Controlling Chemical Equilibrium for Efficient Nanoparticle Conjugation and Release of DNA

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The highly enhanced binding properties of deoxyribonucleic acid (DNA)–gold nanoparticle conjugates (DNA–AuNPs) have attracted researchers’ attention because they could be the fundamental origin of the high sensitivity of detection schemes employing the DNA–AuNPs as functional nanoparticles.1–3 The binding constant of the DNA–AuNPs was determined to be of two orders of magnitude as high as that of free single stranded DNA, which was not the case with sparsely conjugated DNA strands on nanoparticles.4,5 In a typical conjugation procedure, citrate-coated AuNPs are combined with excess thiol DNA, which essentially leads to the limited DNA loading per AuNP and the discard of unconjugated costly thiol DNA (approximately 95%, assuming 100 DNA strands per AuNP).1 To date, several chemical and physical methods have been developed to effectively increase the DNA density on AuNPs. For example, DNA–AuNP conjugation reaction was conducted at high NaCl concentrations,6 with sonication,6 or under acidic (pH 3) conditions7 to increase the density of DNA on AuNPs. While effective and convenient, however, these methods are hampered by the necessity of additional chemicals or instrumentation.

In contrast to the DNA-conjugation reaction, the detailed chemical conditions where the DNA strands are released from the nanoparticle surface have been less frequently investigated. In fact, the complete release of DNA from nanoparticles is as important as the DNA–nanoparticle conjugation, because it is directly associated with the accurate evaluation of DNA loading, a measure of the DNA conjugation efficiency. Conventionally, organic alkylthiol molecules in excess were employed to displace the monothiol DNA, but they were hampered by the poor water solubility and environmental harm.8 Instead of the displacing chemicals, potassium cyanide (KCN) was used to dissolve the gold nanoparticles, whose toxicity, however, could be an even larger concern than that of alkylthiols.9,10 Recently, sodium borohydride (NaBH₄) was used to release organic thiol molecules from AuNPs, potentially applicable to the DNA release.11 Currently, dithiothreitol (DTT) is the most widely used displacing reagent for thiol DNA on AuNPs because of its high water solubility, low toxicity, and facile removal after the displacement.12 The detailed displacement conditions, however, are not fully investigated yet, and are required to be further improved for efficient and optimized quantification of DNA strands conjugated with AuNPs.

Herein, we present a facile and cost-effective method for maximizing DNA loading on AuNPs simply by increasing the AuNP concentration and thus shifting chemical equilibrium of the DNA–AuNP conjugation reaction (Scheme 1(a)). Importantly, this method is extremely simple, straightforward, and can be applied to any DNA sequences. Moreover, it does not require any additional instrumentation or chemical reagents. For the accurate measurement of DNA loading, we first systematically investigated the DTT displacement of DNA strands on AuNPs at various temperatures, DTT concentrations, and incubation time, and determined the optimal displacement condition (Scheme 1(b)).

In order to quantitatively analyze the effect of AuNP concentration on the DNA loading, we first optimized the conventional method to measure DNA loading, in which the fluorophore-labeled thiol DNA is released from the AuNP surface via a displacement reaction induced by DTT, and quantified by photoluminescence and UV–Vis spectroscopies.4 Three key parameters for the DTT displacement of thiol DNA were systematically investigated: (1) incubation time, (2) temperature, and (3) DTT concentration. The optimized DNA release by DTT was then determined by these three parameters (Scheme 1(b)).
DNA were chosen and intensively investigated for the accurate spectroscopic quantification of the DNA–AuNP conjugation: (1) the concentration of DTT, (2) displacement reaction temperature, and (3) displacement reaction time. We first began our investigation by observing the number of displaced DNA strands per AuNPs at three different [DTT]s as a function of the incubation reaction time (25 °C). The DNA–AuNPs were prepared by conjugating thiol DNA and AuNPs at 0.5 M NaCl, and combined at a fixed concentration (1 nM) with DTT at varying concentrations which were 6–8 orders of magnitude higher (1, 10, and 100 mM) than that of the DNA–AuNPs to maximize the displacement efficiency, and were allowed to undergo the DNA displacement at 25 °C. The displaced DNA strands were obtained every 10 min during the incubation until 1 h, and quantitatively analyzed as shown in Figure 1(a). As expected, the efficiency of the DTT-displacement reaction significantly increased as the [DTT] increased. Even after the first 10 min incubation, the displaced DNA strands increased approximately twice and three times as the [DTT] increased by one order and two orders of magnitude. As the incubation time increased, however, the number of displaced DNA slightly increased at 100 mM of DTT (+14 strands), indicating that the displacement reaction almost reached in equilibrium when the [DTT] is high. In contrast, the displacement increased dramatically at 1 mM of DTT after the 1 h incubation (+28 strands), owing to slower kinetics at a lower [DTT]. Although a lower [DTT] resulted in both a faster displacement rate and a larger increase in DTT displacement, the number of displaced DNA after 1 h was still higher at a higher [DTT], implying the greater effect of the [DTT] than the incubation time on the displacement efficiency.

