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Periodic Fluorescent Silver Clusters Assembled by Rolling Circle Amplification and Their Sensor Application

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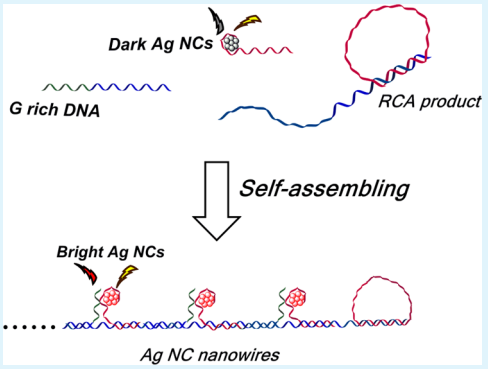
Supporting Information

ABSTRACT: A simple method for preparing DNA-stabilized Ag nanoclusters (NCs) nanowires is presented. To fabricate the Ag NCs nanowires, we use just two unmodified component strands and a long enzymatically produced scaffold. These nanowires form at room temperature and have periodic sequence units that are available for fluorescence Ag NCs assembled which formed three-way junction (TWJ) structure. These Ag NCs nanowires can be clearly visualized by confocal microscopy. Furthermore, due to the high efficiency of rolling circle amplification reaction in signal amplification, the nanowires exhibit high sensitivity for the specific DNA detection with a wide linear range from 6 to 300 pM and a low detection limit of 0.84 pM, which shows good performance in the complex serum samples. Therefore, these Ag NCs nanowires might have great potential in clinical and imaging applications in the future.



KEYWORDS: *silver cluster, rolling circle amplification, self-assembly*

INTRODUCTION

DNA-based assemblies of nanomaterials offer the possibility to fabricate nanobiomaterials with precise structures, versatile functions and numerous applications.[1−4] Due to DNA's remarkable molecular recognition properties, many strategies, such as tile assembly, scaffolded origami and DNA bricks, have been developed to design and produce 1D, 2D, and 3D architectures with sophisticated morphologies.[5−7] Particularly, since DNA origami technology was reported by Rothemund in 2006,[8] this bottom-up strategy had widely used in well-define construction of nanomaterials[9−11] and nanosensor.[12] However, large scale staple strands and precise controlled annealing protocols increase the limitation of this strategy.

Rolling circle amplification (RCA) is an isothermal, enzymatic process mediated by certain DNA polymerases in which long single stranded (ss) DNA molecules are synthesized on a short circular ssDNA template by using a single DNA primer.[13] As an effective DNA amplification tool, RCA was explored as an important technique for ultrasensitive DNA, RNA, and protein detection in diagnostic genomics and proteomics.[14−16] Recently, RCA product was demonstrated as nanoscaffold for nanostructure construction and nanomaterials assembly.[17−20] For example, Sleiman and co-workers fabricated DNA nanotube and AuNP assemblies based on a long continuous scaffold produced by RCA.[21,22] Compare with the long scaffold ssDNA in conventional DNA origami (typically 7249 kb M13 bacteriophage genomic DNA), the RCA product is characteristic of relatively short periodic sequences which can be easily folded into well-defined structure.[23] In addition, these programmable DNA nanostructures based on RCA products were further applied in biomedical applications. Tan et al. and Chen et al. attached the dye-labeled sequence or multi-dye-modified dUTPs on the replicated product for single molecule detection and multiplex fluorescent cellular imaging.[24−27]

