JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Gold Nanoparticle-Enabled Real-Time Ligation Chain Reaction for Ultrasensitive Detection of DNA

Wei Shen, Huimin Deng, and Zhiqiang Gao*

Department of Chemistry, National University of Singapore, Singapore 117543

ABSTRACT: A simple and ultrasensitive colorimetric DNA assay based on the detection of the product of a ligation chain reaction (LCR) and the use of gold nanoparticles (AuNPs) as signal generators has been developed. During LCR, the AuNPs were ligated together, resulting in a distinct color change in real time after a sufficient number of thermal cycles. The cumulative nature of the protocol produced a detection limit of 20 aM with a selectivity factor of 10^3.

Among various gene expression profiling tools, fluorescence-based real-time polymerase chain reaction (PCR) is the most widely used technique, as it combines the excellent amplification power of PCR with quantitative detection of the amplified product.[1] It produces ultrahigh sensitivity and a wide dynamic range while retaining the attractive features of PCR. Although fluorescence-based real-time PCR technology has shown tremendous utility, it suffers from the drawbacks of high cost and complex optics. To circumvent the limitations pertinent to fluorescence detection, it is imperative to exploit alternative ways of quantifying DNA in a simpler and more straightforward manner that is adaptable for both laboratory and point-of-care applications.

The inherent superiorities of colorimetry, such as simplicity, low cost, and freedom from the problems encountered in fluorescence detection systems, make it very attractive for DNA detection.[2] However, the low sensitivity of colorimetry precludes it from being a strong competitor to its fluorescence counterpart.[2] To provide significant enhancement of the sensitivity of colorimetry, an avenue that has recently been explored is the development of nanoparticle-based technologies.[3,4] Utilization of nanoparticles has translated into new assays that improve on current methods of colorimetric DNA quantification. For instance, the ultrahigh extinction coefficient (10^8–10^9) of the surface plasmon resonance (SPR) absorption of gold nanoparticles (AuNPs) offers an excellent opportunity for the development of highly sensitive colorimetric assays.[5] Mirkin's group first demonstrated that AuNPs are valuable platforms for ultrasensitive detection of biomolecules.[4] The sensitivities of AuNP-based colorimetric DNA assays were, however, found to be at nanomolar levels, far inferior to their real-time PCR counterpart.[2] An in situ amplification mechanism is therefore essential to make those assays more attractive. The construction of a powerful amplification platform to produce large amounts of AuNP aggregates with traces of target DNAs is one of the grand challenges facing the development of colorimetric DNA assays.

In this work, the feasibility of utilizing AuNPs as sensitive signal generators together with a ligation chain reaction (LCR)[6] for the colorimetric detection of DNA is demonstrated, leveraged on recent demonstrations of excellent ligation efficiency[7,8] and the formation of AuNP–DNA nanoassemblies during LCR.[9] The assay can be used to monitor DNA-related AuNP ligation processes in real time. DNA can therefore be colorimetrically quantified or semiquantified by visual inspection with high selectivity.

Citrate-capped 20 nm AuNPs were coated with trithiolated capture probes (CPs) using a fluorosurfactant-assisted procedure.[10] In comparison with direct absorption, better stability was observed as a result of the protective effect of the surfactant. Two types of CP-coated AuNPs were prepared, allowing the target DNA to act as a template to align a pair of the CP-coated AuNPs perfectly along it. A surface coverage of 10–12 pmol cm^{-2} was found to be optimal for LCR. Real-time LCR was performed in 100 μL ampligase reaction buffer containing ampligase (100 units), the two types of CP-coated AuNPs (20 nM each), the partial probe pair (0.5 μM each), and the target DNA. Each thermal cycle consisted of a 2 min ligation at 50 °C and a 1 min denaturation at 90 °C. An absorbance ratio of $\geq 10\sigma$ (where σ is the standard deviation of the control) was used to construct the calibration curve. Visual inspection was done when semiquantitative results were needed.

