Supporting Information

Freezing Directed Construction of Bio/nano Interfaces: Reagentless Conjugation, Denser Spherical Nucleic Acids, and Better Nano-flares

Biwu Liu and Juewen Liu*

Department of Chemistry, Waterloo Institute for Nanotechnology

University of Waterloo

Waterloo, Ontario, N2L 3G1, Canada

Email: liujw@uwaterloo.ca

1. Materials and Methods

Chemicals. All of the DNA samples were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). The sequences and modifications of the DNAs are shown in Table S1. Sodium chloride, and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and sodium citrate were from Mandel Scientific (Guelph, ON, Canada). Potassium cyanide was from Sigma-Aldrich. Hydrochloric acid was from VWR (Mississauga, ON). Citrate-capped AuNPs (13 nm) were prepared based on a literature reported method,[1] and the as-prepared concentration was ~10 nM. AuNPs of other sizes (5, 30, 50, and 100 nm diameter) were purchased from BBI Solutions. Milli-Q water was used for all experiments to prepare buffers and solutions.

Instrumentation. The dynamic size and ζ-potential of AuNPs and DNA/AuNPs in the aqueous solutions were measured using a Zetasizer Nano 90 (Malvern) at 25 °C. Typically, 2 nM AuNPs or DNA-functionalized AuNPs were dispersed in HEPES buffer (10 mM, pH 7.6) for measurement. The UV-vis absorption spectroscopy was performed on a UV-vis spectrometer (Agilent 8453A).

Functionalization of AuNPs with DNA. Thiolated DNA samples were used as received without further treatment (i.e. no DTT or TCEP treatment).[2] In a typical experiment, DNA1-FAM (100 μM, 3 μL) was mixed citrate-AuNPs (13 nm, 10 nM, 100 μL). The mixture was then placed in a laboratory freezer (set at -20 °C) for 2 h, followed by thawing at room temperature. For comparison, DNA-functionalized AuNPs using other methods (e.g. salt-aging or low pH) were also carried out. For the salt-aging method,[3] 1 μL of SDS (10% w/v) was added to maintain the colloidal stability of AuNPs. After overnight incubation, NaCl (5 M) was gradually added up to 700 mM over a time period of 10 h

following the literature reported method.[4] The mixture was then incubated overnight again. For the low pH method,[5] 2 μL of citrate buffer (500 mM, pH 3) was added to lower the pH of the sample. After 5 min, another 10 μL of HEPES buffer (500 mM, pH 7.6) was introduced to adjust the pH back to neutral. For AuNPs of different sizes, DNA1-FAM (100 μM, 3 μL) was respectively mixed 100 μL of the AuNP stock solution as received, and the same procedures were then performed. To examine the generality of the method, other DNA sequences were also tested. DNA with a poly-T spacer (DNA2) and a G-rich sequence (DNA3) were used.

Quantification of DNA loading density. To quantify the number of DNA strands attached to each AuNP, 6-carboxyfluorescein (FAM) labeled DNA was used to modify AuNPs. Typically, the stock DNA solution was prepared by dissolving the DNA powder in a buffer or Milli-Q water based on the quantity of DNA provided by the vendor. The DNA solution was then diluted to 1 μM in buffer (10 mM, Tris-HCl, pH 8.5) to measure the actual DNA concentration using UV-vis spectrometry. Using FAM-DNA1 as an example, its extinction coefficients at 260 nm is 233,300 M^{-1} cm^{-1} according to the OligoAnalyzer from IDT (the same as the spec sheet from IDT), and that at 495 nm is 75 000 M^{-1} cm^{-1}. The absorbance at 260 nm and 495 nm were measured to be 0.2363 and 0.0774, respectively. Then, the actual DNA concentration was calculated to be 1.01 μM (260 nm) and 1.03 μM (495 nm). These values are quite close to that based on the DNA quantity provided by IDT (1-3% difference). Therefore, we believe that the concentration was accurate, and the labeling efficiency was close to 100% for our DNA.