DNA stabilized fluorescent silver nanoclusters (DNA-Ag NC) have emerged as new powerful fluorescence nanomaterials in biology.[28−31] Compared with conventional fluorescent materials such as quantum dots and organic dyes, silver nanoclusters (Ag NCs) show advantages of easy preparation[32] and functionalization,[33] smaller size, and lower toxicity.[34,35] It is well-known that DNA template Ag NCs have sequence dependent fluorescence.[36] Some works focus on spatially controlling cluster assembly through DNA nanostructure. Yan first employed DNA origami structure as nanoscaffold for site-specific synthesis of Ag NCs by using Tollens regeant.[37] O'Neill fabricated Ag NC nanotubes using nanotubes assembled from DNA tiles, with hairpin appendages on a subset of the tiles serving as the cluster growth sites.[38] Willner and co-workers controlled assembly of Ag NCs through nucleic-acid-triggered hybridization chain reaction and Y-shape DNA in linear and dendrimer-like structures, respectively.[39,40] However, all these assembly strategies are based on complex nuclear acid design and strict annealing processes. Furthermore, an intrinsic limitation of this type of method is that Ag NC synthesis is an integrated step of the detection process when it is involved in analysis applications, and a few hours are often needed to generate fluorescence, limiting its practical

Received: June 23, 2014
Accepted: August 12, 2014
Published: August 12, 2014

applications.[30] Herein, we report an approach to assemble Ag NCs and a "G-rich helper" strand into nanowires by long repetitive DNA strands synthesized from rolling circle amplification. In this approach, Ag NCs were presynthesized independently. The long enzymatically produced scaffold induces plenty of Ag NCs and the "helper" strands in proximity, which brings the end of the "G-rich helper" to close proximity to Ag NCs, resulting in a significant fluorescence enhancement.[41] Because of the tunable fluorescence intensity of Ag NCs, these nanowires exhibit luminescent function in a sensor application. To the best of our knowledge, the present work is the first constructing Ag NC nanowires as a signal amplification biosensor, based on guanine-rich DNA sequences enhancing fluorescence.

EXPERIMENTAL SECTION

Materials. Silver nitrate ($AgNO_3$) and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (St. Louis, MO). T4 DNA ligase and dNTP were obtained from Takara (Dalian, China), while Phi29 DNA polymerase was purchased from New England Biolabs (Beijing, China). All oligonucleotides with different sequences were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences of the oligonucleotide used in this work are as follows:

primer	5′-GGCGAGACACGGTGCT-GCAG3′
padlock	5′-P-GTGTCTCGCCTTCTT-GTTTCCTTTCCTTGAAACT-TCTTTCTTTCTTCGCTGCAG-CACC-3′
nuclear sequence	5′-TTCTTGTTTCCTTTCCTTG-CCCTTAATCCCC-3′
G-rich helper sequence	5′-GGGTGGGGTGGGGTGGGG-AAACTTCTTTCTTTCTTCG-3′
MT1	5′-GGCGAGACAGGGTGCT-GCAG-3′
MT	5′-GGCGTGACAGGGTGCTG-CAG-3′
MT3	5′-GGCGTGACAGGGTGCTC-CAG-3′

Apparatus. Fluorescence spectra were obtained with a RF-5301PC spectrophotometer (Shimadzu, Japan) equipped with a 150 W xenon lamp (Ushio Inc., Japan). Gels were imaged by using a ChemiDoc X-ray diffraction (XRD) system (Bio-Rad). Morphology observation of Ag NCs and self-assembled Ag NCs in PBS buffer (pH 7.0, 20 mM) was carried via transmission electron microscopy (TEM; JEM-2100 microscope) at an acceleration voltage of 200 kV. The morphology of Ag NC nanowires was characterized with a Veeco Multimode atomic force microscope (Veeco). The fluorescence was analyzed by confocal laser scanning microscopy (Andor RevolutionXD, United Kingdom) equipped with a 543 nm argon laser for elf-assembled Ag NCs.

Preparation of Ag Nanocluster. DNA stabilized Ag nanoclusters were synthesized according to a literature procedure with some modifications.[41,42] Briefly, 15 μL of 100 μM nuclear sequence was mixed with 73 μL of 20 mM phosphate (pH 7.0) buffer, and then 6 μL of 1.5 mM $AgNO_3$ solution was added to provide a Ag^+-to-DNA molar ratio of 6:1. After cooling at 4 °C for 15 min, this mixture was reduced quickly by 6 μL of 1.5 mM $NaBH_4$, followed by vigorous shaking for 1 min. The reaction was kept in the dark at 4 °C for 6 h before use.