Because the DTT displacement of the DNA on the AuNPs led to the destabilization of the AuNPs, we expected to observe irreversible aggregation of the AuNPs with a concomitant red-to-purple color change as a function of [DTT] and incubation time. When DTT was initially combined with the DNA–AuNPs, the mixtures exhibited a red color owing to the surface plasmon resonance (SPR) of the AuNPs (Figure 1(b)). As the incubation proceeded (0.5 h), the DTT displacement took place the most substantially at 100 mM of DTT, resulting in a blue color indicative of the larger aggregate formation. As the [DTT] decreased to 10 and 1 mM, however, the color of the solutions changed to dark purple and purple, respectively, owing to the less displaced DNA at a lower [DTT] and the relatively more stable DNA–AuNPs (Figure 1(b), 0.5 h). After 1 h, the DNA–AuNPs at 100 mM of DTT turned almost colorless, indicating the formation of macroscopic aggregates and largely reduced SPR owing to the significant loss of the DNA strands under our experimental conditions (Figure 1(b), 1 h). Eventually, the complete destabilization and resultant aggregation of AuNPs at all of the [DTT]s was observed after a time period of 5 h (Figure 1(b), 5 h).

Based on the time-dependence of the DTT displacement for the DNA–AuNPs, we determined the incubation time to be 1 h in consideration of both the efficiency of measurement and completion of displacement, and further investigated the effect of (1) incubation temperature and (2) initial DNA loading. We prepared five types of DNA–AuNPs whose DNA loading was systematically controlled at a range of [NaCl] (0.2, 0.4, 0.6, 0.8, and 1.0 M) during the DNA-functionalization, and released their DNA at a range of [DTT] (1, 10, and 100 mM) at three different temperatures (25 °C, 50 °C, and 75 °C) (Figure 2(a)–(c)). Overall, the displacement tended to increase as the temperature increased, indicating the importance of the high reaction temperature for the kinetically and thermodynamically enhanced DNA displacement. In fact, the high temperature itself can be a governing condition to release thiol DNA strands from the AuNP surface. We also noticed that the number of displaced DNA strands from the AuNPs increased as their initial DNA loading increased almost in a linear manner. This strong linearity indicates that the density of DNA on the surface of AuNPs does not affect the kinetics and thermodynamics of the displacement reactions.

![Figure 1](image1.png)

**Figure 1.** (a) The number of DNA strands per AuNP determined as a function of the DTT-displacement reaction time at various [DTT]s at 25 °C. (b) The AuNP solutions containing DTT at various concentrations (1:1 mM, 2:10 mM, and 3:100 mM) at different reaction time periods (0, 0.5, 1, and 5 h).