The obtained DNA/AuNP conjugates using different methods were centrifuged (13 000 rpm, 13 min) to remove the free DNA and washed by HEPES buffer (5 mM, pH 7.6)

for three times. Finally, the DNA/AuNP conjugates were dispersed in HEPES buffer (5 mM, pH 7.6). Then 1 μL of KCN (1 M) was added to each 100 μL of sample to dissolve the AuNPs. It needs to be noted that the number of DNA loaded on AuNPs was determined with standard curves by adding the same DNA of known concentrations in the same buffer condition (instead of based on the absolute absorbance or fluorescence). The comparison of DNA capacity using different methods were always carried out side-by-side to minimize systematic errors. The concentration of AuNPs was quantified based on the UV-vis absorbance at 530 nm,[6] and the extinction coefficients were as follows: 5 nm (1.10×10^7 M^{-1} cm^{-1}), 20 nm (8.60×10^8 M^{-1} cm^{-1}), 30 nm (3.01×10^9 M^{-1} cm^{-1}), 50 nm (1.34×10^{10} M^{-1} cm^{-1}), and 100 nm (1.08×10^{11} M^{-1} cm^{-1}). An average number of DNA on each NP was then calculated. The Student's *t*-test was used to determine the statistical significance of the differences between different methods.

Preparation of nano-flares. For the post-hybridization strategy, capture DNA (DNA1)-modified AuNPs were prepared using the methods mentioned above. Then, the probe DNA (DNA4-FAM) was mixed with the conjugate at designated DNA to NP ratios (10, 25, 50, 100, 200, or 300: 1) in buffer A (25 mM HEPES, pH 7.6, 300 mM NaCl). After overnight incubation at 4 °C, the free probe DNAs were removed by centrifugation. The conjugate was washed with buffer A, and finally dispersed in the same buffer. For the co-adsorption strategy, capture DNA and probe DNA were both mixed with AuNPs. The concentration of the capture DNA was 3 μM, and that of the probe DNA was 0, 0.3, 0.6, 1, 2, and 3 μM. The nano-flares were then obtained using the typical salt-aging or freezing protocol as described above. For the freezing method, NaCl (final concentration = 300 mM) was added to the DNA-AuNPs immediately before thawing to maintain a high salt

concentration needed for duplex DNA. Then the free DNA was removed after thawing by centrifugation and washing.

Signaling of nano-flares. To induce desorption of the probe DNA from nano-flares to produce signal, the prepared nano-flares were dispersed in buffer A. The final AuNP concentration was 1 nM. To quantify the concentration of recoverable probe DNA, excess amount of target DNA (DNA5, 200 nM) was added to induce the fluorescence recovery. The amount of DNA was quantified by comparing with a standard curve. To test the reaction kinetics, 10 nM of the target DNA was added.

Effect of temperature. To examine the role of freezing for DNA attachment, DNA loading at different temperatures was measured. The DNA/AuNP (ratio 300:1) mixture was incubated at different temperatures: -20 ºC in water, -20 ºC with 25% glycerol, 0 ºC, 4 ºC, and 25 ºC in water, 25 ºC with glycerol, and 37 ºC in water for 2 h. Then the loaded DNA was quantified using the method described above. Adding glycerol was to lower the melting point of water and thus inhibit the formation of ice. To test if a fast freezing method can be applied or not, the DNA/AuNP mixture was immersed in a container with dry ice for 2 min, which was sufficient for freezing the sample. Afterwards, the sample were placed on the bench at room temperature for thawing. The color change of AuNPs was recorded by a digital camera.

Effect of pre-loaded DNA on final DNA loading capacity. To probe the reaction mechanism under the freezing process, AuNPs were pre-loaded with DNA1-FAM at designated ratios (0, 25, 50, 100 DNA per AuNP). In a typical experiment, 200 µL of AuNPs (13 nm) were mixed DNA1-FAM of different concentrations. After loading the DNA using the low-pH method as described above, 100 µL aliquot of the mixture was

taken out for DNA quantification. Additional DNA was then added to the remaining solution to make the total DNA concentration the same for all samples. Then freezing was performed, and the final DNA loading capacity was measured.

Table S1. The sequences and modifications of the DNA samples used in this work.