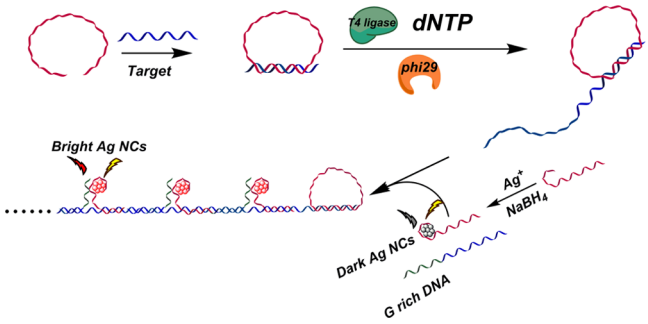
Ligation and RCA Reaction. For the ligation reactions with T4 DNA ligase, the reaction mixture consisted of ligation buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM ATP), 350 U of T4 DNA ligase, 0.5 μM padlock probe, and an appropriate amount of target DNA in a reaction volume of 50 μL. Before the ligase was added, the reaction mixture was heated at 90 °C for 10 min. After the reaction mixture had been cooled to room temperature, the ligase was added and the reaction mixture was incubated at 37 °C for 2h. The product of the ligation reaction was added to the 100 μL RCA reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 200 μM dNTP, and 10 U of phi29 DNA polymerase. The RCA reactions were performed at 37 °C for 4 h and terminated by incubation at 65 °C for 10 min.

Fluorescence Detection. The product of the ligation reaction and 750 nM of Ag NCs and G-rich helper sequence were combined in a 2.5 mL centrifuge tube and diluted to 300 μL with 20 mM phosphate buffer (pH 7.0, 200 mM $NaNO_3$). After incubation for 2h at room temperature, the fluorescence spectra were measured. The excitation wavelength was 560 nm, and the fluorescence emission intensity was measured at 611 nm.

RESULTS AND DISCUSSION

Scheme 1 schematically illustrates the RCA process and its scaffolding for periodical Ag NCs assemblies. The system consists of nanoscaffold preparation and assembling process. For nanoscaffold preparation, the padlock probe was first ligated and circularized with the primer as the template in the presence of T4 DNA ligase. Subsequently, the primer initiates RCA reaction by the high-displacement activity of the phi29 DNA polymerase for producing long enzymatically produced scaffold DNA. In the process of assembly, the Ag NCs and G-rich helper sequences are bind to the periodic sequence of the scaffold DNA. Because of the fluorescence enhancement of silver nanoclusters (Ag NCs) in proximity to guanine-rich DNA sequence, the bright Ag NCs nanowires are form.

Scheme 1. Schematic Illustration of the Preparation of Ag NC Wires by Rolling Circle Amplification



As shown in Figure 1A, Ag NCs of 2 nm diameter were prepared by the classical $NaBH_4$ reduction route.[36] In the presence of the long enzymatic backbone (Scheme 1), plenty of monodisperse Ag NCs are stacking on the DNA nanowires forming self-assembled Ag NCs (Figure 1B). The morphology of the Ag NCs nanowires was investigated by atomic force microscopy (AFM). As shown in Figure 1C, micrometer-long wires are observed with the height of 1.4 nm, resulting from the height of the duplex structure of the DNA in TWJ structure.

The successful preparation of Ag NCs wires by RCA was also confirmed by gel electrophoresis. In the gel, a strong fluorescence band corresponding to DNA with length in excess of 10 000 bp of the marker was observed (Figure 2A, lane a), which would lead to chains of DNA of many micrometers in length. In the absence of target DNA, there was no RCA product observed (Figure 2A, lane b). The successful assembly of Ag NCs onto the scaffold DNA resulted in a remarkable change of the fluorescence spectrum. As shown in Figure 2B, the unassembled Ag NCs show negligible excitation and emission spectra (curves a and b). In the presence of scaffold DNA, monodispersed Ag NCs were tethered in the proximity

