, R.; Zhang, K.; Li, J. Isothermal Amplification for MicroRNA Detection: From the Test Tube to the Cell. *Acc. Chem. Res.* **2017**, *50*, 1059-1068.

(2) Wang, F.; Lu, C.-H.; Willner, I. From Cascaded Catalytic Nucleic Acids to Enzyme-DNA Nanostructures: Controlling Reactivity, Sensing, Logic Operations, and Assembly of Complex Structures. *Chem. Rev.* **2014**, *114*, 2881-2941.

(3) Zhang, H.; Li, F.; Dever, B.; Li, X.-F.; Le, X. C. DNA-Mediated Homogeneous Binding Assays for Nucleic Acids and Proteins. *Chem. Rev.* **2013**, *113*, 2812-2841.

(4) Jans, H.; Huo, Q. Gold Nanoparticle-Enabled Biological and Chemical Detection and Analysis. *Chem. Soc. Rev.* **2012**, *41*, 2849-2866.

(5) Zhang, K.; Deng, R.; Li, Y.; Zhang, L.; Li, J. Cas9 Cleavage Assay for Pre-screening of sgRNAs Using Nicking Triggered Isothermal Amplification. *Chem. Sci.* **2016**, *7*, 4951-4957.

(6) Causa, F.; Aliberti, A.; Cusano, A. M.; Battista, E.; Netti, P. A. Supramolecular Spectrally Encoded Microgels with Double Strand Probes for Absolute and Direct miRNA Fluorescence Detection at High Sensitivity. *J. Am. Chem. Soc.* **2015**, *137*, 1758-1761.

(7) Choi, S.; Dickson, R. M.; Yu, J. Developing Luminescent Silver Nanodots for Biological Applications. *Chem. Soc. Rev.* **2012**, *41*, 1867-1891.

(8) Tao, Y.; Li, M.; Ren, J.; Qu, X. Metal Nanoclusters: Novel Probes for Diagnostic and Therapeutic Applications. *Chem. Soc. Rev.* **2015**, *44*, 8636-8663.

(9) Gong, L.; Kuai, H.; Ren, S.; Zhao, X.-H.; Huan, S.-Y.; Zhang, X.-B.; Tan, W. Ag Nanocluster-Based Label-Free Catalytic and Molecular Beacons for Amplified Biosensing. *Chem. Commun.* **2015**, *51*, 12095-12098.

(10) Guo, W.; Yuan, J.; Dong, Q.; Wang, E. Highly Sequence-Dependent Formation of Fluorescent Silver Nanoclusters in Hybridized DNA Duplexes for Single Nucleotide Mutation Identification. *J. Am. Chem. Soc.* **2010**, *132*, 932-934.

(11) Yeh, H.-C.; Sharma, J.; Han, J. J.; Martinez, J. S.; Werner, J. H. A DNA-Silver Nanocluster Probe that Fluoresces upon Hybridization. *Nano Lett.* **2010**, *10*, 3106-3110.

(12) Yeh, H.-C.; Sharma, J.; Shih, I.-M.; Vu, D. M.; Martinez, J. S.; Werner, J. H. A Fluorescence Light-Up Ag Nanocluster Probe That Discriminates Single-Nucleotide Variants by Emission Color. *J. Am. Chem. Soc.* **2012**, *134*, 11550-11558.

(13) Sharma, J.; Yeh, H.-C.; Yoo, H.; Werner, J. H.; Martinez, J. S. Silver Nanocluster Aptamers: in Situ Generation of Intrinsically Fluorescent Recognition Ligands for Protein Detection. *Chem. Commun.* **2011**, *47*, 2294-2296.

(14) Chen, J.; Ji, X.; He, Z. Smart Composite Reagent Composed of Double-Stranded DNA-Templated Copper Nanoparticle and SYBR Green I for Hydrogen Peroxide Related Biosensing. *Anal. Chem.* **2017**, *89*, 3988-3995.

(15) Deng, R.; Tang, L.; Tian, Q.; Wang, Y.; Lin, L.; Li, J. Toehold-initiated Rolling Circle Amplification for Visualizing Individual MicroRNAs In Situ in Single Cells. *Angew. Chem. Int. Ed.* **2014**, *53*, 2389-2393.