As schematically illustrated in Figure 1A, the assay comprises three principal processes, namely, hybridization at 50 °C, ligation at 50 °C, and denaturation at 90 °C. During hybridization, the target DNA and the two partial probes form duplexes with the two types of the CP-coated AuNPs. Subsequently, ampligase covalently ligates the two AuNPs

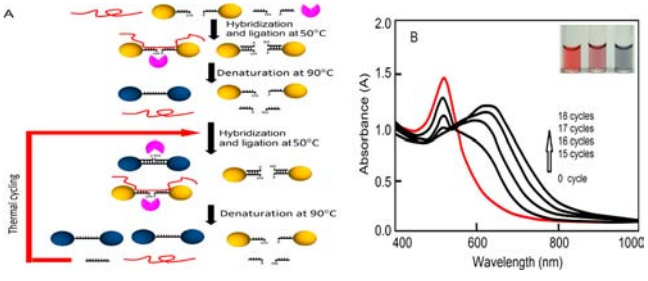

Figure 1. (A) Schematic illustration of the real-time LCR DNA assay. (B) UV–vis spectra of 100 fM target DNA during LCR. Inset: solution color after addition of (left to right) 0, 5, and 50 nM target DNA to 10 nM CP-coated AuNPs.

Received: June 27, 2012
Published: August 27, 2012

dx.doi.org/10.1021/ja306265n | J. Am. Chem. Soc. 2012, 134, 14678–14681

templated by the hybridized target, whereas the duplexes formed between the partial probes and the CP-coated AuNPs remained unligated. When the temperature of the reaction mixture is increased to 90 °C, all of the duplexes are denatured, releasing the target DNA, the ligated AuNPs, and the partial probes. In addition to the target DNA, the ligated AuNP conjugates now also serve as targets for the partial probes. In the second and all subsequent hybridizations and ligations, in addition to the hybridization and ligation process associated with the target, the partial probes are ligated together after hybridizing with the already ligated AuNP conjugates, and these ligated partial probes in turn serve as secondary targets for subsequent hybridization and ligation with the CP-coated AuNPs. Therefore, starting from the second hybridization, repetition of the hybridization and ligation at 50 °C and denaturation at 90 °C exponentially amplifies the number of ligated AuNPs, doubling the amount after each thermal cycle and eventually leading to the color change from wine red to purple/gray. Therefore, the thermal cycling provides the basis for the development of a highly sensitive colorimetric DNA assay.

As shown in the Figure 1B inset, similar to previously reported AuNP-based colorimetric assays,[4,11−13] when the two types of CP-coated AuNPs were mixed with the target solution, depending on the concentration of the target, the solution color changed from wine red to purple at low concentrations and to gray at high concentrations after a very short period of incubation at room temperature, signifying the formation of AuNP aggregates.[4,11−13] In control experiments where no target was present or the target was noncomplementary to the CPs, no solution color changes were observed even after prolonged periods of incubation. Evidently, hybridization between the complementary target and the CP-coated AuNPs prompted the formation of the AuNP aggregates. Such hybridization-induced AuNP aggregation has been exploited by several groups for detection of DNA in connection with color changes.[4,11−13] Our experiments also found a linear relationship between the colorimetric signal and the target concentration. Comparable to most AuNP-based colorimetric DNA assays,[4,11−13] the 20 nm CP-coated AuNPs can be utilized for the detection of DNA from 2 nM to 1.0 μM. This sensitivity is, however, far below the requirement for gene expression profiling. An in situ amplification scheme is needed in order to make it appropriate for use as a gene expression profiling tool. Since the sensitivity of the AuNP-based colorimetric assay is dependent on the amount of AuNP aggregates, the amount of the amplified target/secondary target must be substantially increased in order to lower the detection limit down to a reasonable level. It has been shown that DNA−AuNP nanoassemblies (aggregates) can be produced during LCR.[9] LCR was therefore attempted in order to amplify the target and to ligate the CP-coated AuNPs under thermal cycling conditions. The UV−vis absorption spectra of the LCR amplification during thermal cycling are compiled in Figure 1B. A red shift of ∼110 nm in the SPR absorption peak due to the formation of ligated AuNPs was observed.[4] The absorbance increased progressively as the number of thermal cycles increased. The solution colors after 0, 15, and 18 thermal cycles were similar to those shown left to right, respectively, in the Figure 1B inset. After 30 thermal cycles, the assay was able to distinguish the target from the control at 20 aM with a signal-to-noise ratio of 3.0. Moreover, a linear relationship between the colorimetric signal and the target concentration was established between 50 aM and 20 fM. This sensitivity is now sufficient for direct gene expression profiling. Unfortunately, the narrow dynamic range, due to quick depletion of the reagents (partial probes or/and the CP-coated AuNPs) at high target concentrations, limits its application in gene expression profiling.