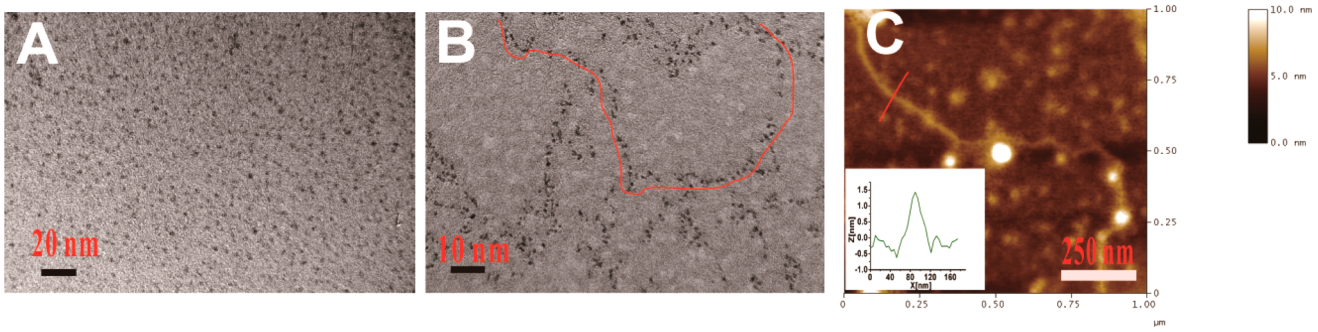


Figure 1. Typical TEM image of the Ag NCs (A) and self-assembled Ag NCs (B). Height-mode AFM image of Ag NCs nanowires (C). Inset: Cross-sectional analysis of the resulting Ag NCs nanowires.

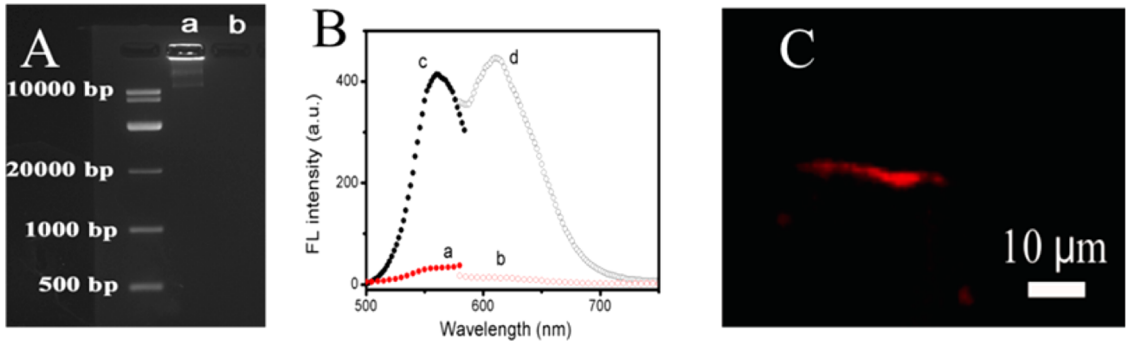


Figure 2. (A) Gel electrophoresis image of Ag NCs nanowires in the presence (a) and absence (b) of target. (B) Fluorescence excitation and emission spectra of Ag NCs before (curves a and b) and after (curves c and d) self-assembly. (C) Confocal microscopy image of the red-emitting Ag NC nanowires. Scale bar is 10 μm.

of guanine-rich DNA sequences, and the significant enhancement of the fluorescent spectrum was observed (curves c and d), which was consistent with the previous reports.[42−44] The resulting DNA-stabilized Ag NCs nanowires were further imaged by confocal fluorescence microscopy. Figure 2C depicts a confocal microscopy image of the resulting red-emitting Ag NC nanowires. Compared with previous report about Ag NC assembly, there is no careful sequence design involved in this method and the room temperature hybridization strategy reduced the errors in hybridization in the tedious annealing process. Meanwhile, the synthesis of Ag NCs is independent of the DNA assembly, which circumvents the interference of unstable $NaBH_4$ during the Ag NC synthesis.

