



HOKKAIDO UNIVERSITY

Title	Controlled Assembly of Gold Nanodiscs via Click Chemistry and DNA Hybridization for Tunable Plasmonic Coupling
Author(s)	Melda, Taspika
Degree Grantor	北海道大学
Degree Name	博士(ソフトマター科学)
Dissertation Number	甲第16797号
Issue Date	2026-03-25
DOI	https://doi.org/10.14943/doctoral.k16797
Doc URL	https://hdl.handle.net/2115/99642
Type	doctoral thesis
File Information	Melda_Taspika.pdf, 全文



Doctoral Dissertation

**Controlled Assembly of Gold Nanodiscs via Click
Chemistry and DNA Hybridization for Tunable
Plasmonic Coupling**

**『クリックケミストリーおよび DNA ハイブリダイ
ゼーションを用いた金ナノディスクの集合制御に
よるプラズモンカップリング調節』**

Melda Taspika

Graduate School of Life Science, Hokkaido University

Division of Soft Matter

March 2026

Table of Contents

Table of Contents	i
Chapter 1 General Introduction	1
1.1 The Importance of Controlled Nanoparticle Assembly	2
1.2 Current Strategies to Control Nanoparticle Assembly	4
1.3 Synthesis, Properties, and Merits of Gold Nanodiscs (AuNDs)	5
1.4 Designs and Purposes of This Research.....	8
Chapter 2 Attempted Assembly and Crosslinking of AuNDs via Click Chemistry	10
2.1 Introduction	11
2.2 Materials and Experiments	13
2.2.1 Materials	13
2.2.2 Instruments for Characterizations	13
2.2.3 Synthesis of AuNDs	13
2.2.4 Surface Modification of AuNDs with C1, COOH, Alkyne, and N3 Ligands	15
2.2.5 Click Reaction between Alk- and N3-modified AuNDs	15
2.2.6 Click Reaction of AuNDs-Alk-(93:5:2) and N3-PEG-N3 Linker	16
2.2.7 Click Reaction of C1, COOH, Alk, and N3-modified AuNDs	16
2.3 Results and Discussions.....	16
2.3.1 Synthesis of AuNDs	16
2.3.2 Surface Modification of AuNDs and Investigation of Thermo-responsiveness.....	19
2.3.3 Click Reaction between Alk- and N3-modified AuNDs	22
2.3.4 Crosslinking of Alk-modified AuNDs with N3-PEG-N3 via Click Reaction.....	27
2.3.5 Thermo-responsiveness of AuNDs Modified with Both Alkyne and N3 Ligands	30
2.3.6 Click Reaction of AuNDs Modified with Both Alkyne and N3 Ligands.....	32
2.4 Conclusions	36
Chapter 3 Preparation of DNA-modified AuNDs for Controlled Assembly	38
3.1 Introduction	39
3.2 Materials and Experiments	40
3.2.1 Materials	40
3.2.2 Instruments for Characterizations	41
3.2.3 Modifying AuNDs with DBCO-DNA via Click Reaction	41
3.2.4 Modifying AuNDs with HS-DNA via Ligand Exchange Reaction.....	42
3.2.5 Calculation of DNA Grafting Density on AuND Surface.....	45
3.3 Results and Discussions.....	46

3.3.1	Surface Modification of AuNDs with DBCO-DNA via Click Reaction	46
3.3.2	Surface Modification of AuNDs with HS-DNA via Salt Aging	53
3.3.3	Surface Modification of AuNDs with HS-DNA via Butanol Dehydration	63
3.3.4	Calculation of Immobilized DNA on AuNDs Surface	69
3.4.	Conclusions	70
Chapter 4 Controlled Assembly of AuNDs via DNA Hybridization.....		72
4.1.	Introduction	73
4.2.	Materials and Experiments	75
4.2.1.	Materials	75
4.2.2.	Instruments for Characterizations	75
4.2.3.	AuNDs Assembly Driven by DNA Hybridization	76
4.2.4.	Numerical Simulation of Extinction Spectra and Near Field Distribution of AuNDs ..	76
4.2.5.	Synthesis of AuNPs	77
4.2.6.	Modifying AuNPs with HS-DNA via Ligand Exchange Reaction	77
4.2.7.	Assembly of AuNPs via DNA Hybridization.....	77
4.2.8.	Reversible Assembly and Disassembly of DNA-modified AuNDs	78
4.2.9.	Determination of Assembly and Disassembly Temperatures	78
4.2.10.	Determination of Interparticle Gap Distance of Assembled AuNDs.....	78
4.2.11.	Suppression of AuND Assembly with Blocking DNA Strands.....	79
4.2.12.	Multimerization of Assembled AuNDs	79
4.2.13.	Separation of Assembled AuNDs by Gel-electrophoresis	79
4.2.14.	Separation of Assembled AuNDs by Centrifugal Force	79
4.2.15.	Crosslinking of Assembled AuNDs	80
4.3.	Results and Discussions.....	81
4.3.1.	Assembly of AuNDs Driven by DNA Hybridization	81
4.3.2.	Dispersion Stability of DNA-modified AuNDs.....	88
4.3.3.	Effect of Curvature on DNA Hybridization.....	94
4.3.4.	Controlled Multimerization Degree of Assembled AuNDs.....	96
4.3.5.	Tuning Interparticle Gap Distance of AuND Assembly by Different DNA Lengths.....	102
4.3.6.	Kinetic and Thermodynamic Effects on AuNDs Assemblies.....	104
4.3.7.	Tuning Assembly and Disassembly Temperatures of Assembled AuNDs	106
4.3.8.	Separation of Assembled AuNDs by Gel-electrophoresis	113
4.3.9.	Size-based Fractionation of AuND Multimer via Centrifugal Separation.....	114
4.3.10.	Crosslinking of Assembled AuNDs with Psoralen Molecule or HS-PEG-HS	117
4.4.	Conclusions	122
Chapter 5 Conclusions and Future Outlooks		124
References.....		127

Acknowledgements	136
Supporting Information	137
I. SEM Images of AuNDs Multimers Prepared with Various Mixing Ratios.....	138
II. SEM Images of AuNDs Multimers Prepared with Various NaCl Concentrations	143
III. SEM Images of AuNDs Multimers Separated by Different Centrifugal Forces.....	145
IV. Cryo-TEM Images of 11 bp-DNA-AuND Multimers with (67:33) Molar Ratio	148
V. Cryo-TEM Images of 20 bp-DNA-AuND Multimers with (67:33) Molar Ratio	151
VI. Cryo-TEM Images of 8 bp-DNA-AuND Multimers with (67:33) Molar Ratio	153
VII. Determination of Assembly (Ta) and Disassembly (Td) Temperatures of AuNDs.....	153

Chapter 1
General Introduction

1.1 The Importance of Controlled Nanoparticle Assembly

Plasmonic gold nanoparticles have been widely used for many applications due to the unique collective oscillation of their conduction electrons, named as plasmon¹. Assembly occurs when individual particles organize into clusters that connect via weak or strong bonds, forming ordered or disordered structures². Energy input or external forces are often required to direct particles into assembled states that are thermodynamically more stable³. Gold nanoparticles can assemble into random or aligned structures through electrostatic interaction⁴, hydrogen bonding⁵, hydrophobic interaction^{6,7}, or deoxyribonucleic acid (DNA) hybridization^{8,9} via ligands or molecules immobilized on their surfaces.

To date, gold nanoparticles have been functionalized with ligands bearing various headgroup structures, which impart different properties^{6,7,10–12}. Among them, DNA ligands enable precise control over the assembled structures¹³, allowing tunable interparticle gap distances^{14–16} through Watson-Crick base-pairing interactions (dA-dT and dC-dG). During assembly, the interaction of individual plasmons leads to the formation of new hybridized modes, enhancing the electric field within the nanogaps. The optical properties of these assemblies are highly sensitive to factors such as cluster sizes⁵, shapes¹⁷, spatial arrangement¹⁸, and interparticle distances¹⁹.

Assembly size plays a critical role in plasmonic coupling and the resulting plasmonic shift. Larger assemblies induce greater plasmonic shifts due to more pronounced near-field interactions⁵. However, uncontrolled assembly growth can lead to aggregation, which obscures clear observation of plasmonic modes¹⁴. In terms of interparticle bonding, aggregation differs from agglomeration. In agglomeration, nanoparticles are weakly bound and can dissociate reversibly. In contrast, aggregation involves strong, irreversible binding between particles²⁰. Aggregation negatively affects the optical properties of nanoparticle assemblies, such as reducing the extinction coefficient and diminishing photothermal efficiency²¹. Therefore, controlled assembly is essential to prevent irreversible aggregation and to regulate plasmonic responses, thereby enhancing the functionality and applicability of assembled nanoparticles in advanced technologies⁷.

Furthermore, plasmonic coupling can be tuned by altering the interparticle gap distance¹⁹ or selectively attaching nanoparticles at desired locations²². When interparticle gap distances decrease, plasmonic interactions intensify, resulting in a larger shift in extinction spectra. However, if particles are too close (~ 1 nm), plasmonic coupling is disrupted due to quantum mechanical electron tunneling²³. Conversely, if the distance is too large, weak

coupling results in negligible plasmonic shifts^{9,24}.

Nanoparticle shape also influences assembly behavior (**Figure 1. 1a**), as curvature affects ligand interactions on the nanoparticle surface^{6,12}. Spherical gold nanoparticles (AuNPs) have high surface curvature, which forces surface ligands to bend or compress during interaction with neighboring particles, leading to reduce thermodynamic stability²⁵. Moreover, isotropic AuNPs tend to assemble into three-dimensional (3D) structures, which often aggregate due to instability arising from high surface energy²⁶. In contrast, gold nanorods (AuNRs) possess varying curvatures along their sides and ends, creating different ligand packing densities. As a result, AuNRs may assemble in a two-step process: first, hydrophobic ligands on the low-curvature sides dehydrate at lower temperatures to promote side-by-side assembly, followed by dehydration of edge ligands, leading to 3D structures¹². Thus, specific interactions^{27,28} at either the ends or sides of AuNRs are necessary to form one-dimensional (1D) or two-dimensional (2D) assemblies²⁹. Gold nanoplates—such as nanocubes (AuNCs), nanotriangles (AuNTs), and nanodiscs (AuNDs)—tend to assemble in a face-to-face configuration due to their large flat surfaces^{7,9,30}. These broad surfaces promote stable ligand interactions without bending²⁵, contributing to enhance assembly stability.

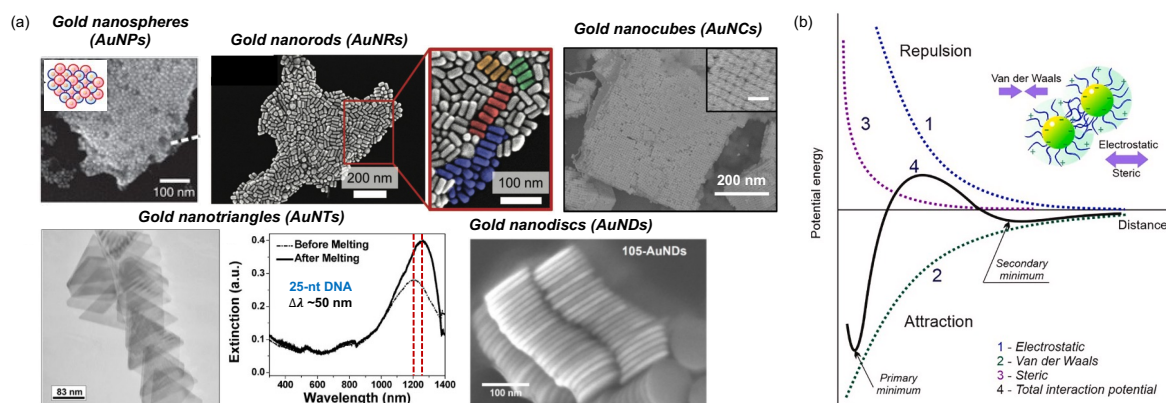


Figure 1. 1. (a) Assembly of different shapes of gold nanoparticles^{7,9,12,26,30}. (b) Total potential energy between colloidal nanoparticles as a function of interparticle gap distance³¹.

Thermodynamically, interactions between colloidal nanoparticles can be described by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, which considers the total potential energy of the system (**Figure 1. 1b**)³¹. At long distances, electrostatic repulsion between nanoparticles is sufficiently strong to keep them stable and dispersed. As nanoparticles approach one another, van der Waals attraction becomes significantly increased. The interplay between electrostatic repulsion and van der Waals attraction forms a characteristic potential energy curve. At very short distances, nanoparticles enter the primary minimum of the total potential energy, resulting in irreversible aggregation. To reach this state, the particles must

overcome an energy barrier by acquiring sufficient kinetic energy. Consequently, nanoparticles typically remain in the secondary minimum, where weaker attraction allows for reversible agglomeration or controlled assembly/disassembly³².

In summary, nanoparticle assembly can be regulated by modulating interparticle interactions and the surrounding environment. For these reasons, controlled assembly—through manipulation of both nanoparticle interactions and external conditions—is essential to: (i) prevent irreversible aggregation, (ii) control assembly size, (iii) regulate assembly structure, and (iv) achieve tunable plasmonic coupling.

1.2 Current Strategies to Control Nanoparticle Assembly

To date, various strategies have been employed to regulate the assembly of gold nanoparticles (AuNPs) by controlling the amount, location, and specificity of surface modifications, enabling the fabrication of one-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) assembly structures. Generally, two main approaches are used: (i) template-based directed assembly and (ii) simple mixing of linker-modified AuNPs. Well-ordered assemblies of AuNPs can be achieved using templates such as glass substrates³³, DNA origami³⁴, and DNA encoders¹⁸. Templates guide AuNPs into linear assemblies with defined particle numbers (**Figure 1. 2a**). However, different oligomer structures require distinct templates and designs, which limits broader applications due to high cost and complex fabrication procedures.

In contrast, simple mixing of complementary functionalized DNA–AuNPs³⁵ (**Figure 1. 2b**) or the use of anionic polymers with oligo(ethylene glycol) (OEG)-coated AuNPs⁵ can control the size of the assemblies. Finite-sized assemblies are achieved by regulating the extent of DNA hybridization between DNA-functionalized AuNPs or hydrogen bonding between anionic polymers and OEG–AuNPs. However, isotropic AuNPs tend to form 3D aggregates, which often precipitate out of solution. Moreover, it is difficult to accurately determine the number of particles within the resulting assembly clusters.

AuNRs can be assembled into one-dimensional (1D) or two-dimensional (2D) structures through selective surface modification at either the ends or the sides. For instance, end-to-end assembly of AuNRs can be achieved by the selective attachment of thiol-terminated polystyrene, which is regulated by solvent conditions (**Figure 1. 2c**)²². However, the linearity of the resulting assemblies decreases as the number of oligomers increases. This limits the formation of long AuNR chains due to the weakness of the connecting points. Additionally, differences in surface curvature lead to varying dehydration temperatures of the surface ligands.

This property allows AuNRs to initially assemble side-by-side into 2D sheets, which can subsequently transform into 3D structures^{12,13}.