As depicted in Figure 2A, the LCR amplification pattern is similar to that of real-time PCR.[1] Since depletion of the

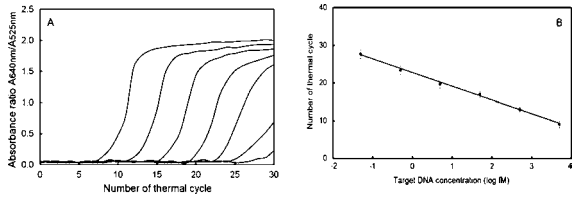

Figure 2. (A) Colorimetric responses of real-time LCR: (left to right) 5000, 500, 50, 5, 0.5, 0.05, and 0 fM target DNA. (B) The corresponding calibration curve.

reagents is exclusively associated with the target concentration, as in real-time PCR, a real-time PCR-like assay should be obtained by adopting the real-time PCR configuration. As mentioned earlier, apart from its exceptional amplification power, real-time PCR offers the widest dynamic range, up to 8 orders of magnitude. It can be seen in Figure 2A that the target could be amplified for up to 30 thermal cycles at 50 aM, whereas only 12 thermal cycles were possible at 5 pM before the signal leveled off. In addition, considerable absorbance was detected for the control after an extended number of thermal cycles, probably as a result of CP-desorption-induced AuNP aggregation and nonspecific ligation.[9] Dithiothreitol displacement tests indicated that up to 60% of the CPs were detached from the AuNPs between 30 and 50 thermal cycles. To minimize this non-target-related background signal, all of the subsequent experiments were performed within 30 thermal cycles. As anticipated, a much wider dynamic range of 50 aM to 5.0 pM with a relative standard deviation of <10% was attained when working with the number of thermal cycles and the target concentration instead of the absorbance and the target concentration (Figure 2B). The detection limit was found to be 20 aM, representing a10^2−10^4-fold improvement over those of previously reported AuNP-based colorimetric assays and the best colorimetric DNA assays.[2−4]

To evaluate the ability of this assay for highly selective DNA detection, a well-studied oncogene, KRAS, was chosen as our target. The CP-coated AuNPs and partial probe pair designed for wild-type (WT) KRAS were used for the detection of WT KRAS in the presence of a large excess of genomic DNA (from salmon sperm) at WT KRAS/genomic DNA base-pair ratios varying from 1:0 to 1:100000. As revealed in Figure 3A, the presence of the genomic DNA with a WT KRAS/genomic DNA base-pair ratio up to 1:100 had no effect on the detection of WT KRAS when readings were taken at room temperature. However, a noticeable increase in the absorbance was observed beyond the ratio of 1:100. For example, as much as ∼25% higher signal was obtained when 100000-fold genomic DNA was present with WT KRAS. The increase was likely caused by the competitive hybridization of the genomic DNA to the CP-coated AuNPs at such a high concentration. Although the genomic DNA is not complementary to the CPs, its much higher concentration may eventually make hybridization possible and stabilize the mismatched genomic DNA−CP-