DNA names	Sequences and modifications (from 5′ to 3′)
DNA1	SH-AAA AAA AAA CCC AGG TTC TCT
DNA1-FAM	SH-AAA AAA AAA CCC AGG TTC TCT-FAM
DNA2	SH-TTT TTT TTT ACG CAT CTG TGA-FAM
DNA3	SH-TTT TGT GGG TAG GGC GGG TTG G
DNA4	ACG CAT CTG TGA AGA GAA CCT GGG
DNA4-FAM	FAM-ACG CAT CTG TGA AGA GAA CCT GGG
DNA5	CCC AGG TTC TCT TCA CAG ATG CGT
DNA6	TCA CAG ATG CGT AAA AAA AAA-SH
DNA7	AAA AAA AAA CCC AGG TTC TCT
DNA7-FAM	FAM-AAA AAA AAA CCC AGG TTC TCT

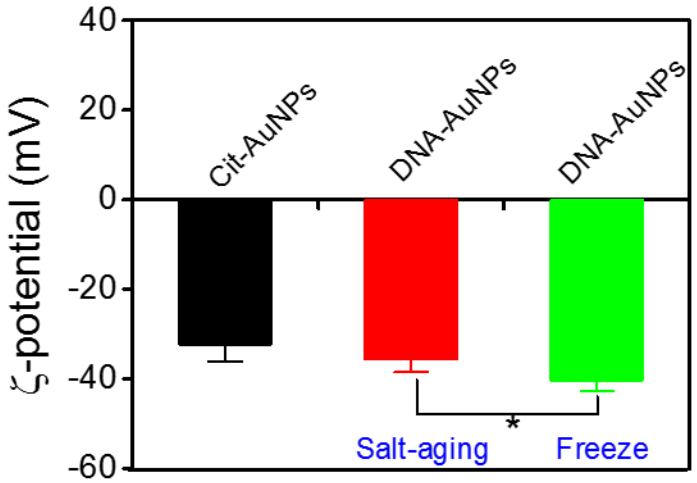

Figure S1. ζ-potential of cit-AuNPs and DNA-capped AuNPs prepared using the salt-aging or freezing method. AuNPs (2 nM) were dispersed in HEPES buffer (pH 7.6, 5 mM) at 25 °C. Both cit-AuNPs and DNA-AuNPs were negatively charged. A higher DNA density should result in a more negatively charged surface. Our data suggested that DNA loading only made the surface slightly more negative, and the ζ-potential changed from -32.4 ± 3.7 mV (cit-AuNPs) to -35.7 ± 2.8 mV (DNA-AuNPs from salt-aging) and -40.4 ± 2.2 mV (DNA-AuNPs from freezing). This might be attributed to that ζ-potential measures only the surface charge, and packing more DNA only slightly increases the surface charge density (e.g. the nucleotides not directly exposed to the surface contribute less to the ζ-potential measurement); (* stands for p value < 0.05, n = 3).

Table S2. Dynamic light scattering (DLS) measurement of AuNPs and their DNA conjugates.

AuNPs	**Average size[a] (nm)**	**PDI[b]**
Cit-AuNPs	14.6 ± 1.4	0.25
DNA-AuNPs from salt-aging	20.6 ± 1.3	0.21
DNA-AuNPs from freezing	21.6 ± 1.4	0.27

[a]: The average sizes and standard deviations are based on the peak value of three independent measurements.

[b]: The tabulated PDI is the mean PDI value from the three measurements, and the PDI is related to the fitting of each DLS spectra (e.g. related to the half-width of the spectra).