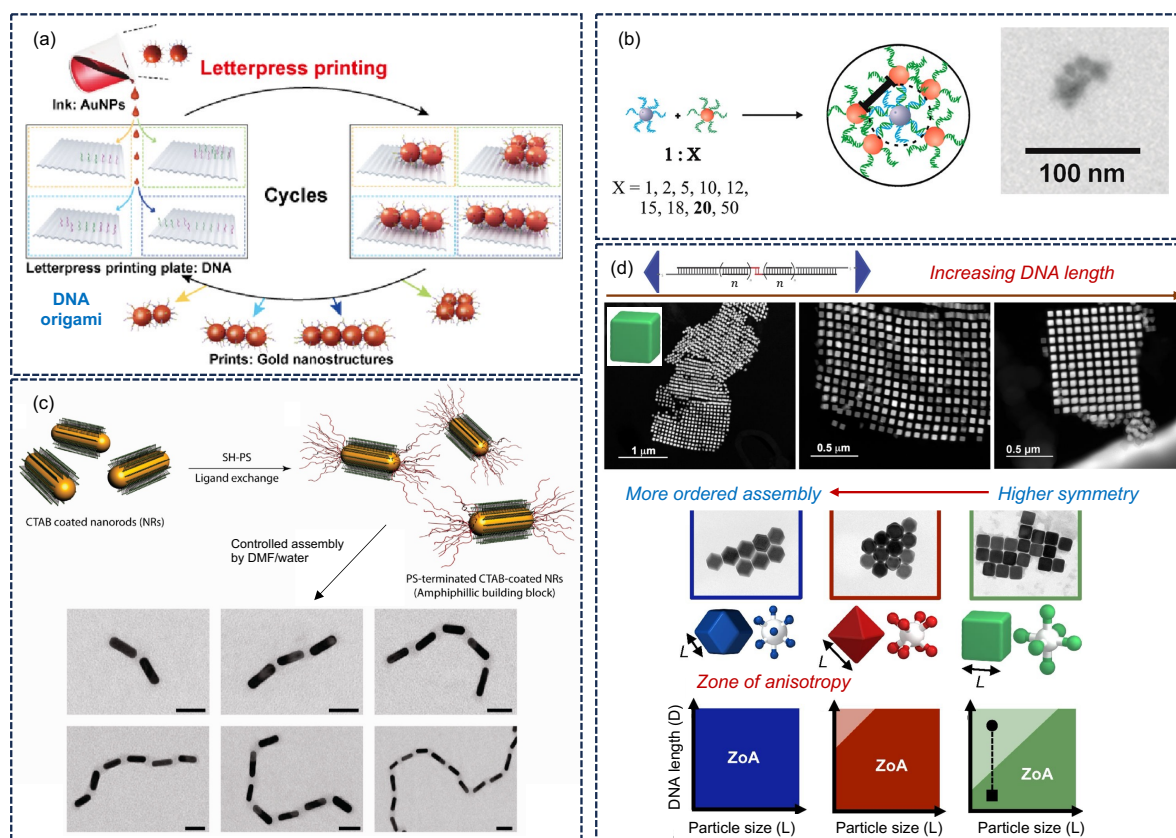


Figure 1. 2. (a) Linear and defined-number assembly of AuNPs by using DNA origami as a template³⁴. (b) Controlled assembly sizes by adjusting the mixing ratio of DNA-modified AuNPs³⁵. (c) Controlled assembly of AuNRs by selective attachment of ligands²². (d) Ordered assembly of anisotropic gold nanoparticles in the zone of anisotropy^{9,13,36}.

In contrast, gold nanoplates preferentially assemble in a face-to-face orientation due to their large flat surfaces^{7,9,30}. For AuNCs and AuNTs, a specific linker length is required to preserve the anisotropy of the particle core (**Figure 1. 2d**). These nanoparticles lose their well-ordered face-to-face arrangement if the particle size and linker length do not fall within the “zone of anisotropy,” which is restricted by their limited symmetry^{36,37}. Among gold nanoplates, gold nanodiscs (AuNDs) possess an effectively infinite anisotropy zone, allowing them to form face-to-face interactions regardless of the ligand length^{17,36}. Moreover, the linear structure of these assemblies facilitates accurate counting of the number of particles within each cluster.

1.3 Synthesis, Properties, and Merits of Gold Nanodiscs (AuNDs)

The synthesis of gold nanodiscs (AuNDs) remains a critical challenge due to difficulties in precisely controlling their size, shape, and composition, which in turn limits their utility in advanced technological applications. Physical methods are commonly employed to fabricate

ordered arrays of AuNDs, owing to their ability to precisely control interparticle gap distances. Among these, lithography is the most widely used technique, forming desired surface patterns by exposing substrates to light, ions, or electrons. Although physical approaches offer high precision in particle arrangement, they often fail to achieve narrow interparticle gap distances and high-symmetry structures³⁸ due to self-shadowing effects during gold deposition, which result in truncated or distorted geometries. Furthermore, additional processing steps are required to obtain dispersible AuNDs in solution.

In contrast, wet chemical methods are well-suited for synthesizing highly symmetrical AuNDs with thicknesses below 10 nm^{7,39}—dimensions that are challenging to achieve using physical techniques. Although reproducibility remains a challenge due to the sensitivity of the growth conditions, chemical synthesis offers a low-cost and scalable route to produce colloidal AuNDs directly. Moreover, chemical methods enable the formation of assemblies with narrow and tunable interparticle gap distances, a feature that is difficult to attain through physical techniques, as discussed later.

In this study, AuNDs were synthesized via the comproportionation reaction of gold nanotriangles (AuNTs), following our previously reported method⁷. AuNDs possess unique structural advantages over other gold nanostructures due to their large flat surfaces and circular symmetry, both of which significantly influence their plasmonic properties. One of the key features of AuNDs is their surface plasmon resonance (SPR), which depends strongly on the size, shape, and uniformity of the discs⁴⁰. SPR refers to the resonant electromagnetic response that arises when surface plasmons oscillate coherently at a frequency matching that of the incident light⁴¹. Upon irradiation by an electric field, the electron cloud in AuNDs becomes polarized, leading to charge separation. The positively charged nuclei exert a restoring force on the displaced electrons, promoting collective oscillation. SPR occurs when the frequency of the incident photons resonates with the natural oscillation frequency of the electrons. This resonance enhances the local electromagnetic field, amplifying radiative properties such as absorption and scattering. When the electric field is tightly confined to the nanoparticle surface due to coherent oscillations, localized surface plasmon resonance (LSPR) occurs^{42,43}. The LSPR peak position depends on both the shape and size of the AuNDs, as well as on their electronic structure and dynamic depolarization effects.

Two-dimensional AuNDs with high aspect ratios typically exhibit only an in-plane dipolar mode, with negligible transverse modes due to their symmetry. The spectral bandwidth of LSPR depends on the disk diameter, influenced by scattering and radiative damping effects. When the diameter exceeds 30 nm, both radiative and nonradiative damping contribute to

LSPR decay, resulting in broader spectral linewidths⁴⁰. Radiative damping arises from photon emission, while nonradiative damping is associated with intraband and interband electronic excitations, leading to the formation of electron-hole pairs. Larger particles exhibit lower LSPR energies and greater radiative damping, causing a red-shift in the LSPR peak.

The geometry of AuNDs also strongly influences their optical absorption and scattering behavior. In stacked AuND dimers composed of discs with different sizes, interactions with incident light produce distinct absorption and scattering modes, observed at approximately 630 nm and 850 nm (**Figure 1. 3a**)⁴⁴. In such dimers, the larger disc contributes predominantly to light scattering, whereas the smaller disc is more effective in light absorption. However, stacked assemblies fabricated by nanoimprint lithography, which lack nanogaps, restrict tunable plasmonic coupling and dynamic hotspot formation.

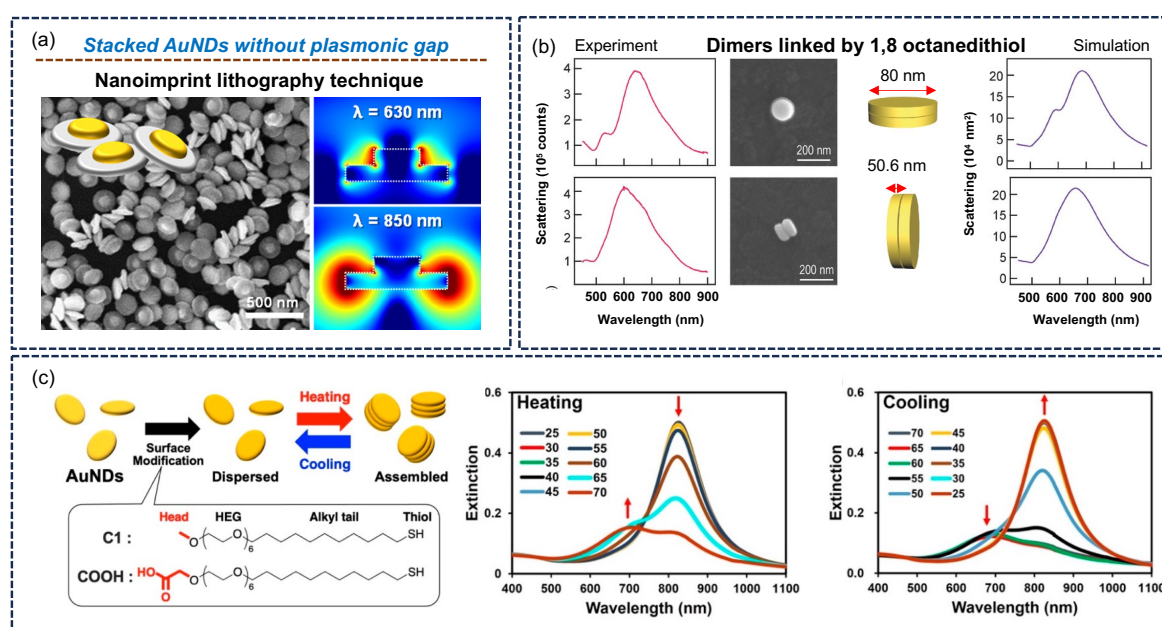


Figure 1. 3. (a) Stacked assembly of AuNDs with different diameters⁴⁴. (b) Assembly of AuND dimers in different geometrical configurations⁴⁵. (c) Reversible assembly/disassembly of AuNDs driven by hydrophobic interaction of thermo-responsive ligand⁷.

The circular symmetry of AuNDs allows them to assemble in face-to-face or side-by-side configurations, resulting in diverse plasmonic modes. In a previous study, AuND dimers linked by octanedithiol were occasionally observed after drying on an indium tin oxide (ITO) substrate⁴⁵ (**Figure 1. 3b**). Although scattering spectra were successfully measured, it proved difficult to reproducibly generate AuND dimers due to the lack of control over the assembly process. Despite the intriguing plasmonic behavior associated with finite-number assemblies, research on AuNDs remains limited. Our group has previously functionalized AuNDs with thermo-responsive and carboxylic ligands, enabling reversible assembly and disassembly of

the particles (**Figure 1. 3c**). Although a significant plasmonic shift was achieved, the assembly process itself could not be reliably controlled in experimental conditions. The use of a single type of ligand on AuNDs restricted the ability to fine-tune both the assembly size and the interparticle gap distance. Therefore, a simple and effective strategy to control the assembly of AuNDs is essential for fully exploiting their plasmonic properties. While size-dependent plasmonic responses of circular AuNDs have been theoretically simulated ^{7,17}, the experimental investigation of their optical properties in finite-size assemblies has not yet been fully explored.

1.4 Designs and Purposes of This Research

The main objective of this research is to control the assembly of AuNDs, including the interparticle gap distances and the degree of multimerization, in order to tune the strength of plasmonic coupling. This is achieved by using two types of ligand-modified AuNDs, which allow to control both assembly size and plasmonic response. To accomplish this purpose, two approaches are employed in this study. First, AuNDs are assembled through cross-linking between alkyne (**Alk**) and azide (**N3**) ligands via a click reaction (**Figure 1. 4a (i)**). The assembly is driven by hydrophobic interactions resulting from the dehydration of the ethylene glycol segments upon heating. The **Alk** and **N3** functional groups on the assembled AuNDs are covalently cross-linked via a copper(I)-catalyzed alkyne–azide cycloaddition (click reaction), thereby stabilizing the multimer structures at temperatures lower than the assembly temperature (T_A). The size of the conjugated AuNDs can be controlled by adjusting the molar mixing ratio of **Alk**- and **N3**-terminated AuNDs. Furthermore, to dynamically control the interparticle gap distances, **Alk**-AuNDs are covalently cross-linked using diazido-PEG (**N3**–PEG–**N3**) with different molecular lengths. This enables to modulate the gap distances through thermal cycling (cooling and heating processes) (**Figure 1. 4a (ii)**).

Secondly, AuND assembly was driven by specific hybridization between complementary single-stranded DNA (ssDNA) ligands in the presence of salt, which screens the electrostatic repulsion (**Figure 1. 4b**). The degree of multimerization can be controlled by adjusting the mixing molar ratio of (–)-DNA-AuNDs to (+)-DNA-AuNDs or by changing the salt concentration to regulate reaction kinetics. In addition, the interparticle gap distances of the assembled AuNDs can be tuned by varying the DNA strand lengths. To stabilize the assembled structures at higher temperatures or under lower salt concentrations, the double-stranded DNA (dsDNA) can be cross-linked using crosslinking agents such as psoralen. Alternatively, the assembled AuNDs can be stabilized by conjugation with dithiol-PEG (HS–PEG–HS) via Au–S bonds. To achieve precise control over AuND assembly—including

interparticle gap distances and the degree of multimerization—for tuning the strength of plasmonic coupling, both approaches are investigated in the following chapters.

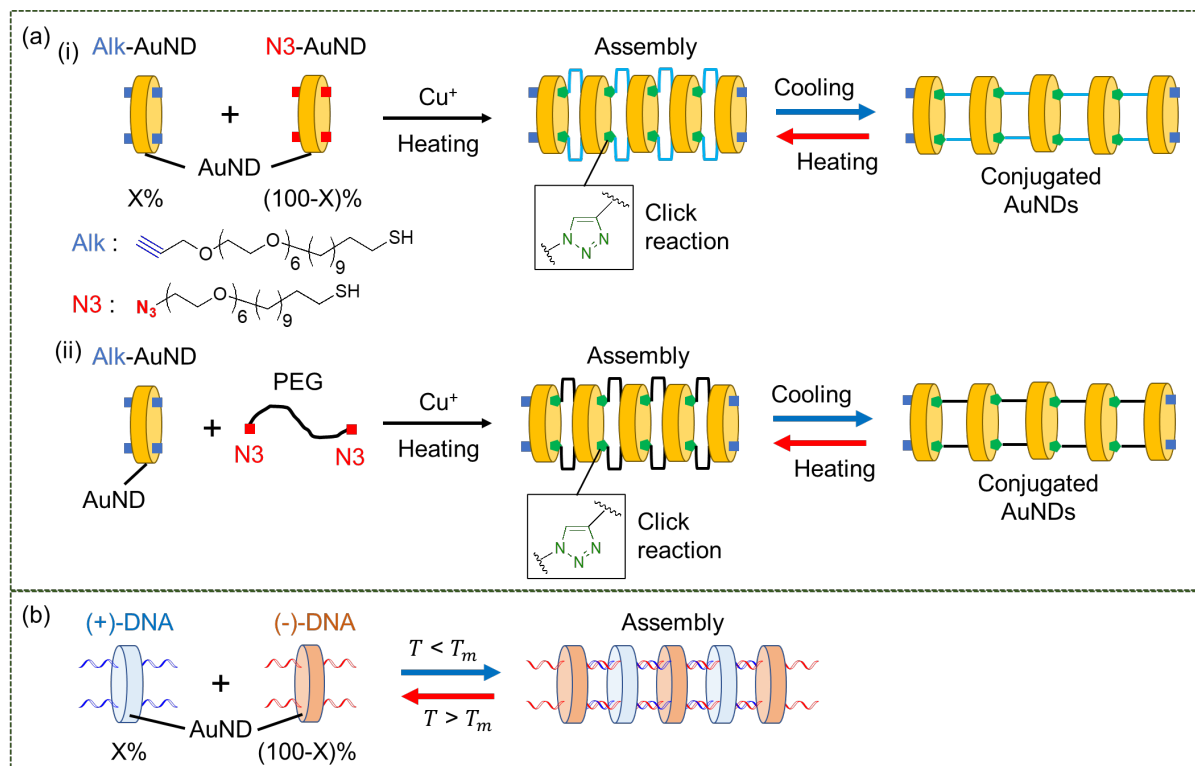


Figure 1. 4. Two approaches used in this study to control the degree of multimerization and interparticle gap distance: (a) (i) Control of multimerization degree by adjusting the mixing molar ratio of Alk- to N3-terminated AuNDs; (ii) dynamic modulation of interparticle gap distance through thermal cycling. (b) Control of multimerization degree by adjusting the mixing molar ratio of (-)-DNA-AuNDs to (+)-DNA-AuNDs.

Chapter 2

Attempted Assembly and Crosslinking of AuNDs

via Click Chemistry

2.1 Introduction

In this chapter, controlled assembly of gold nanodiscs (AuNDs) was investigated utilizing organic ligands combining thermo-responsive and click-reactive functions.