dx.doi.org/10.1021/ja306265n | J. Am. Chem. Soc. 2012, 134, 14678−14681

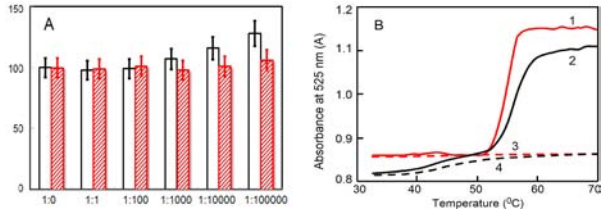

Figure 3. (A) Detection of 50 fM WT KRAS in the presence of increasing amounts of genomic DNA at room temperature (black) and 90 °C (red). (B) Melting behavior of (1) 20 nM WT KRAS–AuNP duplexes, (2) 1 + 200 μM genomic DNA, (3) 1 after ligation, and (4) 2 after ligation.

coated AuNP duplexes, leading to the additional 25% signal. The thermodynamic limitation dictates the selectivity of all hybridization-based assays. On the other hand, the formation of non-ligation-related DNA duplexes is reversible. When the temperature rises, the melting of the DNA–AuNP duplexes leads to the dispersion of the AuNPs back into the solution, and consequently, the solution color changes back to wine red. Figure 3B shows the spectral changes as the melting transitions occurred. Interestingly, the full width at half-maximum (fwhm) of the first derivative of the melting profile of WT KRAS was dependent on the genomic DNA concentration. A very sharp melting curve (fwhm < 3 °C) was observed when no genomic DNA was present, while the addition of an excessively large amount of genomic DNA resulted in spectral changes in the melting behavior. For instance, in the presence of 200 μM genomic DNA, the DNA–AuNP aggregates displayed a melting profile with a fwhm of ~8 °C. Similar dissociation profiles have been observed previously for DNA-functionalized AuNPs conjugated by target DNA strands, where the fwhm was found to be 2.5–4 °C.[10,14–16] Two models, one based on cooperative melting[10,14] and the other on a phase transition,[16] have been proposed to account for the melting behavior of DNA–AuNP aggregates. Such a sharp melting behavior is highly desirable in sequence-specific DNA assays, as it may greatly improve the selectivity.[4,11] However, the severe interference from the coexisting genomic DNA poses a great threat when real-world samples are analyzed. Fortunately, because of the high reversibility of hybridization, the engagement of the ligation reaction, and the thermal cycling, the false-positive response from the interfering genomic DNA can effectively be eradicated by working at the high-temperature end of the thermal cycle rather than at the low-temperature end (Figure 3B).

As shown in Figure 3A, practically no interference from the genomic DNA was observed at WT KRAS/genomic DNA base-pair ratios up to 1:100000 when the readings were taken at 90 °C. Through the incorporation of AuNP-based ligation by LCR, the proposed assay not only offers excellent target amplification but also allows easy elimination of interference from coexisting DNA. All nonligated DNA–AuNP conjugates are dislodged at 90 °C, leaving only the ligated AuNP aggregates in solution. A much-reduced background is therefore produced, extending the dynamic range to 50 aM. When the CP-coated AuNPs and the partial probe sets were further tested for the detection of WT KRAS in the presence of KRAS single-nucleotide polymorphisms (point mutations of C, A, and T at the ligation site), a discounted selectivity factor of 2000 was obtained. In an earlier report, a selectivity factor of 1:100000 was achieved in a AuNP-based hybridization assay when standard solutions of target DNAs were analyzed independently after stringent washing.[11c] However, this selectivity is more a response factor between two pure standard solutions. It may not be applicable to the analysis of genes in real-world samples because of the noticeable competitive binding to the AuNP probes from the large excess of coexisting genomic DNA.