![Figure 2](image2.png)

**Figure 2.** The DNA loading per AuNP determined after their displacement by DTT at various [DTT]s. (a): 100 mM, (b): 10 mM, (c): 1 mM, and (d): 0.1, 0.5, 1, 10, and 100 mM) and temperatures ((a), (b), and (c): 25, 50, and 75 °C; (d): 25 °C). The x-axis indicates the [NaCl] used to conjugate the thiol DNA and AuNPs, which should be proportional to the final DNA loading.
under our experimental conditions, and supports the versatility of the DTT-displacement reaction to quantitatively analyze the DNA loading of DNA–AuNPs regardless of how high the loading is. Interestingly, the numbers of displaced DNA strands at 25 °C and 50 °C were almost the same (Figure 2(a)), proposing that the effect of the temperature on the DTT displacement is negligible when the effect of the [DTT] is dominant. When further increased to 75 °C, however, the temperature eventually plays a determining role in displacing the thiol DNA with DTT (Figure 2(a)). In contrast, the number of displaced DNA strands at 4 °C was almost negligible (data not shown). Importantly, the displacement of the DNA strands was invariably enhanced by a higher [DTT] under any given conditions observed, demonstrating the crucial effect of shifting chemical equilibrium by increasing the [DTT] on the displacement of DNA strands.

The role of DTT to control the chemical equilibrium for displacing DNA was further precisely investigated at a large range of [DTT] (0.1–100 mM) at 25 °C for DNA–AuNPs containing relatively less DNA strands that are typically used as nanoprobe. The [DTT] was reduced down to 0.1 mM to explore the lowest [DTT] required to displace DNA. At [DTT]s from 100 to 1 mM, the displacement reaction resulted in the similar number of DNA strands to those observed in Figure 2(a)–(c) (Figure 2(d)). Interestingly, DTT at 0.5 and 0.1 mM did not induce any displacement of DNA from the DNA–AuNPs that were conjugated at lower [NaCl]s (0.1 and 0.2 M). The displacement gradually increased with more densely loaded DNA–AuNPs ([NaCl] = 0.3, 0.4, and 0.5 M) at 0.5 mM of DTT, showing that the displacement takes place in proportion to the initial DNA loading of the DNA–AuNPs. When the [DTT] is the lowest (0.1 mM), no displacement was observed regardless of the DNA loading. Considering that the displaced DNA strands at 75 °C at 100 mM of DTT from the DNA–AuNPs conjugated at 0.6 M NaCl is almost 100 - 100 mM of DTT, the total DNA concentration of the DNA–AuNPs solution ([DNA–AuNP] = 1 nM) was expected to be approximately 100 nM. Therefore, apparently a [DTT] that is at least three orders of magnitude higher than the [DNA] should be the lowest required for displacement of DNA.

After the investigation of the DTT-displacement conditions for the most optimized measurement of DNA loading, we further investigated the method to shift the chemical equilibrium of the DNA–AuNP conjugation reaction by increasing the [AuNP] at a consistent [DNA]. We prepared eight batches of citrate-modified AuNP solutions containing a series of [AuNPs] from 0.2 to 8 nM,15 and combined each of them with thiol DNA for the DNA–AuNP conjugation (final [DNA] = 4.8 μM). The conjugation was further enhanced by the addition of NaCl (final [NaCl] = 0.5 M), was allowed to proceed for 12 h, and finally analyzed by displacing the DNA strands from each type of DNA–AuNPs at 100 mM of DTT at 75 °C, and measuring their quantity by photoluminescence spectroscopy. Very interestingly, the numbers of displaced DNA strands per AuNP gradually increased as the [AuNP] increased during the DNA-conjugation procedure. For example, the displaced DNA strands per AuNP was 65 when the [AuNP] was 0.2 nM during the DNA conjugation (Figure 3). When the [AuNP] increased to 1 nM, the displaced DNA strands also almost linearly increased to 84, indicating the effect of controlling the [AuNP] on the chemical equilibrium of the DNA–AuNP conjugation, and thus, on the increase in DNA loading (Figure 3, inset). As the [AuNP] further increased to 6 nM, the displacement increased very slowly, indicating the increase in DNA–AuNP conjugation was reaching a plateau. This plateau was expected obviously owing to the limited number of DNA strands during the conjugation. When the [AuNP] was higher (8 nM), eventually the displaced DNA strands per AuNP slightly decreased because of the deficient number of DNA strands to conjugate with AuNPs to achieve the highest possible DNA loading (96 strands per AuNP) which was demonstrated with the AuNPs at 6 nM. This result is highly important, suggesting there is still enough room to easily increase the DNA loading in the current scheme of the DNA–AuNP conjugation by increasing the [AuNP]. The demonstrated increase in conjugation efficiency in this work is 15%.