Ligands for metal nanoparticles are used to stabilize the surface, enhance dispersibility in solution, and functionalize the surface. Generally, ligands are classified into two categories: organic and inorganic. One of the major classes of organic ligands for gold nanoparticles is alkanethiols, which have the structure $\text{HS}-(\text{CH}_2)_n-\text{X}$, where the alkyl chain length and terminal functional group (X) can vary to provide specific properties such as carboxylic ($-\text{COOH}$), amine ($-\text{NH}_2$), thiol ($-\text{SH}$), or hydroxyl ($-\text{OH}$) groups. The strong chemical affinity between thiol groups and gold is exploited as the initial driving force for the self-assembled monolayer formation of alkanethiol ligands on gold surface⁴⁶. In our group, thermo-responsive alkanethiol ligands functionalized with hexa(ethylene glycol) derivatives and alkyl head groups have been employed to modify gold nanoparticles, enabling reversible assembly and disassembly controlled by temperature^{5,7,12,47}. Upon heating, the thermo-responsive ligands undergo dehydration at specific temperatures, enhancing hydrophobic interactions and promoting nanoparticle assembly. The alkyl head groups significantly influence the surface hydrophobicity of nanoparticles, thereby affecting their assembly temperature^{6,12,48}.

Click chemistry—particularly copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC)—has been widely applied in bioconjugation and nanostructure functionalization due to its high selectivity, excellent yield, and minimal side reactions^{49,50}. The CuAAC reaction conjugates alkyne (**Alk**) and azide (**N3**) functional groups in the presence of Cu^+ as a catalyst, lowering the activation energy and accelerating the reaction. Specifically, the **Alk** group forms an alkynyl-copper(I) intermediate, which then reacts with the **N3** group. The reaction proceeds via a [3+2] cycloaddition followed by protonation to form a 1,2,3-triazole product^{51,52}, resulting in a covalent linkage through the triazole ring (**Figure 2. 1a**).

In this research, AuNDs were modified with hexa(ethylene glycol) derivatives possessing methyl, alkyne, azide, or carboxylic acid terminal groups and an alkyl-thiol tail (**Figure 2. 1b**), designated as **C1**, **Alk**, **N3**, and **COOH**, respectively. **C1** is a thermo-responsive ligand that facilitates AuND assembly and promotes interparticle contact. **COOH** is deprotonated to COO^- in the basic solution, providing electrostatic repulsion to maintain nanoparticle dispersion. **Alk** and **N3** are click-reactive ligands that undergo a Cu^+ -mediated click reaction, forming covalent cross-links via a 1,2,3-triazole ring. The combination of these ligands enables the formation of AuND assemblies crosslinked via click chemistry and

thermally promoted contact. Moreover, by using hetero-functionalized AuNDs modified with **Alk** and **N3**, the degree of multimerization can be tuned by adjusting the molar mixing ratio of **Alk**- to **N3**-modified AuNDs (**Figure 2. 1c(i)**). Additionally, crosslinking **Alk**-functionalized AuNDs with diazido-PEG (**N3**–PEG–**N3**) allows dynamic control over interparticle gap distances in the assembled structures (**Figure 2. 1c(ii)**), utilizing reversible assembly and disassembly through thermal cycling (heating and cooling).

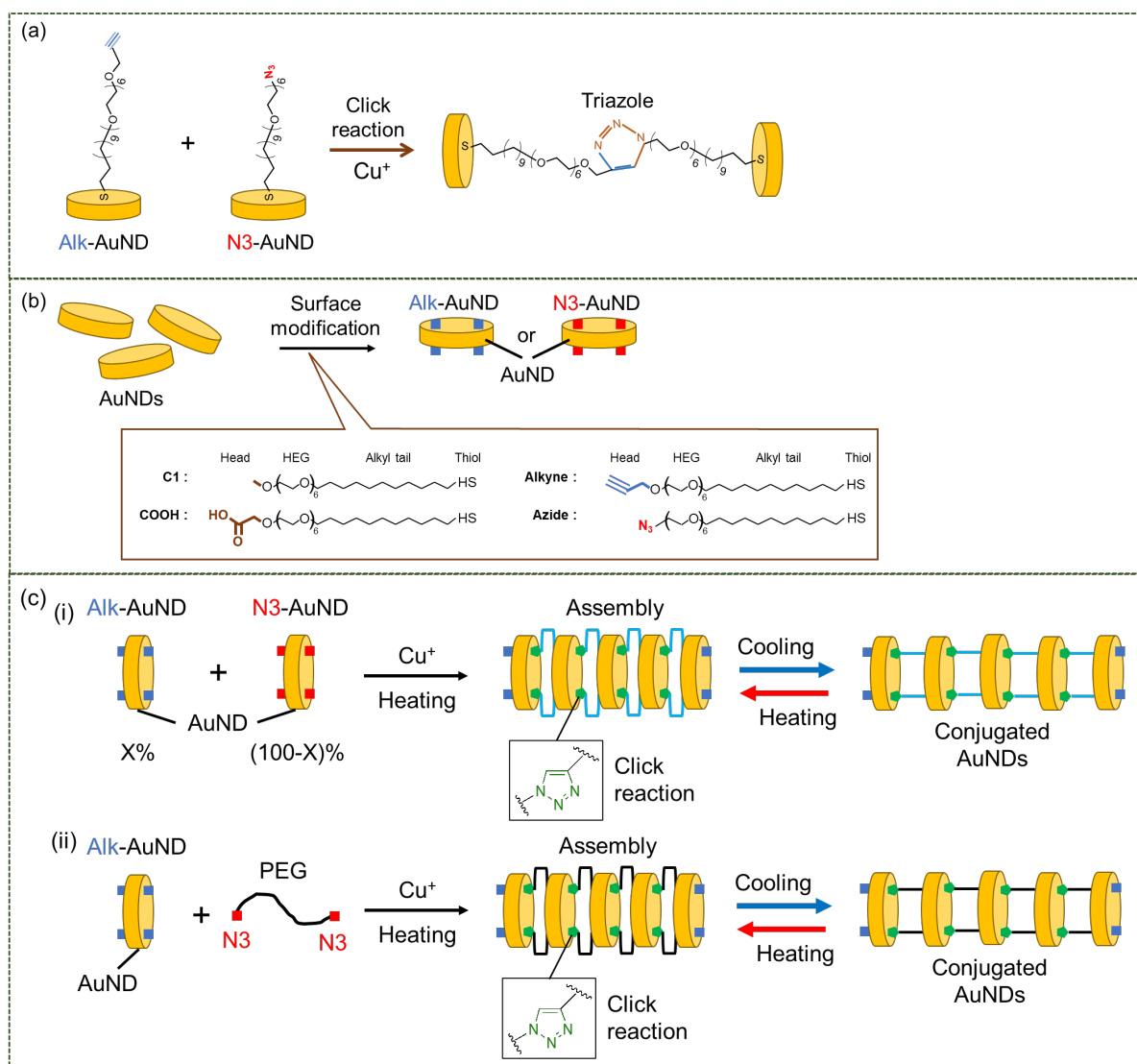


Figure 2. 1. Schematic illustrations of attempted assembly and crosslinking of AuNDs via click chemistry. (a) Click reaction between **Alk** and **N3** functional groups. (b) Chemical structures of ligands used to modify AuNDs surfaces. (c) Controlled AuNDs assembly by click reaction: (i) control of multimerization degree by adjusting the mixing molar ratio of **Alk**- to **N3**-terminated AuNDs; (ii) dynamic modulation of interparticle gap distance through thermal cycling.

2.2 Materials and Experiments

2.2.1 Materials

The carboxylic acid-terminated ligand, 20-(11-mercaptoundecanyloxy)-3,6,9,12,15,18-hexaoxaicosanoic acid (**COOH**) was purchased from Dojindo Laboratories Co., Ltd. (Japan). Thermo-responsive ligand with a methyl-terminated head (**C1**) and surface ligands with **Alkyne** and **N3** head were prepared in our laboratory. Hydrogen tetrachloroaurate (III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), sodium borohydride (NaBH_4), hexadecyl trimethylammonium bromide (CTAB), sodium iodide (NaI), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) were purchased from Merck (Sigma Aldrich Chemie GmbH, München, Germany). Sodium hydroxide (NaOH , 1 mol/L), L(+)-ascorbic acid sodium salt ($\text{C}_6\text{H}_7\text{NaO}_6$), HEPES, and copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were purchased from Wako Pure Chemical Industries (Japan). Tris (3-hydroxypropyl-triazolylmethyl) amine 95% (THPTA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). **N3**-PEG-**N3** linker ($M_w = 1050$ g/mole) was synthesized in our laboratory. All commercial reagents were used without further purification. Ultrapure water was used to prepare all solutions for the experiments (Milli-Q reference system).

2.2.2 Instruments for Characterizations

UV-Vis-NIR spectrophotometer (V-730 or V-770) with a PAC-743R Automatic 6 position Peltier cell changer (JASCO Corp., Tokyo, Japan) was used to record the extinction spectra. A heating rate of 1 °C/min and a waiting time of 5 min were used for temperature-dependent sample measurements upon heating and cooling. STEM images were captured by an HD-2000 (Hitachi High-Tech, Tokyo, Japan) with a 200 kV accelerating voltage. The sample was prepared by dropping 5 μL of the concentrated sample solution onto a copper TEM-grid and dried at room temperature. To observe AuND thickness, 15 μL of 1 nM AuNDs dispersed in 1 mM CTAB was deposited on silicon substrate. Then, sample was dried at 25 °C to increase CTAB concentration. Consequently, AuNDs gradually aggregated due to the enhancement of surface hydrophobicity. Then, SEM images were captured by Ultimate Field Emission Scanning Electron SU-8230 (Hitachi High-Tech, Tokyo, Japan) under 10 kV and 10 μA emission current without tilting. Zeta potentials were measured by ZetaSizer Nano ZS (Malvern Panalytical Ltd., Morvin, UK).

2.2.3 Synthesis of AuNDs

AuNDs stabilized with CTAB were synthesized from gold nanotriangles (AuNTs) via a comproportionation reaction according to our previous report⁷. AuNTs was prepared by seed-

based method. Seed is a small nucleus (typically <5 nm), which serves as a starting point to produce an anisotropic structure. Seed should be monodispersed and display crystallographic habit by adding an excess amount of strong reducing agent as quick as possible and under vigorous stirring. Firstly, 0.5 mL of 20 mM hydrogen tetrachloroaurate (III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) solution was added to 36.5 mL of water to prepare AuNTs. Next, 1 mL of 10 mM sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$) and 1 mL of 100 mM ice-cold solution of sodium borohydride (NaBH_4) were added under vigorous stirring at room temperature for about 2 minutes. NaBH_4 is a strong reducing agent which leads the in-situ formation of seeds through partial reduction of the gold precursor (Au^{3+}) into Au^0 . The seed solution was incubated at 30 °C for 4 hours. Notably, longer incubation promoted larger seeds and heterogeneous nucleation, leading to grow AuNTs with low monodispersity. Homogeneous nucleation is required to avoid the formation of new nuclei during the growth step.

The growth solution was prepared by adding 54 μL of 0.1 M sodium iodide (NaI) to 108 mL of 0.05 M CTAB. High concentration of NaI is necessary to block the isotropic growth of nanoparticle. The mixed solution was divided into three containers named with 1, 2, and 3. Container 1 and 2 contained 9 mL of the solution and container 3 carried 80 mL of the solution. Then, 125 μL of 20 mM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 50 μL of 100 mM sodium hydroxide (NaOH), and 50 μL of 100 mM citric acid ($\text{C}_6\text{H}_8\text{O}_6$) were added respectively to container 1 and 2. After that, 1.25 mL of 20 mM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 0.5 mL of 100 mM NaOH , and 0.5 mL of 100 mM $\text{C}_6\text{H}_8\text{O}_6$ were added respectively to container 3. Subsequently, 1 mL of the seed solution was added to container 1 with shaking for complete mixture. Then, 1 mL of container 1 solution was added to container 2. After complete mixing in short time, all solution from container 2 was poured into container 3. Four chloride ligands in AuCl_4^- will be eventually replaced by bromide ions (Br^-) from CTAB through the reaction of $[\text{AuCl}_4]^- \rightleftharpoons [\text{AuCl}_3\text{Br}]^- \rightleftharpoons [\text{AuCl}_2\text{Br}_2]^- \rightleftharpoons [\text{AuClBr}_3]^- \rightleftharpoons [\text{AuBr}_4]^-$. The addition of ascorbic acid to the growth solution will reduce the Au^{3+} to Au^+ which is more stable species, preventing the comproportionation reaction when the seed solution was injected and inhibiting the second nucleation during the growth step. After storing at 30 °C for 24 h, all solution was poured out and the as-produced AuNTs were dispersed in 20 mL of 25 mM CTAB.

To synthesize AuNDs, 20 μL of 10 mM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was added to 5 mL AuNTs under vigorously mixing by vortex for 60 s. Then, the solution was incubated at 30 °C for 13 h. The as-produced AuNDs was purified by 2 cycles of centrifugation (9400 g, 8 min, 30 °C) to stop comproportionation reaction with removing as much as supernatant in every cycle. Lastly, AuNDs were dispersed in 10 mM CTAB up to 5 mL. The AuNDs solution was stored

at 30 °C for further experiments.

2.2.4 Surface Modification of AuNDs with C1, COOH, Alkyne, and N3 Ligands

To modify AuNDs with ligands, 1 mL of CTAB-stabilized AuNDs was purified by centrifugation (9,400 g, 8 min, 30 °C) to remove excess CTAB. Then, 900 μ L of supernatant was removed before changing the solution to 700 μ L of H₂O. Next, after reducing **C1**, **Alk**, and **N3** ligands with TCEP at 30 °C for 1 h, 100 μ L of 5 mM concentrated ligands (**C1**, **COOH**, **Alk**, and **N3**) and 100 μ L of 2 mM CTAB, was added to 800 μ L of AuNDs solution, followed by mixing with vortex mixer before incubation at 30 °C for 15 hours to promote ligand exchange reaction. The second step modification was conducted with the same ligand concentration and incubated at 30 °C for 24 hours. Next, the modified AuNDs were washed with 10 mM HEPES buffer (pH 8.0) followed by 10 mM NaOH to stabilize AuNDs. After centrifugation, 900 μ L supernatant was removed and the same volume of HEPES or NaOH was added to the precipitated AuNDs. Then, AuNDs were continuously washed by HEPES with the same procedure twice to completely remove the excess ligands. Finally, AuNDs were dispersed in HEPES and stored at 4 °C before characterization and click reaction.

2.2.5 Click Reaction between Alk- and N3-modified AuNDs

For click reaction experiments, the **Alk**- to **N3**-modified AuNDs were mixed with the same molar ratio (1:1, $X = 50\%$). The ratio was controlled by using the same extinction intensity and volume of **Alk**- and **N3**-modified AuNDs. The total volume of **Alk**- and **N3**-terminated AuNDs was 500 μ L. Firstly, 250 μ L of 20 mM THPTA ligand was added to 50 μ L of 20 mM CuSO₄ · 5H₂O followed with vigorously mixing. Then, 250 μ L of **Alk**- and **N3**-modified AuNDs were added to the mixed solution, respectively. Finally, 200 μ L of 10 mM L-ascorbic acid was mixed to the solution. After addition of the reducing agent, the solution colour was changed from blue to colourless, indicating the oxidation state of Cu was reduced from (+2) to (+1). Then, the sample was heated from 25 to 70 °C in UV spectrophotometer (1 °C/min and a waiting time of 5 min before each measurement) to promote the assembly and click reaction, followed by cooling from 70 to 25 °C. The extinction spectra during heating and cooling were recorded and analyzed to study the assembly properties. As control experiments, samples without Cu, **Alk**, or **N3** were prepared with the same procedure. Reaction time, reaction temperature, or Cu⁺¹ concentration was increased to produce larger assembly. After click reaction in the heating block, click solution was replaced to 10 mM HEPES buffer (pH 8.0) to acquire clear extinction spectra and TEM images.

2.2.6 Click Reaction of AuNDs-Alk-(93:5:2) and N3-PEG-N3 Linker

For the CuAAC reaction experiments, the final concentration of N3-PEG-N3 linker ($M_w = 1050$ g/mole) was varied (0; 0.1; 1; 5; 10; 20; 30; and 40 mM) with the same amount of AuNDs-Alk-(93:5:2). The concentration of AuNDs-Alk-(93:5:2) was controlled by measuring its extinction intensity. Firstly, 250 μ L of 100 mM THPTA ligand was added to 50 μ L of 100 mM CuSO₄ · 5H₂O following with vigorously mixing. Then, 400 μ L of AuNDs-Alk-(93:5:2) was added to the mixed solution following with the addition of 100 μ L of N3-PEG-N3 linker. Finally, 200 μ L of 50 mM L(+)-ascorbic acid sodium salt was mixed to the solution to reduce the oxidation state of Cu from (+2) to (+1). Then, the samples were heated at 80 °C overnight to proceed click reaction. Next, click solution was removed to stop the reaction and obtain a clear extinction spectrum. Finally, AuNDs-Alk-(93:5:2) was dispersed in 1 mL of 10 mM of HEPES buffer (pH 8.0). The extinction spectra and structures of AuNDs-Alk-(93:5:2) were investigated after overnight incubation at 4 °C to promote dissociation of assembled AuNDs.