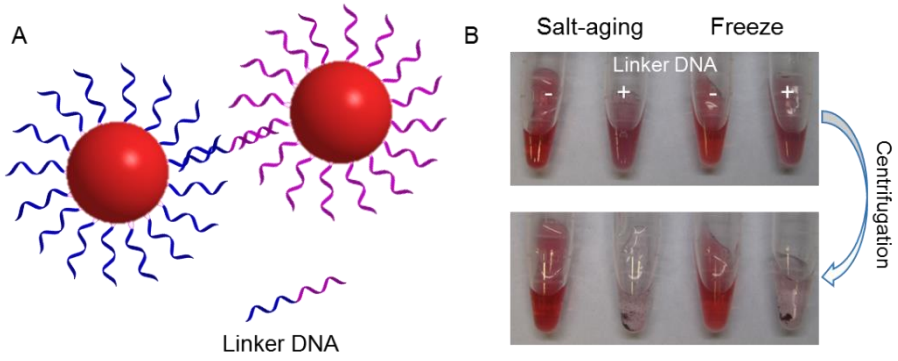

Figure S2. (A) Schemes and (B) photographs showing the assemly of DNA-functionalized AuNPs by a linker DNA. DNA1 and DNA6 were respectively attached to 13 nm AuNPs using the salt-aging and the freezing method. The initial concentration of these DNAs was 3 μM. After removing the non-attached free DNA, the DNA/AuNPs were dispersed in HEPES buffer (5 mM, pH 7.6). To achieve DNA-directely assemly, the linker DNA (DNA4, 200 nM) was incubated with DNA1-AuNPs (5 nM) and DNA6-AuNPs (5 nM) in HEPES buffer (10 mM, pH 7.6, with NaCl 300 mM). As shown in the photograph (B), adding the linker DNA resulted in the color change from red to purple, and the sample easily precipitated after a mild centrifution (5000 rpm for 5 min). As a control, the DNA-functionalized AuNPs without the linker DNA were still red and well dispersed. This indicated that the conjugates prepared by freezing were functional and can perform DNA-directed assembly.

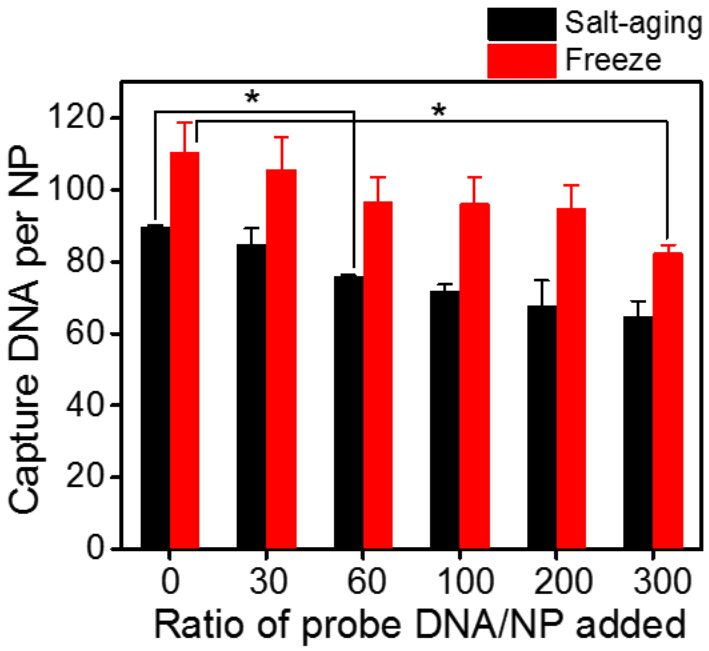

Figure S3. The number of capture DNA on each AuNP as a function of the probe DNA concentration using the co-adsorption strategy. DNA1-FAM (3 μM) and DNA4 (0, 0.3, 0.6, 1, 2, and 3 μM) were used to modify AuNPs (stock, ~ 10 nM) to prepare nano-flares. Two methods, salt-aging and freezing, were respectively perfomed to obtain the conjugates. For both methods, fewer capture DNA strands were attached as more probe DNAs was added. This decrease was attributable to the limited surface area on the AuNPs. Also, hybridization of the probe DNA with the capture DNA inhibited the attachment of capture DNA to AuNPs, again due to limited surface area on the AuNPs. (* stands for p value < 0.05, n = 3.)