Due to the highly effective RCA reaction in signal amplification, we combined the Ag NC assembly and RCA reaction for the specific DNA detection. The primer of 20mer oligonucleotide sequence was chosen as a target DNA. The feasibility of the design is presented in Figure 3. In the presence of the target DNA, the product of the RCA reaction as a nanoscaffold puts the two tailored sequence fragments in proximity. As a result, the fluorescence of Ag NCs enhances greatly. The resulting Ag NCs featured an emission band centered at 611 nm when excited at 560 nm (Figure 3a). In contrast, almost no fluorescence at 611 nm was observed (Figure 3b, c) without the target DNA and phi29 DNA polymerase, respectively, as there was no long tandem repeated sequence produced by the RCA reaction. Thus, the Ag NCs and enhancer probes could not be gathered in proximity, and no fluorescence enhancement was observed. Although many papers have reported some DNA detection methods based on fluorescent Ag NCs,[45−47] the sensitivity of Ag NC nanosensors might be limited if each target DNA generates only one

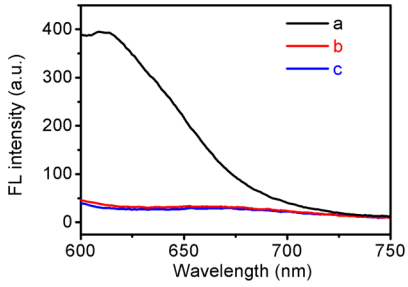


Figure 3. Fluorescence profiles of self-assembled Ag NCs nanowires for the specificity analysis with (a) target and phi29 polymerase; (b) target only; (c) phi29 polymerase only. Experimental conditions: target DNA, 0.3 nM; phi29 polymerase, 10 U; Ag NCs, 750 nM; enhance probe, 750 nM.

fluorescent AgNC. Meanwhile, few reports combine the signal amplification and Ag NCs, higher background signal results in poor determination limit.[48,49] In addition, target-triggered isothermal amplification strategy can achieve ultrahigh sensitivity;[48,50,51] the unstable $NaBH_4$ limits the reproducibility of the assay. Herein, we combine the RCA product and Ag NCs assembled for constructing Ag NCs nanowires and also show good performance in sensing amplification, which circumvents the disadvantage mentioned above.

In this strategy, RCA was a crucial step, which mediated the generation and amplification of the fluorescence signal. In order to achieve the system's best sensing performance, several experimental parameters were investigated (Figure S1, Supporting Information). The amount of phi29 DNA polymerase and RCA reaction time were set at 10 U and 4 h, respectively. We found that the concentration of Ag NCs and the self-assembly equilibrium

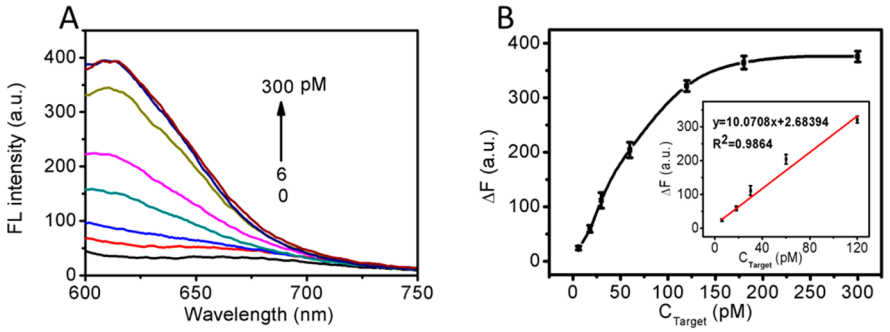


Figure 4. (A) Fluorescence response to the different concentrations of the target DNA: 6, 18, 30, 60, 120, 180, 300 pM (from bottom to top). (B) Corresponding calibration curve of the Ag NC nanowire strategy for the DNA assay. Inset: Linear response at low concentrations of the target DNA.