2.2.7 Click Reaction of C1, COOH, Alk, and N3-modified AuNDs

The concentration of AuNDs-(97:3) or AuNDs-Alk-N3-(93:3:2:2) was adjusted by measuring their extinction intensity (extinction value: ~0.2-0.4). Firstly, 250 μ L of 100 mM THPTA ligand was added to 50 μ L of 100 mM CuSO₄ · 5H₂O following with vigorously mixing. Then, 500 μ L of AuNDs-Alk-N3-(93:3:2:2) in 10 mM of HEPES (pH 8.0) was added to the mixed solution following with the addition of 200 μ L of 50 mM L(+)-ascorbic acid sodium salt. HEPES concentration was maintained at 10 mM. After the addition of the reducing agent, the solution colour was changed from blue to colourless, indicating the oxidation state of Cu was reduced from (+2) to (+1). Then, the samples were heated in UV spectrophotometer from 25 to 80 °C to proceed click reaction, followed by cooling down to 25 °C to dissociate assembled AuNDs. Next, click solution was removed to stop the reaction. Finally, AuNDs-Alk-N3-(93:3:2:2) was dispersed in 1 mL of 10 mM of HEPES buffer (pH 8.0). The extinction spectra and structures were investigated after overnight incubation at 4 °C to promote dissociation of assembled AuNDs.

2.3 Results and Discussions

2.3.1 Synthesis of AuNDs

AuNTs was prepared by seed-based method to produce thin AuNTs. However, this method has limitations, including low reproducibility, low yield, and a strong dependence on the time interval, which affects particle size and uniformity^{53,54}. The extinction spectrum of the as-produced AuNTs (**Figure2.2a**) showed a broad peak which indicates AuNTs has low

monodispersity⁵⁵. A small peak appears around 1050 nm, indicating LSPR peak of AuNTs. TEM image of AuNTs (**Figure 2.2b**) showed most of particles did not form a perfect triangle shape, suggesting the low shape-yield of AuNTs. The side length of AuNTs was determined by measuring 20 particles and the average to be 167 ± 16 nm.

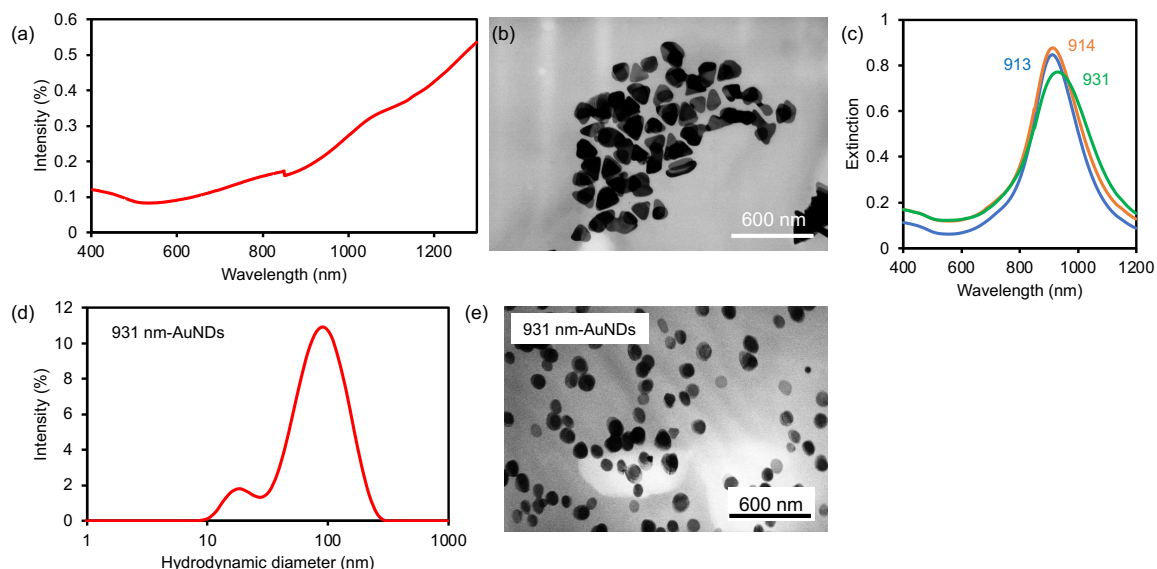


Figure 2. 2. Characterization of as-synthesized AuNTs and AuNDs prepared using a non-optimized method. (a) Extinction spectrum and (b) STEM image in transmission electron (TE) mode of CTAB-dispersed AuNTs. (c) Extinction spectra of AuNDs from different synthesis batches. (d) Hydrodynamic diameter of AuNDs measured by DLS. (e) STEM image in TE mode of AuNDs from the batch exhibiting a peak at 931 nm.

Subsequently, a comproportionation reaction was carried out to convert AuNTs into AuNDs by adding Au^{3+} in the presence of CTAB. In this reaction, Au^{3+} selectively oxidizes the surface atoms of AuNTs with the lowest metal coordination number⁵⁶. After the reaction, the extinction spectrum became sharper with a peak centered at approximately 931 nm (**Figure 2. 2c**), indicating the formation of AuNDs. DLS analysis showed two peaks around 10 and 100 nm (**Figure 2. 2d**), which are attributed to the rotational and translational diffusion modes of the anisotropic nanoparticles. The diameter of the resulting anisotropic AuNDs was determined by measuring 75 particles from a TEM image (**Figure 2. 2e**), yielding an average diameter of 105 ± 17 nm, consistent with our previous report⁷. Notably, different batches of AuNDs exhibited slight variations in their extinction spectra (**Figure 2. 2c**), even though they were synthesized from the same seed solution. This suggests that the seed-mediated method is highly sensitive to the timing of seed solution addition, which significantly affects the final nanoparticle properties⁵⁷. When the addition duration exceeds 5 seconds, it results in poor monodispersity and broader extinction peaks in the resulting AuNTs. Therefore, further

optimization of the synthesis protocol is necessary to achieve better reproducibility. Additionally, different plasmonic peaks of AuNDs (**Figure 2. 2c**) indicated that LSPR peaks are highly sensitive to the aspect ratio (diameter and thickness), as discussed in previous report⁵⁸.

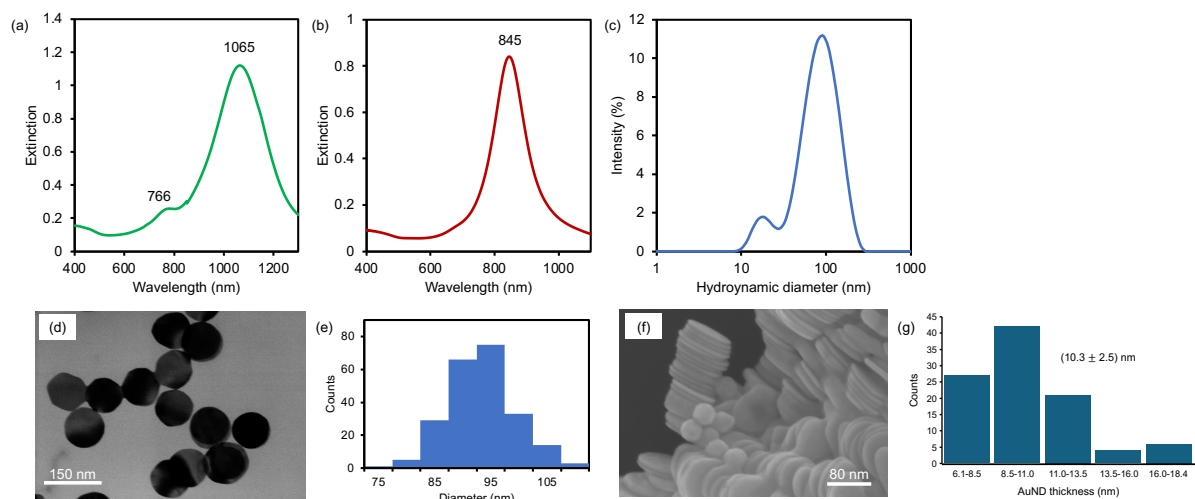


Figure 2. 3. Characterization of the as-synthesized AuNTs and AuNDs using an improved method. (a) Extinction spectrum of CTAB-dispersed AuNTs. (b) Extinction spectrum of CTAB-dispersed AuNDs. (c) Hydrodynamic diameter of CTAB-dispersed AuNDs measured by DLS. (d) STEM image in transmission electron (TE) mode of CTAB-dispersed AuNDs dried at 25 °C. (e) Size distribution of AuNDs calculated using ImageJ software. (f) SEM image of aggregated AuNDs, formed by increased CTAB concentration upon drying at 25 °C. (g) Thickness distribution of AuNDs measured using ImageJ software.

Consequently, the synthesis procedure was improved. By reducing the incubation time of the seed solution from 6 to 4 hours, the uniformity of the resulting AuNTs was significantly enhanced. The increased monodispersity was confirmed by a sharper extinction peak with an LSPR centered at 1065 nm (**Figure 2. 3a**). Following the comproportionation reaction, the extinction spectrum shifts to 845 nm (**Figure 2. 3b**), indicating the formation of AuNDs. DLS measurement showed two peaks around 18 and 91 nm (**Figure 2. 3c**), corresponding to the rotational and translational diffusion modes of the anisotropic nanoparticles. The size of the AuNDs was determined from a STEM image (**Figure 2. 3d**) using ImageJ software. The average diameter of 226 particles is (91 ± 6) nm (**Figure 2. 3e**). AuND thickness was obtained from SEM image (**Figure 2. 3f**) by measuring 100 particles of assembled AuNDs. Upon drying, the concentration of CTAB gradually increased, which enhanced both the attractive depletion forces and repulsive electrostatic interactions, thereby promoting the one-dimensional (1D) assembly of AuNDs. The average thickness was determined to be 10 ± 3 nm (**Figure 2. 3g**). Notably, accurately measuring the thickness of isolated particles in SEM images was

challenging due to densely stacked structures and limited image resolution. Based on the molar extinction coefficient in the previous study,⁵⁹ the concentration of AuNDs was estimated to be approximately 0.29 nM.

2.3.2 Surface Modification of AuNDs and Investigation of Thermo-responsiveness

To reduce the interparticle distance and promote the click reaction, AuNDs were modified with the thermo-responsive ligand (**C1**). Conversely, disassembly is also essential for dynamically altering the interparticle spacing. In our previous study, AuNDs modified with 100% **C1** did not exhibit reversible thermo-responsiveness due to strong hydrophobic and van der Waals interactions between the flat surfaces of the AuNDs⁷. To address this issue, a **COOH** ligand was introduced to impart electrostatic repulsion and facilitate disassembly. As a result, AuNDs were modified with 97% **C1** and 3% **COOH** ligand, named as AuNDs-(97:3). After modification, zeta potential of AuNDs was changed from +55.6 mV for CTAB-stabilized AuNDs to -13 mV for AuNDs-(97:3). Upon heating, the extinction intensity began to decrease at 35 °C (**Figure 2. 4a**), indicating the onset of AuND assembly. Further heating to 70 °C led to a significant decrease in extinction intensity, suggesting precipitation due to the formation of large aggregates.

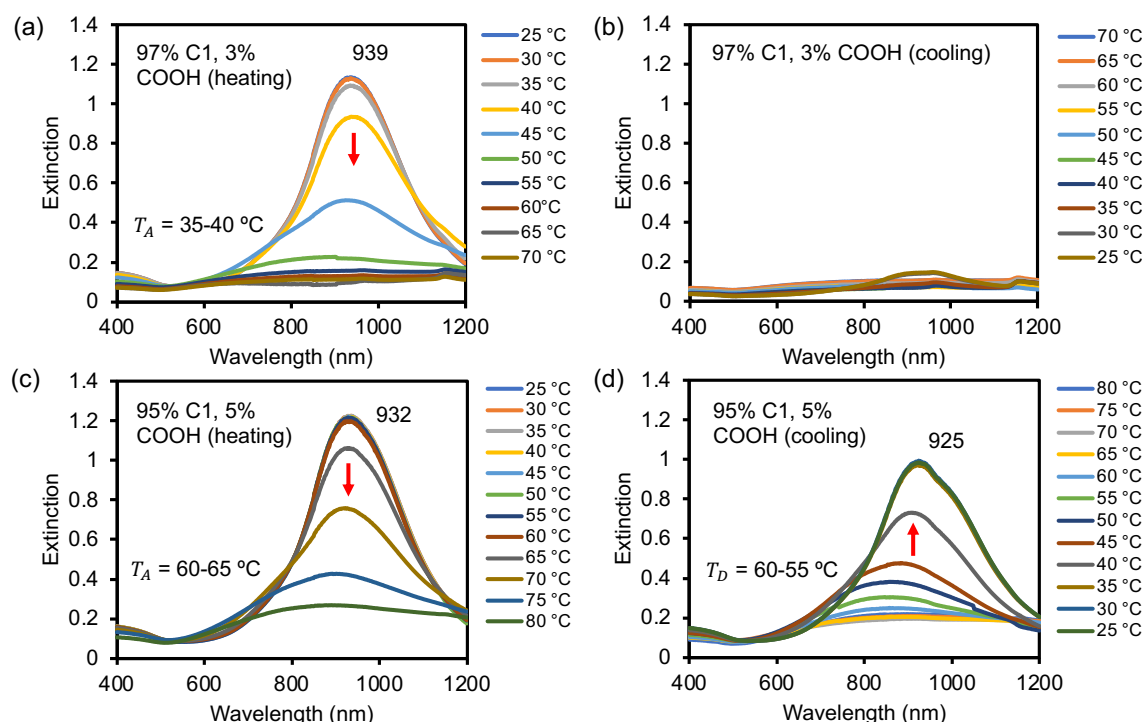


Figure 2. 4. (a, b) Extinction spectra of AuNDs-(97:3) in 10 mM HEPES buffer (pH 8.0) upon (a) heating from 25 to 70 °C and (b) cooling from 70 to 25 °C. (c, d) Extinction spectra of AuNDs-(95:5) in 10 mM HEPES buffer (pH 8.0) upon (c) heating from 25 to 80 °C and (d) cooling from 80 to 25 °C.

When cooled, AuNDs-(97:3) did not return to their initial extinction spectrum (**Figure 2. 4b**), indicating that the electrostatic repulsion was insufficient to fully dissociate the assemblies. To enhance charge repulsion and achieve reversible assembly behavior, the proportion of **COOH** ligand increased to 5%, resulting in AuNDs-(95:5). This modification enabled reversible assembly and disassembly, as confirmed by temperature-dependent spectral changes (**Figure 2. 4c** and **Figure 2. 4d**). The assembly temperature of AuNDs-(95:5) was higher (between 60 and 65 °C) compared to AuNDs-(97:3), due to the stronger electrostatic repulsion. This trend was consistent with the zeta potential, which increased from –13 mV for AuNDs-(97:3) to –15 mV for AuNDs-(95:5).

Next, AuNDs were modified with [90% **C1**, 5% **COOH**, 5% **Alk**] or [90% **C1**, 5% **COOH**, 5% **N3**], denoted as AuNDs-**Alk**-(90:5:5) and AuNDs-**N3**-(90:5:5), respectively. The extinction intensity of AuNDs-**N3**-(90:5:5) was approximately half that of AuNDs-**Alk**-(90:5:5) at 25 °C prior to heating (**Figure 2. 5a,c**), suggesting lower dispersibility of the **N3**-functionalized AuNDs at room temperature. This observation is consistent with previous reports, which documented aggregation and poor colloidal stability of **N3**-functionalized Au nanoparticles in buffer solutions due to their neutral and hydrophobic nature^{60,61}.