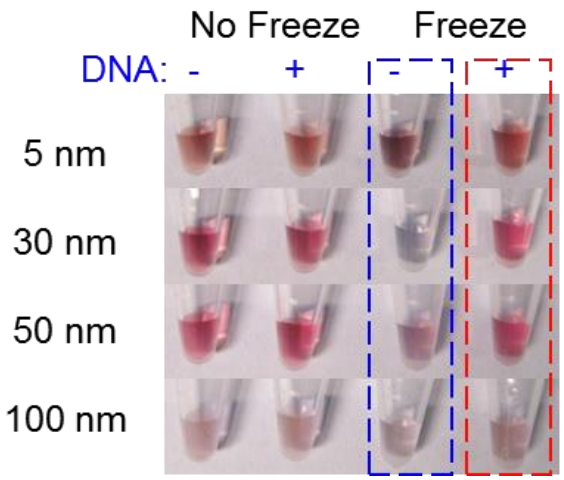

Figure S4. Photographs showing the color of AuNPs with different sizes before and after freezing in the presence or absence of DNA1. The DNA concentration was 3 μM for all the samples. It clearly shows that the DNA was able to protect the AuNPs from freezing-induced aggregation. In the absence of the DNA, AuNPs of all sizes aggregated, indicated by the color change (blue box). In the presence of DNA1, the color of the AuNPs was retained for all the sizes. Furthermore, the UV-vis absorption spectra of the prepared DNA/AuNP conjugates were also measured and are shown in Figure S5.

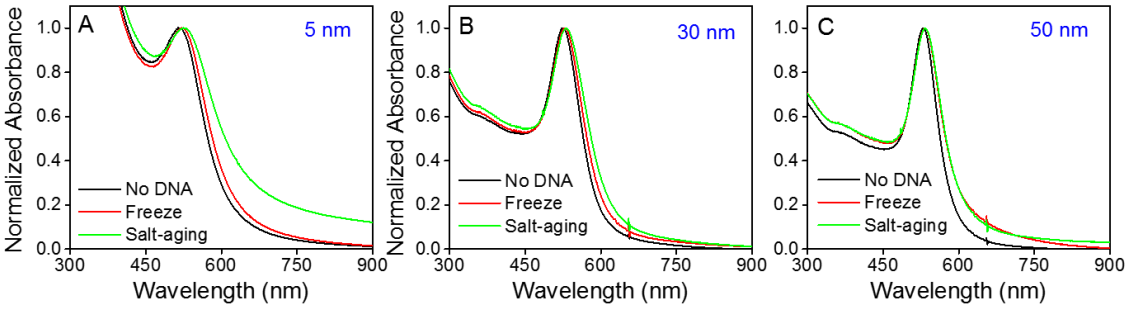

Figure S5. Normalized UV-vis absorption spectra of citrate-capped AuNPs and DNA-AuNPs prepared using the salt-aging method and the freezing method for AuNPs of (A) 5 nm, (B) 30 nm, and (C) 50 nm. In general, freezing supported better colloidal stability of the AuNPs as indicated by the smaller shift of the spectra.

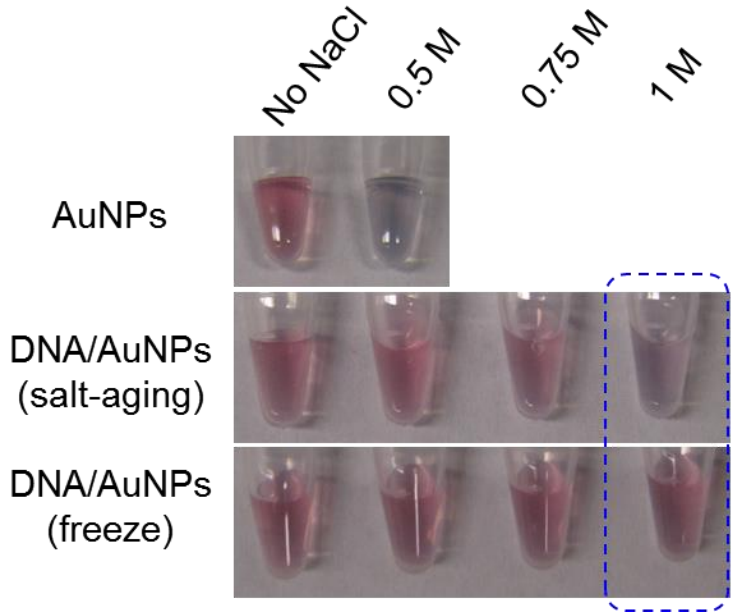

Figure S6. Stability of 50 nm AuNPs after DNA (DNA1) modification using the salt-aging or the freezing method. Photographs showing the color change of the AuNPs or DNA/AuNPs after adding NaCl of different concentrations for 10 min. Without DNA protection, the AuNPs aggregated quickly after mixing with NaCl (0.5 M). After modifying the AuNPs with the DNA using the salt-aging method, the color was retained with up to 0.75 M NaCl, but changed to blue at 1 M NaCl. Using the freezing method, the conjugate was still stable with up to 1 M NaCl. Freezing DNA/AuNPs not only serves as simpler method to functionalize AuNPs with DNA, but also it provides more stable conjugates against salt.