To understand the capability of the assay for profiling genes in real-world samples, KRAS genes in two genomic DNA samples extracted from cultured cells (SW480 and K562) were analyzed. Mutation-T (MT-T) KRAS was unambiguously confirmed in SW480 cells (homozygous GTT mutant), and no mutation was detected in the genomic DNA from K562 cells (WT KRAS codon 12 DNA). For further confirmation, the same samples were analyzed by direct DNA sequencing. The results obtained by the proposed assay agreed well with the direct sequencing data. The genomic DNA extracted from K562 cells was further diluted with genomic DNA from SW480 cells to produce decreasing WT/MT-T gene ratios. Samples in which WT KRAS represented from 50 to 0.01% of the total genomic DNA were then analyzed. It was observed that the WT was detectable down to a WT/MT-T ratio of 1:1500. In addition, there was a direct correlation between the thermal cycle threshold and the logarithmic dilution factor of the WT gene in the samples. This excellent selectivity implies that each quantified result represents only a specific quantity of a single allele, not the combined quantities of the entire gene family.

In conclusion, it has been demonstrated that AuNP-based LCR offers new possibilities in the development of ultrasensitive DNA assays. The ligation of CP-coated AuNPs leads to aggregation of the AuNPs, generating a visible color change. DNA can therefore be quantified at ultralow levels with excellent selectivity. Such a real-time amplification strategy may eventually lead to the development of a simple and portable gene profiling system.

AUTHOR INFORMATION

Corresponding Author

chmgaoz@nus.edu.sg

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the A*STAR-ANR Program.

REFERENCES

(1) Van Guilder, H. D.; Vrana, K. E.; Freeman, W. M. *Biotechniques* **2008**, *44*, 619–626.
(2) Lodeiro, C.; Capelo, J. L.; Mejuto, J. C.; Oliveira, E.; Santos, H. M.; Pedras, B.; Nuñez, C. *Chem. Soc. Rev.* **2010**, *39*, 2948–2976.
(3) Tansil, N. C.; Gao, Z. Q. *Nano Today* **2006**, *1*, 28–37.
(4) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547–1562.
(5) Link, S.; El-Sayed, M. A. *J. Phys. Chem. B* **1999**, *103*, 8410–8426.
(6) Barany, F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 189–193.
(7) Chen, X. J.; Ying, A.; Gao, Z. Q. *Biosens. Bioelectron.* **2012**, *36*, 89–94.
(8) Psifidi, A.; Dovas, C.; Banos, G. *PLoS One* **2011**, *6*, No. e14560.
(9) Claridge, S. A.; Mastroianni, A. J.; Au, Y. B.; Liang, H. W.; Fréchet, J. M. J.; Alivisatos, A. P. *J. Am. Chem. Soc.* **2008**, *130*, 9598–9605.
(10) Zu, Y. B.; Gao, Z. Q. *Anal. Chem.* **2009**, *81*, 8523–8528.
(11) (a) Elghanian, R.; Storhoff, J. J.; Mucic, R. C.; Letsinger, R. L.; Mirkin, C. A. *Science* **1997**, *277*, 1078–1081. (b) Reynolds, R. A.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **2000**, *122*, 3795–

3796. (c) Park, S. J.; Taton, T. A.; Mirkin, C. A. *Science* **2002**, *295*, 1503−1506.
(12) Li, H.; Rothberg, J. *J. Am. Chem. Soc.* **2004**, *126*, 10958−10961.
(13) Wilson, R. *Chem. Soc. Rev.* **2008**, *37*, 2028−2045.
(14) Jin, R. C.; Wu, G. S.; Li, Z.; Mirkin, C. A.; Schatz, G. C. *J. Am. Chem. Soc.* **2003**, *125*, 1643−1654.
(15) Long, H.; Schatz, G. C. *J. Phys. Chem. B* **2006**, *110*, 2918−2926.
(16) (a) Lukatsky, D. B.; Frenkel, D. *Phys. Rev. Lett.* **2004**, *92*, No. 068302. (b) Park, Y.; Stroud, D. *Phys. Rev. B* **2003**, *67*, No. 212202.