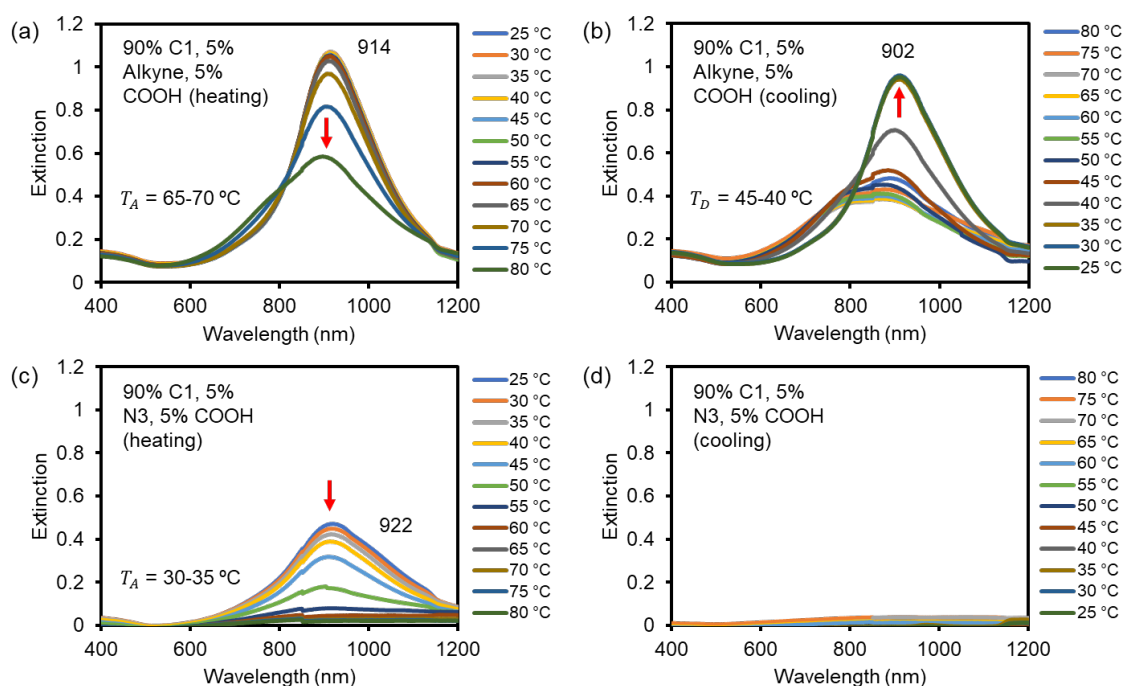


Figure 2. 5. Extinction spectra of click ligand–modified AuNDs (5%) for evaluating thermo-responsiveness in 10 mM HEPES buffer (pH 8.0). (a, b) AuNDs-**Alk**-(90:5:5) upon (a) heating from 25 to 80 °C and (b) cooling from 80 to 25 °C. (c, d) AuNDs-**N3**-(90:5:5) upon (c) heating from 25 to 80 °C and (d) cooling from 80 to 25 °C.

The incorporation of 5% **Alk** or 5% **N3** significantly influenced the thermo-responsive

assembly and disassembly behavior of AuNDs. Compared to AuNDs-(95:5) (**Figure 2. 4c**), the assembly temperature of AuNDs-**Alk**-(90:5:5) was slightly higher, occurring between 65 and 70 °C upon gradual heating from 25 to 80 °C (**Figure 2. 5a**). In contrast, the inclusion of 5% **N3** drastically lowered the assembly temperature to between 30 and 35 °C (**Figure 2. 5c**). This suggests that the assembly temperature is strongly influenced by ligand hydrophobicity, with more hydrophobic ligands dehydrating more readily and thus lowering the temperature at which assembly occurs. After heating to 80 °C, the samples were cooled back to 25 °C to induce disassembly. For AuNDs-**Alk**-(90:5:5), the extinction spectrum nearly returned to its initial state, with a disassembly temperature observed between 45 and 40 °C (**Figure 2. 5b**). In contrast, the extinction spectrum of AuNDs-**N3**-(90:5:5) did not recover, even after cooling to 25 °C (**Figure 2. 5d**), indicating that the AuNDs remained in an assembled state. This irreversible behavior may be attributed not only to hydrophobic interactions but also to strengthened hydrogen bonding under hydrophobic conditions, which hinders disassembly. Consequently, **N3** concentration was reduced to produce reversible assembly and disassembly of **N3**-modified AuNDs.

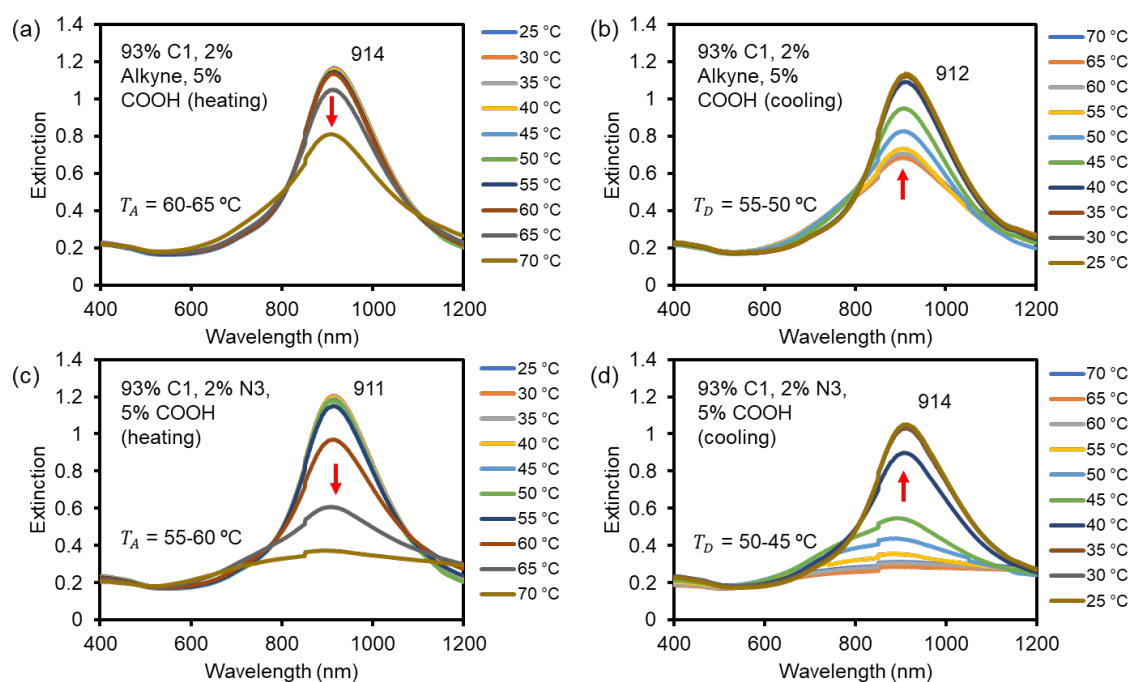


Figure 2. 6. Extinction spectra of click ligand-modified AuNDs (2%) for evaluating thermo-responsiveness in 10 mM HEPES buffer (pH 8.0). (a, b) AuNDs-**Alk**-(93:5:2) upon (a) heating from 25 to 80 °C and (b) cooling from 80 to 25 °C. (c, d) AuNDs-**N3**-(93:5:2) upon (c) heating from 25 to 80 °C and (d) cooling from 80 to 25 °C.

Next, the concentration of **Alk** or **N3** was reduced to 2%, and the corresponding samples were designated as AuNDs-**Alk**-(93:5:2) and AuNDs-**N3**-(93:5:2), representing AuNDs

modified with 2% **Alk** or 2% **N3**, respectively. Reducing the **Alk** concentration (AuNDs-**Alk**-(93:5:2)) led to a decrease in the assembly temperature (60-65 °C; **Figure 2. 6a**) and an increase in the disassembly temperature (55-60 °C; **Figure 2. 6b**), thereby narrowing the temperature gap between assembly and disassembly. In contrast, decreasing **N3** concentration (AuNDs-**N3**-(93:5:2)) induced higher assembly temperature (**Figure 2. 6c** vs. **Figure 2. 5c**). Additionally, the lower **N3** content improved the dispersibility of AuNDs- **N3**-(93:5:2) at room temperature prior to heating. Notably, AuNDs-**N3**-(93:5:2) exhibited disassembly upon cooling from 70 to 25 °C (**Figure 2. 6d**) with a greater reduction in extinction intensity compared to that of AuNDs-**Alk**-(93:5:2), suggesting that **N3** is more hydrophobic than **Alk**. Due to their improved thermo-responsiveness, AuNDs-**Alk**-(93:5:2) and AuNDs-**N3**-(93:5:2) were selected for the subsequent click reaction experiment aimed to conjugating AuNDs.

2.3.3 Click Reaction between **Alk**- and **N3**-modified AuNDs

The click reaction between **Alk**- and **N3**-modified AuNDs was performed by mixing equimolar amounts of AuNDs-**Alk**-(93:5:2) and AuNDs-**N3**-(93:5:2) in a click reaction solution. The concentration of AuNDs was adjusted by controlling the final extinction intensity of AuNDs-**Alk**-(93:5:2) or AuNDs-**N3**-(93:5:2) to an absolute value of 0.6. After mixing the AuNDs with the click solution, the samples were heated from 25 to 70 °C in a UV-Vis-NIR spectrophotometer to promote assembly and the click reaction. Extinction spectra were measured every 5 °C after a 5-minute equilibration period at each temperature.

AuNDs began to assemble at 50 °C, as indicated by a decrease in extinction intensity (**Figure 2. 7a**). Similar phenomena were observed in the samples containing only **Alk**- or **N3**-modified AuNDs (**Figure 2. 7g,j**). In contrast, the mixture of **Alk**- and **N3**-modified AuNDs without Cu⁺ in the click solution exhibited only a slight decrease in intensity (**Figure 2. 7d**), indicating that no significant assembly occurred. These results suggest that Cu⁺ may affect the surface charge of AuNDs by reducing the electrostatic repulsive forces induced by **COOH** ligands. Upon cooling from 70 to 25 °C, the extinction spectra of samples containing Cu⁺ did not recover (**Figure 2. 7b,h,k**), indicating that the AuNDs remained assembled. For the mixture of **Alk**- and **N3**-modified AuNDs (**Figure 2. 7b**), the successful progress of the click reaction is inferred from the retention of the assembled structure below the assembly temperature. On the other hand, the samples containing only **Alk**- or **N3**-AuNDs also showed irreversible spectral changes (**Figure 2. 7h,k**), despite the absence of a click reaction without both **N3** and **Alk**. These results support the hypothesis that Cu⁺ induces a change in surface charge, leading to strong interactions between AuNDs. Further investigation is needed to

elucidate the underlying mechanism.

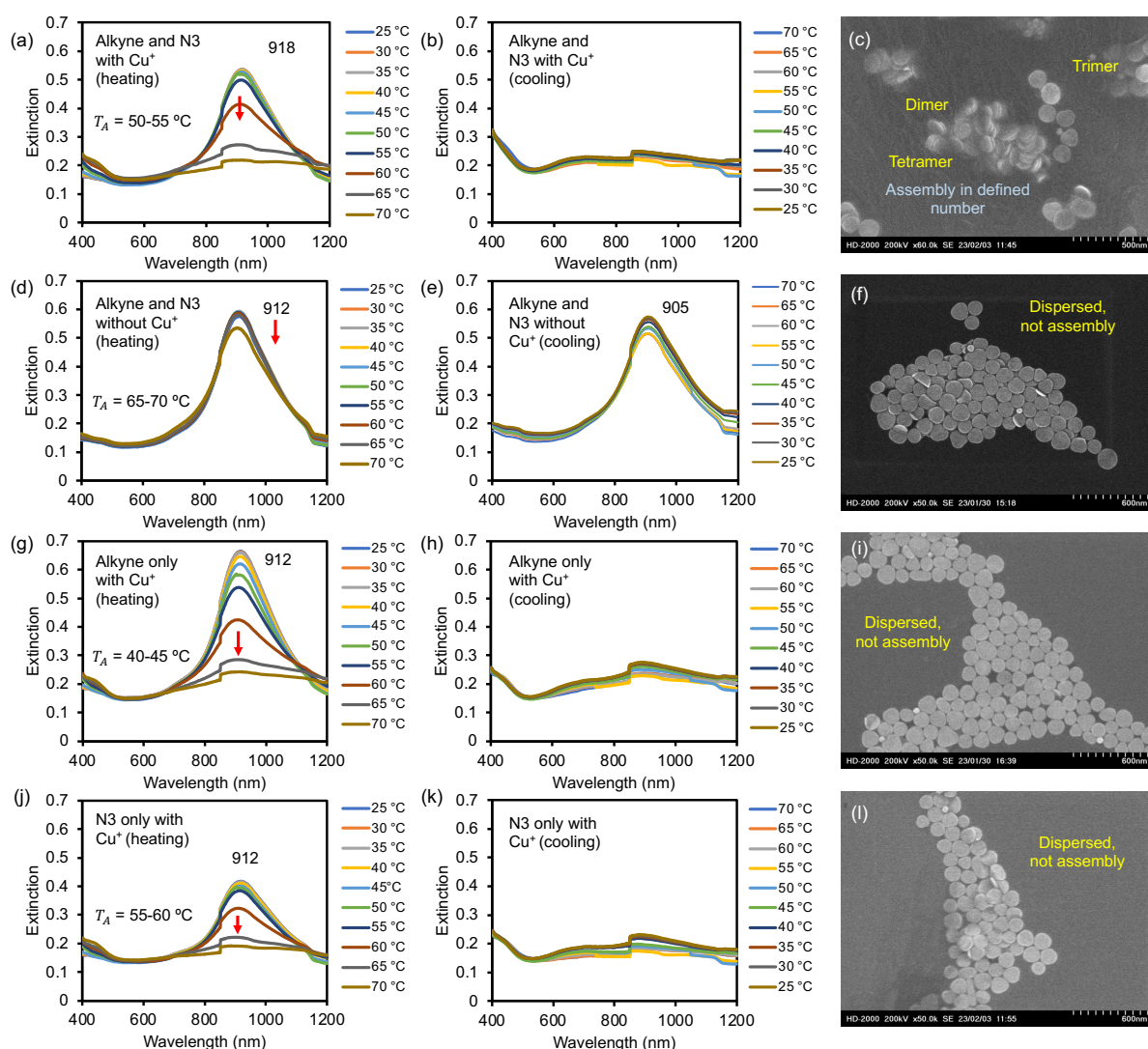


Figure 2. 7. Click reaction of Alk- and N3-modified AuNDs through thermal assembly. (a, b) Extinction spectra of a mixture of Alk- and N3-modified AuNDs in the click reaction solution upon (a) heating from 25 to 70 °C and (b) cooling from 70 to 25 °C. (c) STEM image (SE mode) of AuNDs after the click reaction. (d, e) Extinction spectra of a mixture of Alk- and N3-modified AuNDs in the click solution without Cu⁺ upon (d) heating from 25 to 70 °C and (e) cooling from 70 to 25 °C. (f) STEM image (SE mode) of AuNDs after heating and cooling without Cu⁺. (g, h) Extinction spectra of Alk-modified AuNDs in the click solution upon (g) heating from 25 to 70 °C and (h) cooling from 70 to 25 °C. (i) STEM image (SE mode) of Alk-modified AuNDs after heating and cooling. (j, k) Extinction spectra of N3-modified AuNDs in the click solution upon (j) heating from 25 to 70 °C and (k) cooling from 70 to 25 °C. (l) STEM image (SE mode) of N3-modified AuNDs after heating and cooling.

To confirm the occurrence of the click reaction, each sample was examined by STEM after removing the click solution. All samples were dried at 25 °C, below their assembly temperature. In the absence of Cu⁺, as well as in the samples containing only Alk-modified or only N3-modified AuNDs, the AuNDs remained dispersed (**Figure 2. 7f,i,l**), indicating that no covalent linkages were formed via the click reaction. In contrast, the mixture of Alk-

modified AuNDs exhibited stacked structures (**Figure 2. 7c**), confirming covalent linkage through the click reaction. However, the assembled structures were primarily small oligomers such as dimers, trimers, and tetramers, suggesting that the reaction time was insufficient for extensive linkage formation. To obtain larger oligomers as the main product after the click reaction, optimization of the reaction time and temperature is necessary.

Accordingly, the reaction time of click reaction was extended to 24 h using a heating block at different reaction temperatures (60, 70, and 80 °C). After performing the click reaction with an equimolar mixture of AuNDs-**Alk**-(93:5:2) and AuNDs-**N3**-(93:5:2) in the click solution, the samples were purified by centrifugation and sonication to remove the reaction solution. The purified samples were then redispersed in fresh HEPES buffer and analyzed by extinction spectroscopy and STEM imaging to investigate the assembly properties of the AuNDs (**Figure 2.8**).