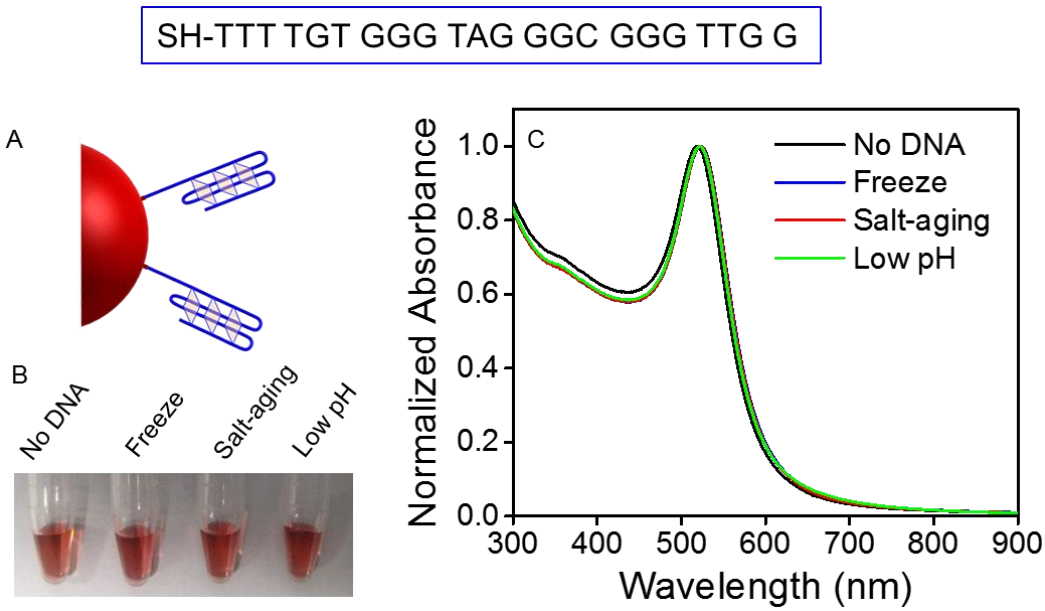

Figure S7. Attaching a G-rich DNA (DNA3, sequence in th top) to AuNPs using different methods. The DNA concentration was 3 μM, and the 13 nm AuNPs were ca. 10 nM (the stock solution). (A) A scheme showing the AuNPs with a G-rich DNA on the surface, and the DNA likely formed a G-quadruplex structure. (B) Photographs (C) normalized UV-vis absorption spectra showing the color of AuNPs and DNA3/AuNPs using the three different methods. With the protection of the DNA, the AuNPs were still red showing the typical absorption peak at ca. 520 nm, indicating the successful loading of the G-rich DNA.

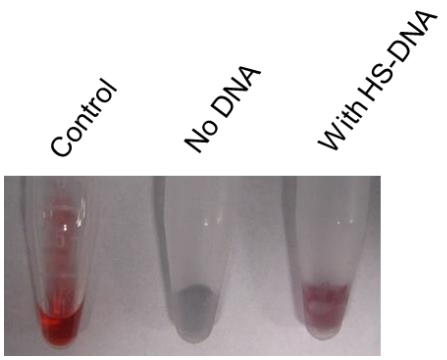

Figure S8. A photograph showing the color of the 13 nm AuNPs and DNA/AuNPs under the frozen state. Without DNA, the color of the AuNPs turns to blue, suggesting aggregation. With the addition of a thiolated DNA (DNA1, 3 μM), the color of AuNPs remained reddish during freezing.