between the RCA product and Ag NCs were two key factors in this strategy. As shown in Figure S1C, the fluorescence intensity increased with the increase of Ag NC concentration. When the concentration of Ag NCs reached 750 nM, the maximum fluorescence intensity was achieved. Thereafter, the fluorescence response exhibited a gradual decrease with a further increase in the concentration of Ag NCs. As a result, 750 nM of Ag NCs was selected for further investigation. We also studied the non-specificity of fluorescence enhancement of Ag NCs due to the high concentration of "G-rich helper" DNA in the reaction system (Figure S2). In the absence of target DNA, with increasing the concentration of the "G-rich helper" DNA, the background signal showed negligible enhancement. On the other hand, the time of forming the three-way junction influenced the signal-to-noise of this approach. The fluorescence intensity increased with increasing the time of self-assembly in the range 0−120 min and reached the plateau at 120 min. Thus, 120 min of hybridization time was selected for this study.

Figure 4 shows representative fluorescence responses with different concentrations of target DNA under the optimized experimental conditions. A linear dependence between the fluorescence intensity and the concentration of the target DNA was obtained in the range from 6 to 120 pM with a limit of detection (LOD) as low as 0.84 pM (3σ).

To evaluate the selectivity of this method, four DNA sequences, including the target, the single-base mismatched target (MT1), the two-base mismatched target (MT2), and the three-base mismatched target (MT3) at the same concentration (0.3 nM) were selected, and the results are shown in Figure 5. Taking advantage of a padlock probe in highly specific single nucleotide polymorphism detection,[52] the Ag NC nanowires exhibit good selectivity to the mismatch target. The fluorescence intensity of the target DNA is approximately 2-fold higher than that in response to MT1, about 3-fold higher to MT2, and about 4-fold higher to MT3. All these observations indicate that the present biosensor can easily differentiate between perfect matched and mismatched targets and, therefore, provides a great opportunity for single-nucleotide polymorphism analysis.

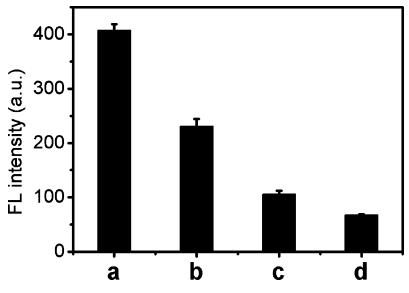


Figure 5. Relative fluorescence response from different inputs of (a) target DNA, (b) one-base mismatched sequence (MT1), (c) two-base mismatched sequence (MT2), and (d) three-base mismatched sequence (MT3). Target DNA and other mismatched strands were all 0.3 nM.

To demonstrate the feasibility of the practical application of this sensor, three concentrations of target (9, 16, and 23 pM) were spiked into diluted human serum. The serum with high concentration shows a remarkable emission peak under experimental conditions which influences the Ag NC fluorescence. Thus, 1% serum was chosen as the detection condition for practical application. The recovery values were in the range of 94.5−102.5% (Table S1), indicating that the sensor is capable of analyzing real biological samples with a high serum percentage by diluting the real sample.

CONCLUSIONS

In conclusion, we have developed a facile method to prepare Ag NC nanowires. They are made from two tailored single strands that will come together only in the presence of an enzymatically produced backbone, via addressable binding regions. Furthermore, the tunable fluorescence intensity of these nanowires can be used as a nanosensor for specific DNA detection with RCA reaction. This novel approach has shown some unique features. First, the assay does not involve any chemical modification. Second, due to the G-rich sequence enhanced fluorescence of Ag NCs, the assay does not require troublesome separation procedures. In view of these advantages, this fluorescent DNA nanomaterial has great potential in DNA diagnostics and clinical analysis.

ASSOCIATED CONTENT

Supporting Information

Effects of the incubation time of the RCA reaction, the amount of phi29 polymerase; amount of Ag NCs and the time of hybrization on the fluorescent intensity of the sensing system; recovery of the target DNA in spiked serum samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the National Key Scientific Program Nanoscience and Nanotechnology (2011CB933600), the Suzhou Nanotechnology Special Project (ZXG2013028), and the National Natural Science Foundation of China (21275109 and 21205089).

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