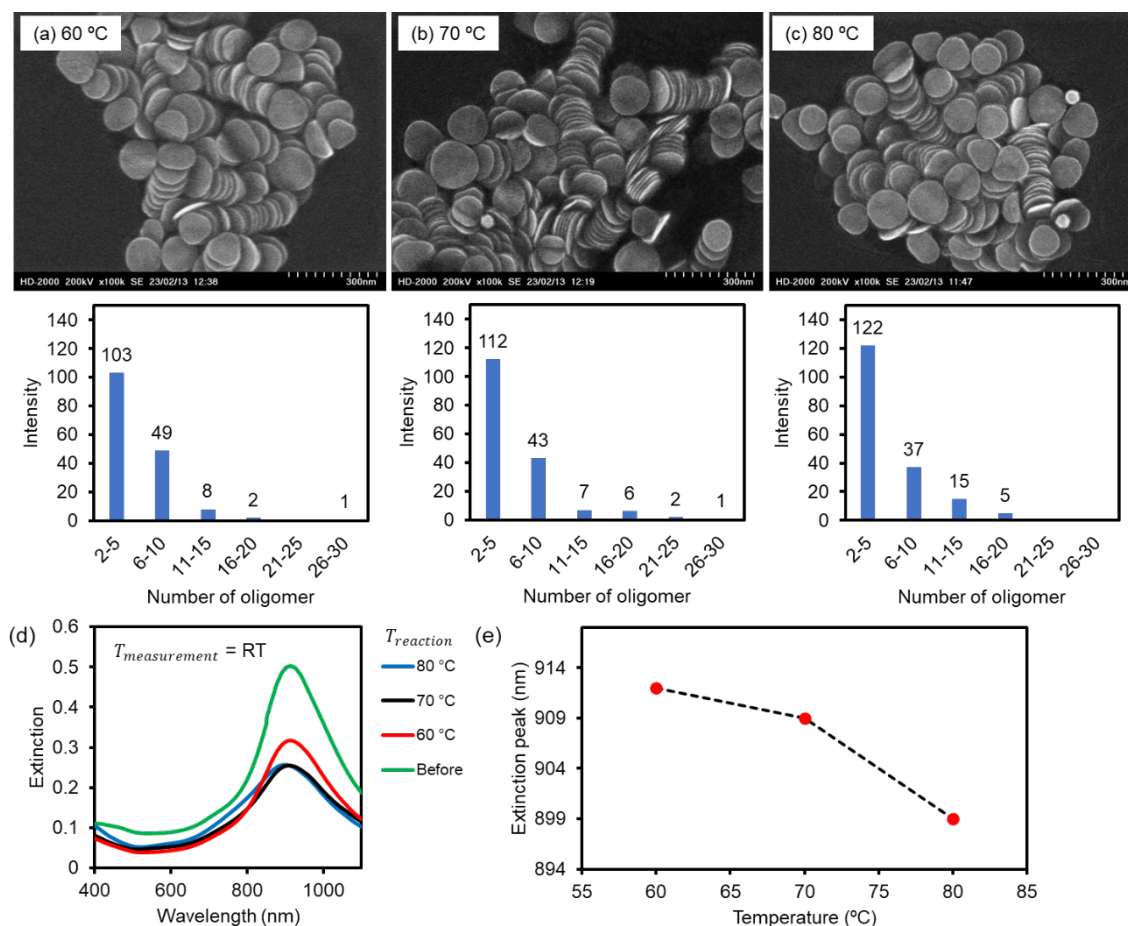


Figure 2. 8. STEM images in SE mode of a mixture of **Alk**- and **N3**-modified AuNDs after the click reaction at (a) 60 °C, (b) 70 °C, and (c) 80 °C. The number of oligomers was quantified from eight different regions in the STEM images of each sample and is presented as histograms. (d) Extinction spectra and (e) extinction peak positions of the samples after the click reaction and subsequent redispersion in fresh HEPES.

All samples incubated at 60, 70, and 80 °C formed large assembly structures after redispersion in fresh HEPES (**Figure 2. 8a-c**), indicating a successful click reaction. Compared to the extinction intensity of AuNDs-**Alk**-(93:5:2) before heating in the click solution, the percentages of assembled AuNDs were 33%, 50%, and 51% at 60, 70, and 80 °C, respectively (**Figure 2. 8d**). These results suggest that higher reaction temperatures promoted greater assembly, reducing the population of dispersed AuNDs in the solution (as indicated by lower extinction intensities). The number of assembled structures was quantified from eight different regions in the STEM images for each sample (see histogram in **Figure 2. 8a-c**). The extinction spectrum correlated well with the structural observations: higher reaction temperatures resulted in broader extinction peaks (**Figure 2. 8d**), indicating increased polydispersity due to the formation of assemblies composed of a wider range of oligomer sizes. Additionally, a blue shift in the extinction spectra was observed at higher temperatures, attributed to face-to-face arrangements formed by increasing numbers of AuNDs (**Figure 2. 8e**).

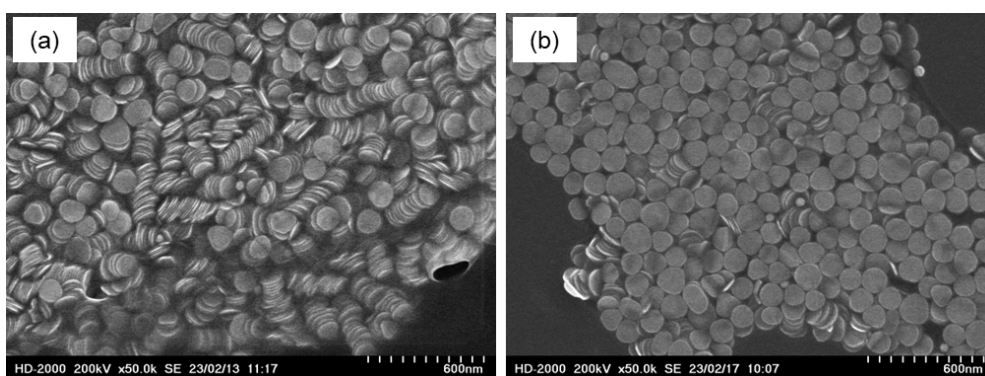


Figure 2. 9. STEM images in SE mode of a mixture of **Alk**- and **N3**-modified AuNDs heated at 80 °C for 24 h without Cu⁺: (a) after cooling to room temperature and replacing the solution with fresh HEPES; (b) after storage at 4 °C for 4 days.

As a control experiment, a sample without Cu⁺ was prepared and heated at 80 °C for 24 h. Unfortunately, the STEM image shows that the majority of AuNDs were assembled after purification and replacement of the solution with fresh HEPES (**Figure 2. 9a**). This result indicates that the large assembly structures, even when formed through hydrophobic and van der Waals interactions, are difficult to fully disassemble due to the strong binding between the flat surfaces of AuNDs via multivalent interactions. Furthermore, hydrogen bonding is stabilized in a hydrophobic environment, which also contributes to the inhibition of disassembly. Accordingly, to promote complete disassembly of the AuNDs, the sample without Cu⁺ was stored at 4 °C for 4 days, followed by centrifugation and sonication. However, even under these thorough disassembly conditions, some AuNDs remained assembled, while most were dispersed as monomers (**Figure 2. 9b**). Probably, it is caused by prolonged

hydrophobic interactions between AuND surfaces, inducing a closely packed interface that significantly excluded hydrating water molecules, thereby inhibiting rehydration of the ethylene glycol moiety by blocking access of water to the inner surfaces.

As another factor strengthening interaction between AuNDs, Cu^+ ions in the click solution can mediate the interaction between ionic bonds and **COOH** groups on AuNDs surface. Subsequently, the click reaction between **Alk**- and **N3**-modified AuNDs was performed at 60 °C using lower concentrations of Cu^+ (0, 1, 4, and 5 mM) to avoid strong stacking of AuNDs that is not linked via the click reaction (**Figure 2. 10**).

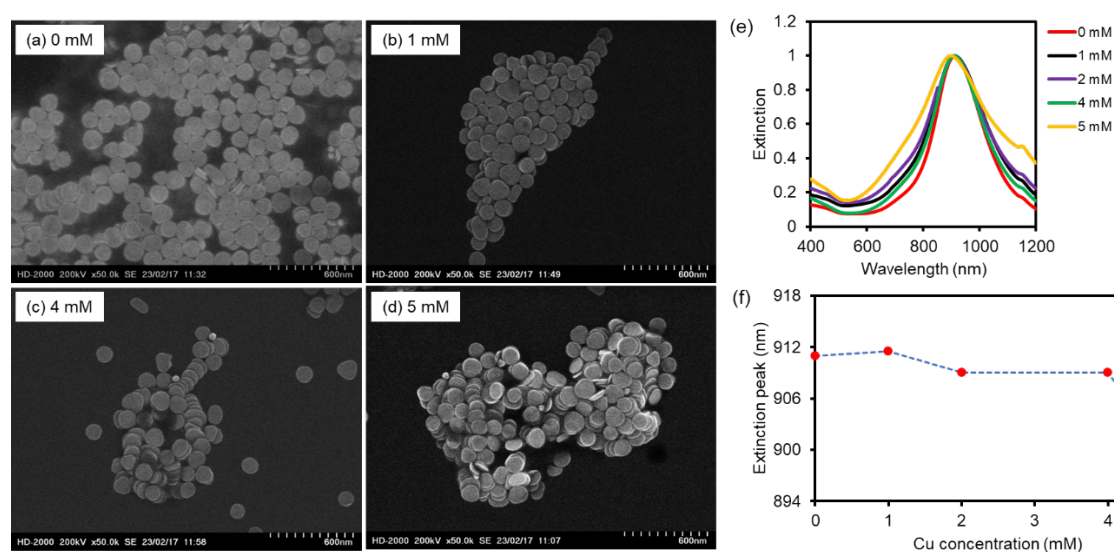


Figure 2. 10. STEM images in SE mode of AuNDs after the click reaction with various Cu^+ concentrations: (a) 0 mM, (b) 1 mM, (c) 4 mM, and (d) 5 mM. (e) Extinction spectra and (f) corresponding peak wavelengths of the samples in 10 mM HEPES buffer (pH 8.0). The click reaction solution was replaced with fresh HEPES buffer prior to STEM imaging and extinction measurements.

As a result, higher Cu^+ concentrations tended to form larger assembly structures (**Figure 2. 10a-d**). This is likely due to the increased reaction rate of the click reaction at higher Cu^+ concentrations. The extinction spectra at higher Cu^+ concentrations exhibited broader peaks (**Figure 2. 10e**), suggesting higher polydispersity in the assembled structures with varying oligomer sizes. A blue shift in the peak (**Figure 2. 10f**) further supports the increased number of AuNDs forming face-to-face assemblies. Nevertheless, ionic interactions between Cu^+ and **COOH** groups may still contribute to the assembly. Additionally, even the sample without Cu^+ showed some degree of assembly (**Figure 2. 10a**), suggesting that hydrophobic interactions between AuNDs without covalent linkage can partially contribute to the formation of assembly structures. Further experiments are necessary to confirm whether these assemblies result from successful click reactions. Another factor that may reduce the formation of larger assemblies

is the significant difference in the assembly behavior between **Alk**- and **N3**-modified AuNDs (**Figure 2. 5a-d**). This likely hindered the pairing between **Alk**- and **N3**-modified AuNDs, thereby decreasing the probability of click reactions occurring. In the later sections (**2.3.5-2.3.6**), AuNDs were modified with both **Alk** and **N3** ligands to overcome this issue, ensuring all AuNDs would exhibit the same assembly temperature. Another possible factor contributing to the observed face-to-face overlapping structures in the STEM images (**Figure 2. 10**) is entropic effects. These effects may lead to the formation of 1D assemblies because of a spontaneous transition from a disordered isotropic phase to an ordered nematic phase due to the significantly increased concentration upon drying^{13,62,63}.

2.3.4 Crosslinking of **Alk**-modified AuNDs with **N3**-PEG-**N3** via Click Reaction

The click reaction between **Alk**-modified AuNDs and **N3**-PEG-**N3** linker was conducted in a click solution with varying concentrations of **N3**-PEG-**N3** linker. Compared to the initial extinction spectrum of AuNDs-**Alk**-(93:5:2) dispersed in 10 mM HEPES buffer (pH 8.0), the spectra of AuNDs-**Alk**-(93:5:2), both with and without the **N3**-PEG-**N3** linker, were not fully restored after replacing the click solution with fresh HEPES and incubating overnight at 4 °C (**Figure 2. 11a-e**). This suggests that some AuNDs remained assembled even after the medium was changed to fresh 10 mM HEPES (pH 8.0). Regarding plasmonic shifts, higher concentrations of the **N3**-PEG-**N3** linker induced greater blue shifts in the extinction peaks (**Figure 2. 11b-e**), which correlates with STEM images (**Figure 2. 11g-j**) showing longer assembled structures of AuNDs-**Alk**-(93:5:2). In addition to the alkyl chains in the ligands, the ethylene glycol segments of the ligands and the **N3**-PEG-**N3** linker exhibit hydrophobic behavior upon dehydration at elevated temperatures, which promotes assembly. Ideally, the click reaction cannot proceed without **N3**-PEG-**N3** linker. Therefore, AuNDs should disassemble when the temperature falls below their assembly temperature (T_A). Interestingly, the extinction peak of AuNDs-**Alk**-(93:5:2) without **N3**-PEG-**N3** linker (**Figure 2. 11a**) also shows a blue shift, even though the corresponding STEM image (**Figure 2. 11f**) indicates a dispersed state. This may be due to close-packed assemblies driven by strong hydrophobic and van der Waals interactions, especially considering the large flat surfaces of the AuNDs. These interactions may inhibit water molecules from rehydrating the ethylene glycol moieties between particles, resulting in slow disassembly. Taken together, the persistence of assembled AuNDs-**Alk**-(93:5:2) structures after replacing the solution with fresh HEPES may be attributed to covalent linkages formed via the click reaction, strong hydrophobic interactions, van der Waals forces, or a combination of these factors.

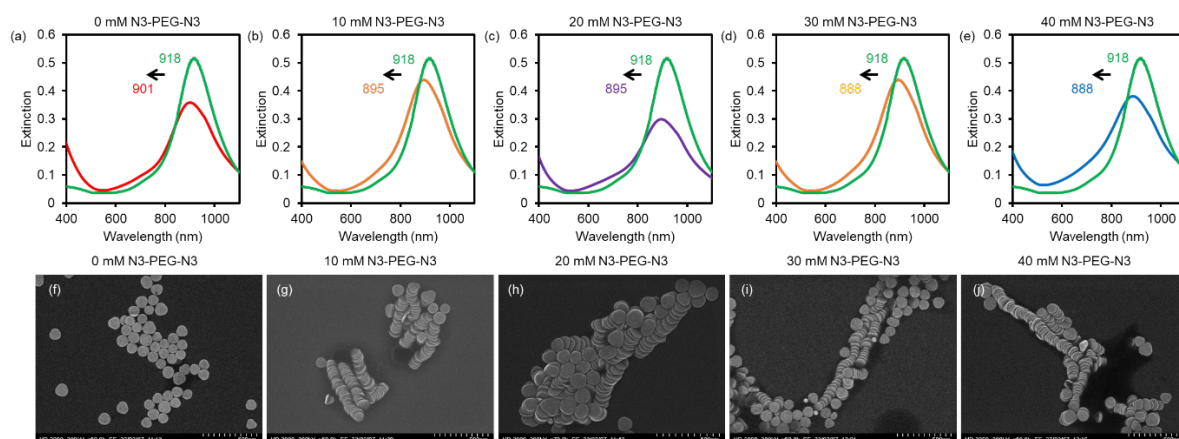


Figure 2. 11. Characterization of AuNDs-Alk-(93:5:2) after the click reaction with various concentrations of N3-PEG-N3 linker (0, 10, 20, 30, and 40 mM), followed by replacement of the solution with fresh HEPES buffer (10 mM, pH 8.0). (a–e) Comparison of extinction spectra of AuNDs-Alk-(93:5:2) dispersed in 10 mM HEPES (pH 8.0) before (green line) and after the click reaction with (a) 0 mM, (b) 10 mM, (c) 20 mM, (d) 30 mM, and (e) 40 mM N3-PEG-N3 linker. (f–j) STEM images in SE mode of AuNDs-Alk-(93:5:2) dispersed in 10 mM HEPES (pH 8.0) after reaction with (f) 0 mM, (g) 10 mM, (h) 20 mM, (i) 30 mM, and (j) 40 mM N3-PEG-N3 linker.