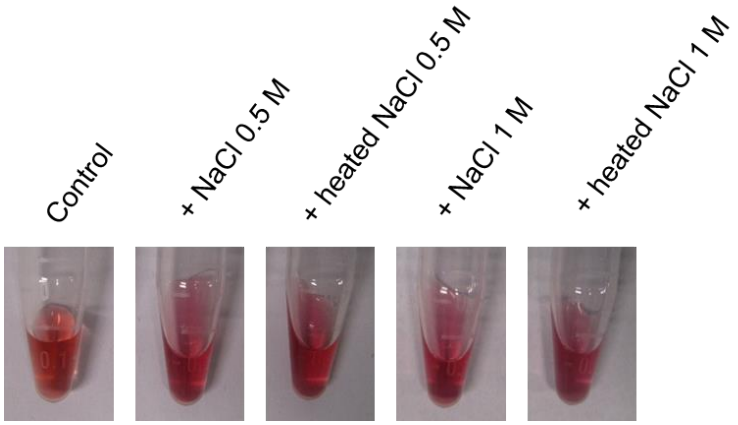

Figure S9. Photographs of DNA-functionalized upon adding NaCl solutions at different temperatures. NaCl (1 M or 2 M, 100 µL) was heated under 80 °C for 10 min before a quick mixing with the frozen DNA/AuNPs (100 µL). NaCl with the same concentration at room temperarure was also introduced for comparison. The fact that all the samples remained red indicated that the DNAs were attached to the AuNPs during freezing instead of during thawing.

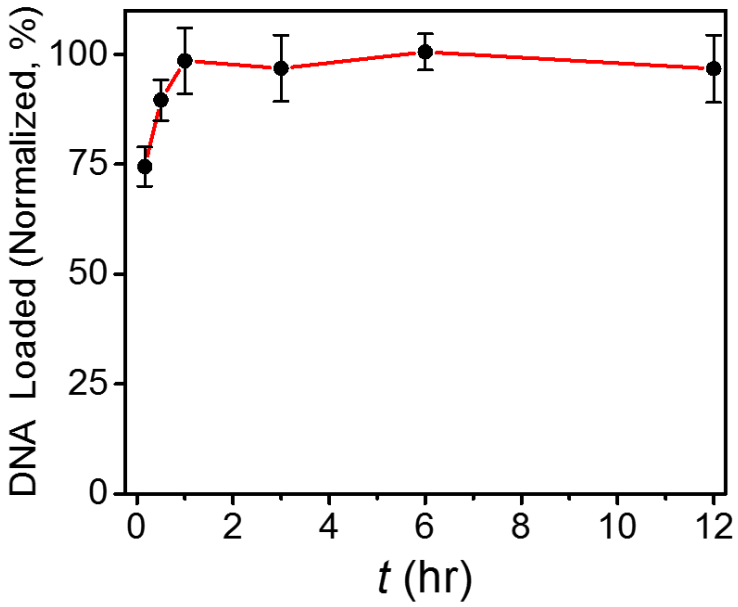

Figure S10. DNA loading density measured as a function of time using DNA1-FAM. The initial DNA/AuNP ratio was 300:1. Samples were thawed at different time points (before 1 h, the sample remained in the liquid phase). It shows that after 1 h or long freezing (up to 12 h), the number of adsorbed DNA remained constant. The change in the first hour was attributed to the time it takes for freezing (e.g. by putting a 2 mL sample in a 4 mL glass vial at room temperature to the -20 ºC freezer, it takes around 1 h to freeze). For most experiments, we performed the freezing for 2 h to make sure the reaction has reached saturation. As shown in the main paper, freezing by using dry ice can shorten the process to around 2 min.