In this work, two important results are reported: (1) we systematically optimized the measurement conditions of DNA loading on AuNPs based on the placement of DNA strands by DTT; and (2) we enhanced the DNA loading on AuNPs by shifting the chemical equilibrium of the DNA–AuNP conjugation reaction easily by increasing the AuNP concentration. These two results are closely correlated with each other, because analytical development of measuring DNA loading on AuNP should precede the enhancement of DNA–AuNP conjugation for accurate and precise evaluation. Although this strategy is expected to increase the total number of AuNP-conjugated DNA strands, obviously the DNA loading “per” AuNP would not indefinitely increase. At least, however, the amount of discarded thiol DNA is expected to be minimized. Importantly, the novel method presented in this work for enhancing the DNA loading on AuNPs would offer substantial convenience and cost-effectiveness to researchers.

Note

Figure 3. The number of DNA strands per AuNP as a function of the [AuNP]. The DNA–AuNP were conjugated at 0.5 M NaCl, and analyzed by displacing the DNA at 100 mM DTT at 75 °C. As the [AuNP] increases up to 1 nM, the DNA loading also increases almost in a linear manner (inset).
who develop the synthesis and applications of DNA conjugates of other nanoparticles, including silver, palladium, platinum, and silica.16–20

Experimental

Materials and Instruments. Gold(III) chloride trihydrate (Cat. # 520918), sodium citrate tribasic dihydrate (Cat. # S4641), TWEEN® 20 (T20, Cat. # 9005-64-5), and dithiothreitol (DTT) (Cat. # 43815) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluoroecein-modified thiol DNA sequence (5'- HS-A10'-AGTGAATAAT-FAM 3'; FAM indicates fluorescein amide) was purchased from GenoTech Corp. (Daejeon, Republic of Korea). The NAP-5 Sephadex columns were purchased from GE healthcare (Amersham, UK). The 96-well black clear-bottom plates (Costar 3603) were purchased from Corning, Inc (Corning, NY, USA). Ultrapure water was obtained from Direct-Q3 system (18.2 MΩ·cm, Millipore (Billerica, MA, USA)). The UV–Vis spectrometer (Agilent 8453, Agilent Technologies, Santa Clara, CA, USA) and the fluorescence reader Gemini XPS microplate reader, Molecular Devices, Sunnyvale, CA, USA were used to obtain the DNA loading on AuNPs. Synthesis of Gold Nanoparticles (AuNPs). Forty-nine milliliter of ultrapure water was heated to 100 °C. When it was boiling, 1 mL of gold chloride solution (12.7 mM) and 0.94 mL of trisodium citrate solution (38.8 mM) were rapidly injected with stirring. After 5 min of reaction, the color of solution was red, indicating that AuNPs with 15 nm were synthesized, and then as-synthesized AuNPs were cooled to 25 °C. Conjugation of Thiol DNA with AuNPs. Thiol DNA was mixed with 0.10 M DTT solution in a phosphate buffer (10 mM phosphate, pH 7.4, 0.01% T20) and incubated for 12 h at 25 °C. Once DTT occupies the AuNP surface, there is no electrostatic repulsion between the AuNPs because DTT is not charged. This change in interparticle interactions leads to the aggregation of the AuNPs. Displacement of DNA and Fluorescence Measurement. For the release of the DNA strands from the AuNP surfaces, 100 µL of the DNA–AuNPs (1 nM) were mixed with desired concentrations of DTT solutions (the final [DTT] = 100, 10, and 1 mM) at three different temperatures (25 °C, 50 °C and 75 °C, respectively) for various time periods (10–60 min). After the displacement reaction, the reddish mixture turned colorless because the reducing of surface protection results in the destabilization and the aggregation of AuNPs. Acknowledgment. This research was supported by the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIP (Grant No. 2015M3A9D7031015). References

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