To confirm the occurrence of the click reaction, AuNDs-Alk-(93:5:2) were reacted with 40 mM N3-PEG-N3 linker in click solution both in the presence and absence of Cu⁺. In both cases, the extinction peak shifted to the shorter wavelengths after replacing the solution with fresh HEPES buffer (10 mM, pH 8.0) (**Figure 2. 12a-b**), indicating that the AuNDs remained assembled. STEM images (**Figure 2. 12e-f**) confirmed the presence of assembly structures. These results suggest that the N3-PEG-N3 linker reinforces adhesion between AuNDs, making disassembly difficult once assembled. Subsequently, an exhaustive dissociation protocol was applied, involving overnight incubation at low temperature (4 °C) followed by multiple rounds of vigorous washing. If the AuNDs were covalently conjugated through the click reaction, they would maintain their assembled structure even under these harsh conditions. In contrast, AuNDs without Cu⁺—in which the click reaction does not proceed—would be expected to disassemble under these conditions, as their adhesion is solely based on hydrophobic interactions. As the result, both extinction spectra shifted to the longer wavelength (**Figure 2. 12c-d**), indicating disassembly of AuNDs and the absence of covalent cross-linking via the click reaction. However, in the Cu⁺-containing sample, a partial retention of the short-wavelength peak was observed, resulting in peak broadening. This retained component is likely derived from covalently linked AuNDs formed via the click reaction. STEM images of both Cu⁺-containing and Cu⁺-free samples (**Figure 2. 12g-h**) showed that most AuNDs were dispersed as monomers, consistent with the spectral red shift, although small oligomers were still present. Further quantitative analysis of oligomer frequency is necessary to conclusively

demonstrate that click linkages formed more frequently in the Cu^+ -containing sample than in the Cu^+ -free control. These results suggest that a substantial portion of AuND stacking within larger assemblies originated from hydrophobic interactions without covalent linkage, while covalent linkages via the click reaction may have formed to a limited extent.

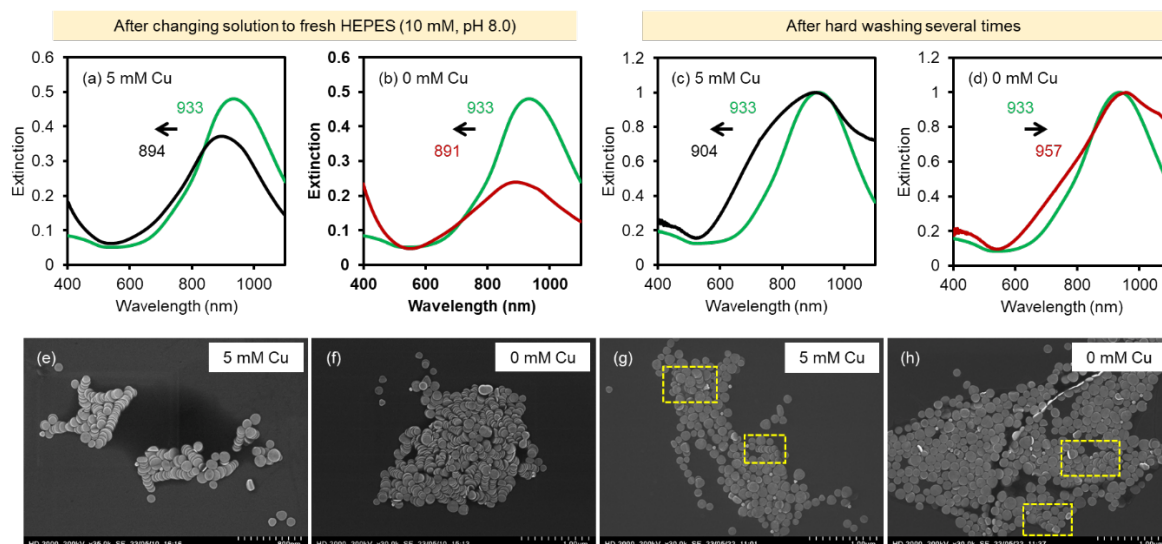


Figure 2. 12. Reaction of AuNDs-Alk-(93:5:2) with 40 mM N3-PEG-N3 linker in the presence and absence of Cu^+ , followed by replacement of the click solution with fresh HEPES buffer (10 mM, pH 8.0) and subsequent intensive washing to dissociate assembled AuNDs. (a–b) Comparison of extinction spectra of AuNDs-Alk-(93:5:2) dispersed in 10 mM HEPES (pH 8.0) before (green line) and after the click reaction: (a) with Cu^+ and (b) without Cu^+ . (c–d) Comparison of normalized extinction spectra of AuNDs-Alk-(93:5:2) dispersed in 10 mM HEPES (pH 8.0) before (green line) and after repeated washing: (c) with Cu^+ and (d) without Cu^+ . (e–h) STEM images in SE mode of AuNDs-Alk-(93:5:2): (e–f) After replacement with fresh HEPES: (e) with Cu^+ and (f) without Cu^+ . (g–h) After repeated washing: (g) with Cu^+ and (h) without Cu^+ .

The adhesive effects contributing to the assembly structures—including click linkages, hydrophobic interactions, and van der Waals interactions—were investigated by varying N3-PEG-N3 concentrations (0.1, 1, and 5 mM). Higher concentrations of N3-PEG-N3 tended to induce a larger spectral shift (Figure 2. 13a), indicating the formation of a greater number of oligomers (Figure 2. 13b–d). The number of oligomers was quantified from five separate STEM images for each condition (Figure 2. 13b–d, bottom). Based on the observed assembly structures (Figure 2. 13b–d), higher N3-PEG-N3 concentrations promoted more extensive assembly, likely due to enhanced adhesive effects. Even at low N3-PEG-N3 concentrations, it was still difficult to dissociate AuNDs once assembled. To investigate whether heat-induced assembly promotes irreversible adhesion, reactions between AuNDs-Alk-(93:5:2) and 40 mM N3-PEG-N3 in click solution were carried out at 30 °C and 80 °C—temperatures below and above the assembly temperature (T_A), respectively. After replacing the click solution with

fresh HEPES buffer, the extinction spectra of both samples did not return to their original states (**Figure 2. 13e**), indicating that the AuNDs remained assembled. These findings were supported by STEM images (**Figure 2. 13f-g**) which showed that both samples incubated at 30 °C and 80 °C formed extended assembly structures, with no significant differences between the lower and higher temperatures relative to T_A . These results indicate that heat-induced assembly does not lead to irreversible adhesion. Rather, they suggest that **N3-PEG-N3** plays a key role in promoting adhesion.

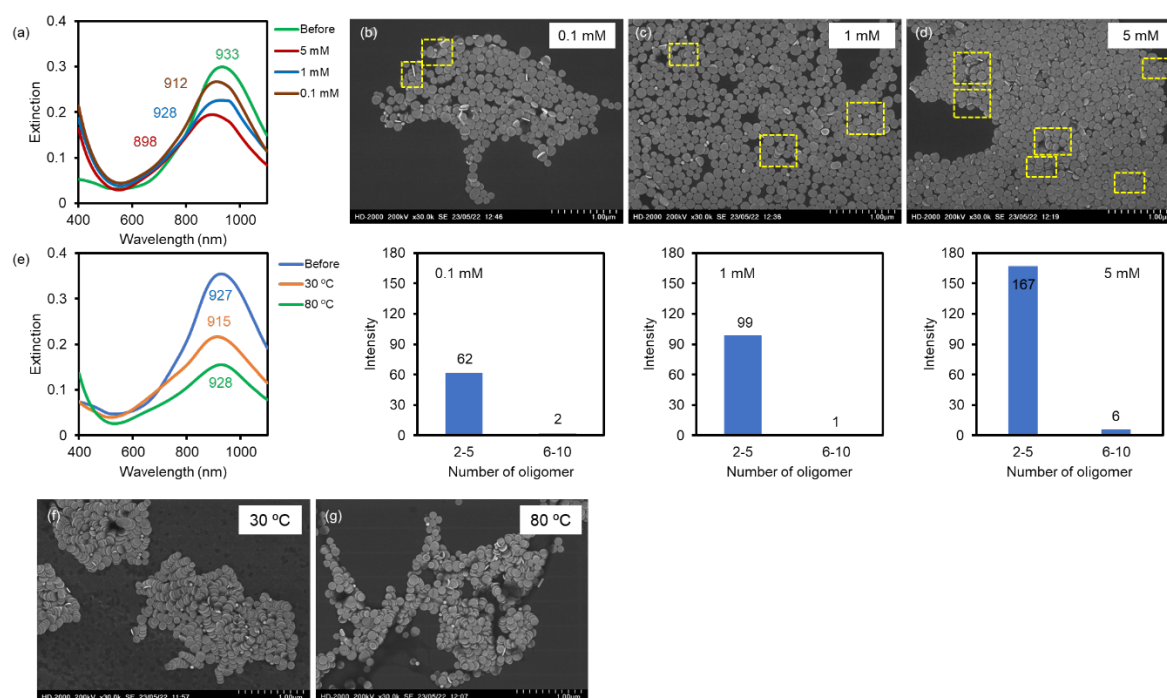


Figure 2. 13. (a–d) Click reaction between AuNDs-**Alk**-(93:5:2) and low concentrations of **N3-PEG-N3** linker. (a) Comparison of extinction spectra of AuNDs-**Alk**-(93:5:2) dispersed in 10 mM HEPES (pH 8.0) before (green line) and after the click reaction with low **N3-PEG-N3** concentrations. (b–d) STEM images in SE mode of AuNDs dispersed in 10 mM HEPES (pH 8.0) after the click reaction with low **N3-PEG-N3** concentrations: (b) 0.1 mM, (c) 1 mM, and (d) 5 mM. (e) Comparison of extinction spectra of AuNDs-**Alk**-(93:5:2) dispersed in 10 mM HEPES (pH 8.0) before (blue line) and after the click reaction with 40 mM **N3-PEG-N3** at different temperatures. (f–g) STEM images in SE mode of AuNDs dispersed in 10 mM HEPES (pH 8.0) after the click reaction at different temperatures: (f) 30 °C and (g) 80 °C.

2.3.5 Thermo-responsiveness of AuNDs Modified with Both Alkyne and N3 Ligands

In the above sections, the click reaction between **Alk**- and **N3**-modified AuNDs was attempted, but the results did not clearly confirm the occurrence of a click reaction between **Alk** and **N3** functional groups. This may be due to the significantly different assembly temperatures between **Alk**- and **N3**-modified AuNDs, which likely prevented their paired assembly (**Figure 2. 6a-d**), thereby reducing the probability of the click reaction. Additionally, cross-linking between **Alk**-modified AuNDs and **N3-PEG-N3** linker was not clearly confirmed,

likely due to the limited access of **N3** to **Alk**, which decreased the click reaction efficiency. To address these issues, AuNDs were modified simultaneously with both **Alk** and **N3** ligands to ensure contact between **Alk** and **N3** during assembly, thereby increasing the probability of click reaction. As a first step, the thermo-responsiveness of AuNDs modified with both **Alkyne** and **N3** ligands was investigated.

AuNDs-(97:3), used as a benchmark reference, showed reversible assembly and disassembly (**Figure 2. 14a,e**). Upon heating, the extinction intensity began to decrease at 75 °C (**Figure 2. 14a**), indicating the formation of assembled AuNDs. This temperature is 15 °C higher than previously reported⁷, suggesting that AuNDs-(97:3) are less hydrophobic. Further heating to 80 °C caused a decrease in the extinction intensity at ~830 nm and the appearance of a new plasmon peak around 709 nm. These spectral features were maintained upon cooling from 80 to 50 °C (**Figure 2. 14e**). When the temperature reached 45 °C, the extinction intensity at ~830 nm increased and the 709 nm peak began to diminish, indicating disassembly. The extinction spectrum fully returned to its initial state at 35 °C, suggesting that the AuNDs were completely disassembled and redispersed.

In contrast, AuNDs-**Alk-N3**-(93:3:2:2) showed a gradual decrease in extinction intensity upon heating from 55 to 80 °C (**Figure 2. 14b**). The addition of 2% **Alk** and 2% **N3** lowered the assembly temperature (T_A) due to the increased hydrophobicity, which promoted dehydration at lower temperatures. Compared to AuNDs-(97:3), the lower extinction intensity at 80 °C suggests that a larger number of AuNDs formed assemblies. The intensity ratio of the 709 nm to 830 nm peaks was also lower for AuNDs-**Alk-N3**-(93:3:2:2), indicating the formation of fewer small oligomers. Upon cooling to 25 °C, the extinction spectrum did not return to its initial state (**Figure 2. 14f**), likely due to strong hydrophobic interactions between **Alk** and **N3** ligands, which prevented rehydration. Furthermore, hydrogen bonding is greatly enhanced under hydrophobic conditions, making disassembly more difficult. AuNDs-**Alk-N3**-(87:3:5:5) began to assemble at a similar temperature to AuNDs-**Alk-N3**-(93:3:2:2), but showed lower extinction intensity at 80 °C (**Figure 2. 14c**), consistent with its greater hydrophobicity. This sample also showed a lower relative intensity of the 830 nm peak compared to the ~700 nm region, along with broader spectral features, suggesting the formation of larger oligomers and multimer assemblies. Upon cooling, only a slight increase in extinction intensity was observed, indicating that most AuNDs retained their assembled state (**Figure 2. 14g**). Further increasing the **Alk** and **N3** content to 10% resulted in complete assembly of AuNDs-**Alk-N3**-(77:3:10:10) at 80 °C (**Figure 2. 14d**). These assembled structures were maintained upon cooling to 25 °C (**Figure 2. 14h**) due to even stronger hydrophobic

interactions, resulting in irreversible assembly. These results demonstrate that the addition of **Alk** and **N3** significantly lowers the assembly temperature, increases the number of assembled AuNDs, and reduces the reversibility of assembly/disassembly due to enhanced hydrophobic interactions.

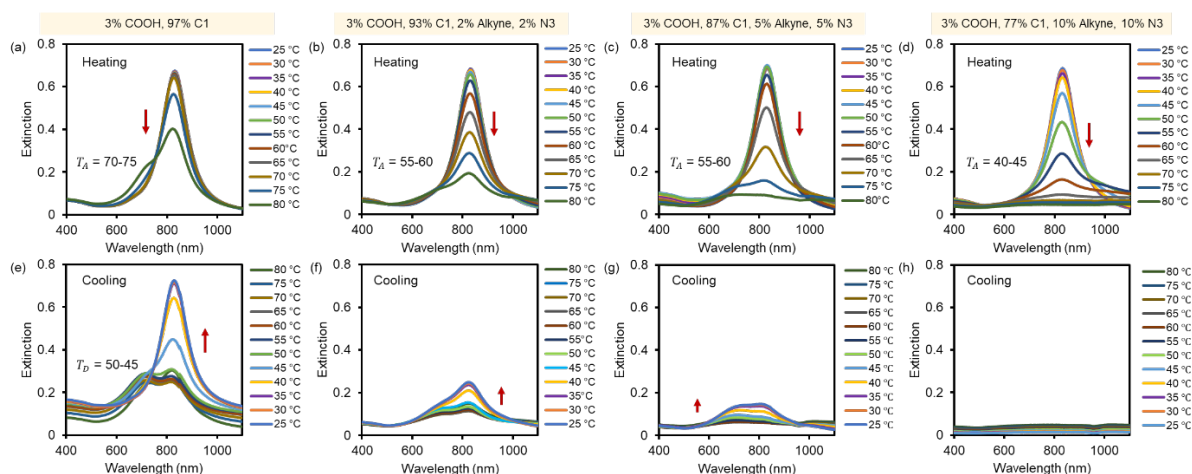


Figure 2. 14. Extinction spectra of **C1**, **COOH**, **Alk**, and **N3**-modified AuNDs for investigation of thermo-responsibility in HEPES buffer (10 mM, pH 8.0). (a, e) AuNDs-(97:3) during (a) heating from 25 to 80 °C and (e) cooling from 80 to 25 °C. (b, f) AuNDs-**Alk**-**N3**-(93:3:2:2) during (b) heating from 25 to 80 °C and (f) cooling from 80 to 25 °C. (c, g) AuNDs-**Alk**-**N3**-(87:3:5:5) during (c) heating from 25 to 80 °C and (g) cooling from 80 to 25 °C. (d, h) AuNDs-**Alk**-**N3**-(77:3:10:10) during (d) heating from 25 to 80 °C and (h) cooling from 80 to 25 °C.