(16) Wang, F.; Lu, C.-H.; Liu, X.; Freage, L.; Willner, I. Amplified and Multiplexed Detection of DNA Using the Dendritic Rolling Circle Amplified Synthesis of DNAzyme Reporter Units. *Anal. Chem.* **2014**, *86*, 1614-1621.

(17) Deng, R.; Zhang, K.; Sun, Y.; Ren, X.; Li, J. Highly Specific Imaging of mRNA in Single Cells by Target RNA-initiated Rolling Circle Amplification. *Chem. Sci.* **2017**, *8*, 3668-3675.

(18) Yin, P.; Choi, H. M. T.; Calvert, C. R.; Pierce, N. A. Programming Biomolecular Self-Assembly Pathways. *Nature* **2008**, *451*, 318-322.

(19) Dirks, R. M.; Pierce, N. A. Triggered Amplification by Hybridization Chain Reaction. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 15275-15278.

(20) Yin, F.; Liu, H.; Li, Q.; Gao, X.; Yin, Y.; Liu, D. Trace MicroRNA Quantification by Means of Plasmon-Enhanced Hybridization Chain Reaction. *Anal. Chem.* **2016**, *88*, 4600-4604.

(21) Su, F.-X.; Yang, C.-X.; Yan, X.-P. Intracellular Messenger RNA Triggered Catalytic Hairpin Assembly for Fluorescence Imaging Guided Photothermal Therapy. *Anal. Chem.* **2017**, *89*, 7277-7281.

(22) Li, B.; Jiang, Y.; Chen, X.; Ellington, A. D. Probing Spatial Organization of DNA Strands Using Enzyme-Free Hairpin Assembly Circuits. *J. Am. Chem. Soc.* **2012**, *134*, 13918-13921.

(23) Tang, Y.; Lin, Y.; Yang, X.; Wang, Z.; Le, X. C.; Li, F. Universal Strategy To Engineer Catalytic DNA Hairpin Assemblies for Protein Analysis. *Anal. Chem.* **2015**, *87*, 8063-8066.

(24) Zheng, A.-X.; Li, J.; Wang, J.-R.; Song, X.-R.; Chen, G.-N.; Yang, H.-H. Enzyme-Free Signal Amplification in the DNAzyme Sensor via Target-Catalyzed Hairpin Assembly. *Chem. Commun.* **2012**, *48*, 3112-3114.

(25) Orbach, R.; Guo, W.; Wang, F.; Lioubashevski, O.; Willner, I. Self-Assembly of Luminescent Ag Nanocluster-Functionalized Nanowires. *Langmuir* **2013**, *29*, 13066-13071.

(26) Li, B.; Ellington, A. D.; Chen, X. Rational, Modular Adaptation of Enzyme-Free DNA Circuits to Multiple Detection Methods. *Nucleic Acids Res.* **2011**, *39*, e110-e110.

(27) Wu, Z.; Fan, H.; Satyavolu Nitya Sai, R.; Wang, W.; Lake, R.; Jiang, J. H.; Lu, Y. Imaging Endogenous Metal Ions in Living Cells Using a DNAzyme-Catalytic Hairpin Assembly Probe. *Angew. Chem. Int. Ed.* **2017**, *56*, 8721-8725.

(28) Zadeh, J. N.; Steenberg, C. D.; Bois J. S.; Wolfe B. R.; Pierce M. B.; Khan A. R.; Dirks R. M.; Pierce N. A. NUPACK: Analysis and design of nucleic acid systems. *J. Comput. Chem.* **2010**, *32*, 170-173.

(29) Lu, W.; Chen, Y.; Liu, Z.; Tang, W.; Feng, Q.; Sun, J.; Jiang, X. Quantitative Detection of MicroRNA in One Step via Next Generation Magnetic Relaxation Switch Sensing. *ACS Nano* **2016**, *10*, 6685-6692.

(30) Lu, J.; Getz, G.; Miska, E. A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebert, B. L.; Mak, R. H.; Ferrando, A. A.; Downing, J. R.; Jacks, T.; Horvitz, H. R.; Golub, T. R. MicroRNA Expression Profiles Classify Human Cancers. *Nature* **2005**, *435*, 834-838.

(31) Chang, K.; Elledge, S. J.; Hannon, G. J. Lessons from Nature: microRNA-Based shRNA Libraries. *Nat. methods* **2006**, *3*, 707-714.

Table of Contents use only