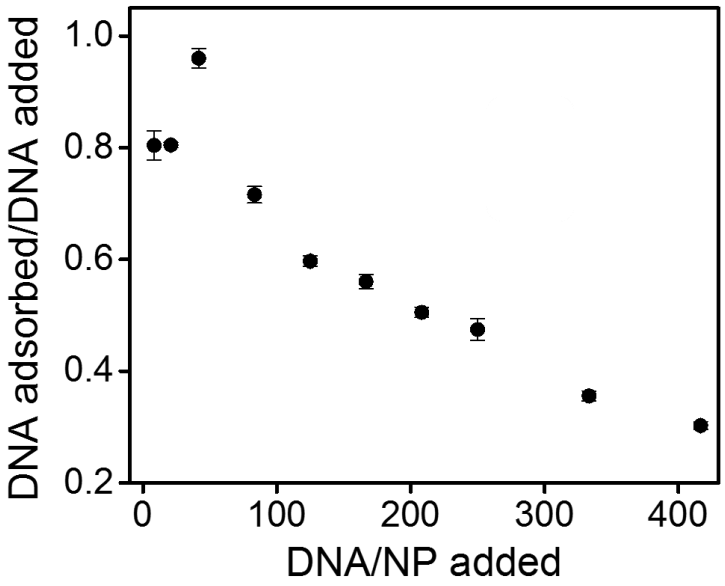

Figure S11. Fraction of DNA loading as a function of the intial DNA to AuNP ratio using the freezing method. With increase of the starting DNA concentration, more DNAs were loaded on each AuNP as shown in Figure 4C in the main paper. Here, we plotted the adsorption efficiency. At low initial DNA concentrations (e.g. DNA:AuNP < 50:1), the DNA loading efficiency was quite high (e.g. more than 80% DNA adsorbed). However, since the total DNA concentration was low, the final DNA density was far from saturation. With further increasing the initial DNA concentration, the fraction of DNA adsorbed decreased, but the final DNA density was increase as shown in Figure 4C. This can be explained by the Langmuir monolayer adsorption model especially with lateral ligand repulsion.

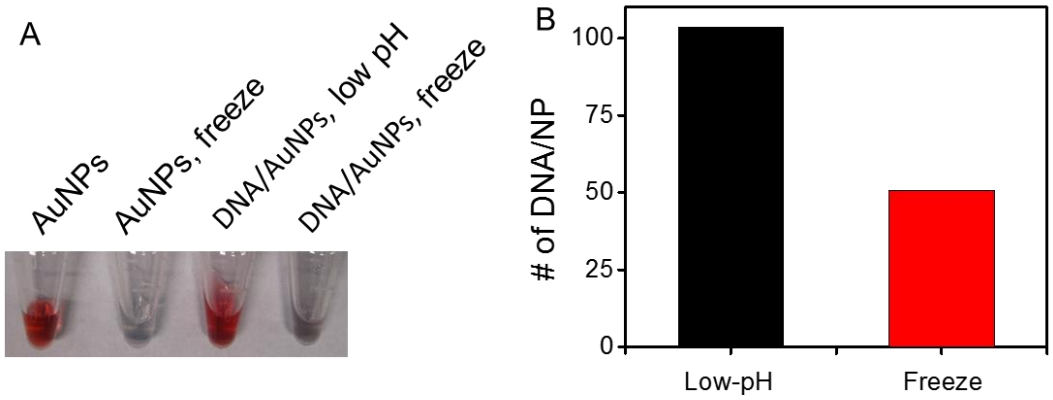

Figure S12. Non-thiolated DNA (DNA7-FAM) was used to functionalize AuNPs via the freezing method and the low pH method. The (A) photograph and (B) loading density were compared. DNA7-FAM (3 μM) failed to protect the AuNPs from freezing-induced aggregation. Each AuNP absorbed only ca. 50 strands of DNA7-FAM. For comparison, loading the same DNA sequence using the low pH method was performed, and each NP adsorbed ca. 100 DNAs and the AuNPs remained stable with the low pH method. Therefore, having a thiol group is critical for the freezing method to work, and this experiments tells us the importance of adsorption through the end and the freezing method seems to be able to increase the difference between thiol and base for AuNP adsorption kinetically.

Additional References

(S1) Liu, J.; Lu, Y. *Nat. Protoc.* **2006**, *1*, 246.

(S2) Liu, B.; Liu, J. *Anal. Methods* **2017**, *9*, 2633.

(S3) Storhoff, J. J.; Elghanian, R.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **1998**, *120*, 1959.

(S4) Hurst, S. J.; Lytton-Jean, A. K. R.; Mirkin, C. A. *Anal. Chem.* **2006**, *78*, 8313.

(S5) Zhang, X.; Servos, M. R.; Liu, J. *J. Am. Chem. Soc.* **2012**, *134*, 7266.

(S6) Hill, H. D.; Millstone, J. E.; Banholzer, M. J.; Mirkin, C. A. *ACS Nano* **2009**, *3*, 418.