2.3.6 Click Reaction of AuNDs Modified with Both Alkyne and N3 Ligands

The Click reaction of **Alk**, **N3**-modified AuNDs was promoted by heating from 25 to 80 °C in a UV spectrophotometer. Compared to the click reaction between **Alk**-modified AuNDs and **N3**-PEG-**N3** linker, the incubation time was shortened to prevent the formation of larger polymers, which tend to irreversibly aggregate, and to facilitate the dissociation of AuNDs. In addition, the HEPES concentration was maintained at 10 mM to minimize electrostatic interactions caused by salts in the click solution, which could otherwise induce irreversible assembly. Upon heating from 25 to 80 °C, both AuNDs-**Alk**-**N3**-(93:3:2:2) in click solution with and without Cu⁺ exhibited assembly (**Figure 2. 15a,c**). The lower extinction intensity observed for AuNDs-**Alkyne**-**N3**-(93:3:2:2) in the click solution with Cu⁺ indicates that Cu⁺ reduced the electrostatic repulsion from **COOH** groups, promoting assembly more readily. Upon cooling from 80 to 25 °C, the extinction spectra of AuNDs-**Alk**-**N3**-(93:3:2:2) in the click solution with and without Cu⁺ did not return to the initial intensities (**Figure 2. 15b,d**), indicating that the AuNDs maintained their assembled structures. Several factors—such as salts in the solvent, large flat surfaces, hydrophobic interactions, and hydrogen bonding—likely

contributed to the close-packed assembly of AuNDs. The electrostatic repulsion from 3% **COOH** modification was not strong enough to dissociate the assembled AuNDs.

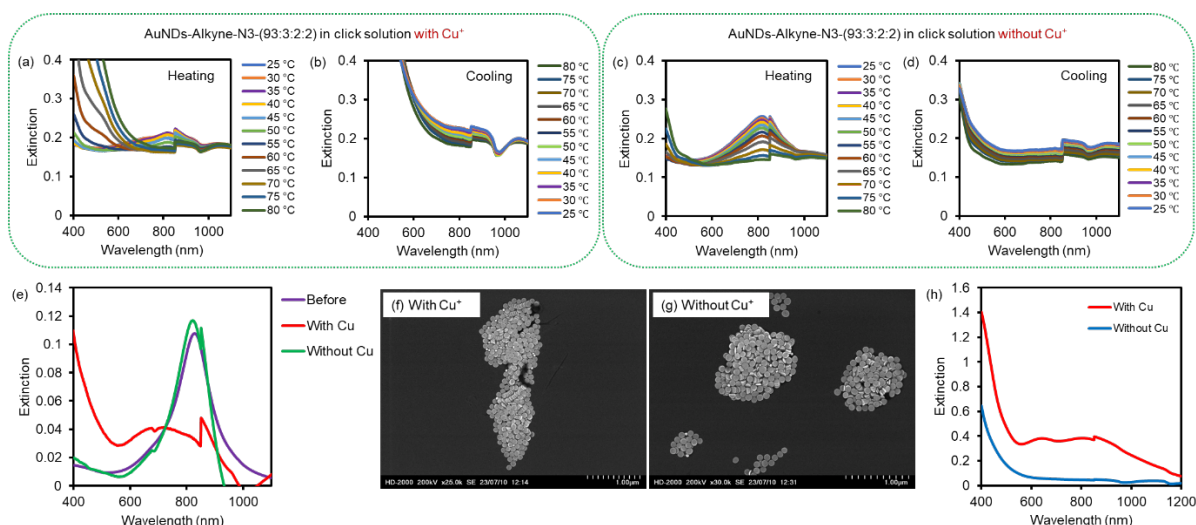


Figure 2. 15. (a–e) Extinction spectra of AuNDs-**Alk-N3**-(93:3:2:2) in the click solution and after replacement with HEPES buffer (10 mM, pH 8.0). (a, b) In click solution with Cu^+ : (a) during heating from 25 to 80 °C and (b) during cooling from 80 to 25 °C. (c, d) In click solution without Cu^+ : (c) during heating from 25 to 80 °C and (d) during cooling from 80 to 25 °C. (e) After removal of the click solution and redispersion of AuNDs-**Alk-N3**-(93:3:2:2) in HEPES buffer (10 mM, pH 8.0). (f, g) STEM images in SE mode of AuNDs-**Alk-N3**-(93:3:2:2) after removal of the click solution and redispersion in HEPES buffer (10 mM, pH 8.0): (f) with Cu^+ and (g) without Cu^+ . (h) Extinction spectra of the click solution itself with and without Cu^+ .

Subsequently, the click solution was removed, and AuNDs-**Alk-N3**-(93:3:2:2) were dispersed in 10 mM HEPES buffer (pH 8.0). After sonication, the extinction spectra were recorded and compared with that of dispersed AuNDs-**Alk-N3**-(93:3:2:2) in 10 mM HEPES buffer (pH 8.0) before the click reaction. The extinction spectrum of AuNDs-**Alk-N3**-(93:3:2:2) in the click solution without Cu^+ was identical to that before the click reaction (**Figure 2. 15e**, green vs purple), suggesting that all AuNDs were dissociated by washing and sonication. In contrast, the extinction spectrum of AuNDs-**Alk-N3**-(93:3:2:2) in the click solution with Cu^+ did not recover but shifted to a shorter wavelength around 700 nm (**Figure 2. 15e**, red). In the context of the click reaction, this shift suggests that AuNDs were covalently conjugated via the click linkage. However, this conclusion remains ambiguous, as a similar peak around 650 nm was also observed in the click solution after heating (**Figure 2. 15h**, red), suggesting that the click solution was not completely removed from the AuNDs-**Alk-N3**-(93:3:2:2) with Cu^+ . To clearly confirm the click reaction, the structures of AuNDs were examined by STEM after drying at room temperature. Both AuNDs-**Alk-N3**-(93:3:2:2) with and without Cu^+ formed elongated oligomers (**Figure 2. 15f,g**), indicating that the AuNDs

remained assembled after the click solution was removed. This makes it difficult to definitively conclude whether covalent bonding occurred.

Similar results were observed for AuNDs-(97:3), which lack functional groups necessary for the click reaction, in the click solution with Cu^+ . After assembly upon heating from 25 to 80 °C (**Figure 2. 16a**), the extinction spectra did not recover upon cooling from 80 to 25 °C (**Figure 2. 16b**). Although the click reaction cannot occur in the absence of **Alk** and **N3** functional groups, the assembled structures remained even after the solution was replaced with 10 mM HEPES buffer (pH 8.0) (**Figure 2. 16c**, blue), as supported by STEM image (**Figure 2. 16d**) showing AuND oligomers. These results suggest that the click solution inhibited the reversible disassembly of AuNDs. It is likely that salt in the click solution reduced the electrostatic repulsion provided by **COOH**, thereby preventing the disassembly of AuNDs. Based on these findings, the progress of the click reaction in AuNDs-**Alk-N3**-(93:3:2:2) remains unclear, since the effects of strong hydrophobic interactions and covalent bonding on the assembly could not be distinguished from each other.

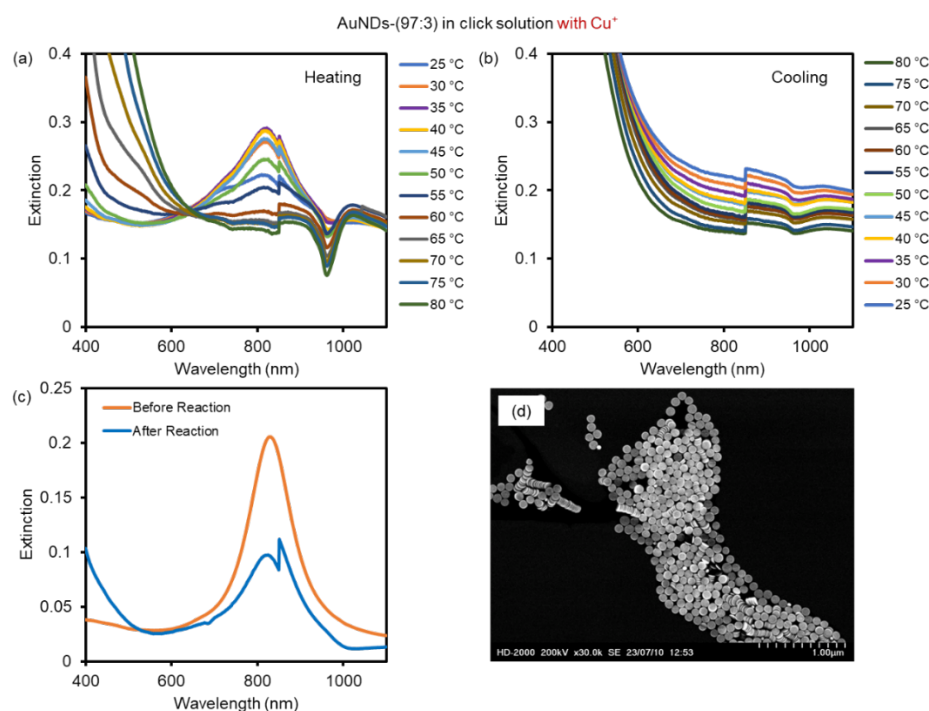


Figure 2. 16. (a–c) Extinction spectra of AuNDs-(97:3) in click solution and after replacing the click solution with HEPES buffer (10 mM, pH 8.0). (a, b) In click solution with Cu^+ : (a) during heating from 25 to 80 °C and (b) during cooling from 80 to 25 °C. (c) After removing the click solution and redispersion of AuNDs-(97:3) in HEPES buffer (10 mM, pH 8.0). (d) STEM image (SE mode) of AuNDs-(97:3) after removal of the click solution with Cu^+ and redispersion in HEPES buffer (10 mM, pH 8.0).

The effect of Cu^+ in the click solution on assembly was investigated using AuNDs-(97:3), AuNDs-**Alk-N3**-(93:3:2:2), and AuNDs-**Alk-N3**-(87:3:5:5). To clearly distinguish this effect,

assembly was monitored during incubation at 30 °C, a temperature low enough to avoid thermally induced assembly, over an extended period. Extinction spectra were recorded throughout a one-week incubation. After one week, all samples showed a decrease in the extinction peak at 830 nm (**Figure 2. 17a-f**), indicating that AuNDs assembled in both click solutions—with and without Cu⁺. In samples with Cu⁺, an additional extinction peak appeared at 650 nm, corresponding to a color change in the click solution caused by Cu ions. Compared to the samples without Cu⁺ (**Figure 2. 17b,d,f**), those with Cu⁺ (**Figure 2. 17a,c,e**) exhibited a more rapid decrease in intensity, suggesting that Cu⁺ reduced the electrostatic repulsion provided by COOH groups and thereby promoted assembly. The addition of 2% **Alk** and 2% **N3** (**Figure 2. 17c,d**) increased the hydrophobicity of the AuND surface, enhancing the assembly rate in both Cu⁺-containing and Cu⁺-free click solutions. Further increasing the proportions to 5% **Alk** and 5% **N3** accelerated the assembly rate even more (**Figure 2. 17g,h**). These results demonstrate that both the presence of Cu⁺ in the click solution and the increased hydrophobicity of the ligands promote AuND assembly, even in the absence of thermal induction.

After one week, the click solution was removed, followed by repeated washing of the AuNDs with 10 mM HEPES buffer (pH 8.0) and 10 mM NaOH. The extinction spectrum of AuNDs-(97:3) incubated in click solution without Cu⁺ was fully restored to its initial state (**Figure 2. 17h**), indicating that all AuNDs were successfully redispersed. This result suggests that the interactions responsible for the stacked structure—such as hydrophobic interactions among ethylene glycol units, click ligands, and salts in the click solution—are relatively weak and reversible, allowing the AuNDs to dissociate completely. In contrast, the extinction spectrum of AuNDs-(97:3) incubated in click solution with Cu⁺ was only partially recovered (**Figure 2. 17g**), implying that ionic bonds between Cu⁺ and deprotonated COOH groups on adjacent AuNDs were sufficiently strong to preserve the assembled structures even after washing with HEPES buffer and NaOH. Compared to AuNDs-(97:3), AuNDs-**Alk-N3**-(93:3:2:2) and AuNDs-**Alk-N3**-(87:3:5:5) exhibited significantly lower extinction intensities after removing the click solution (**Figure 2. 17i,j,k,l**), suggesting that the increased hydrophobicity due to **Alk** and **N3** ligands contributed to enhance hydrophobic interactions, stronger ionic bonding, and possibly hydrogen bonding between AuNDs within the assembled structures, thereby inhibiting disassembly.

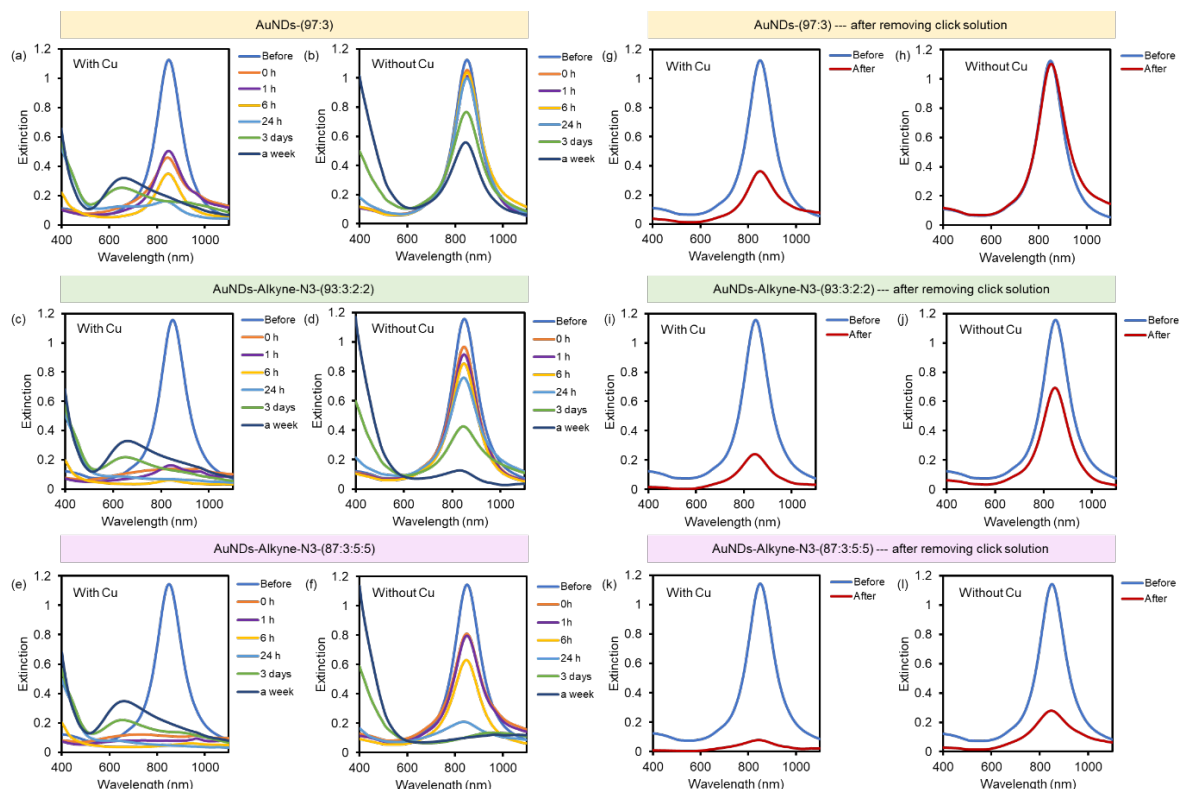


Figure 2. 17. (a–f) Extinction spectra of AuNDs-(97:3), AuNDs-**Alk-N3**-(93:3:2:2), and AuNDs-**Alk-N3**-(87:3:5:5) incubated in click solution with or without Cu^+ at 30 °C for one week to investigate the effect of Cu^+ on assembly. (a, b) AuNDs-(97:3): (a) in click solution with Cu^+ and (b) without Cu^+ . (c, d) AuNDs-**Alk-N3**-(93:3:2:2): (c) in click solution with Cu^+ and (d) without Cu^+ . (e, f) AuNDs-**Alk-N3**-(87:3:5:5): (e) in click solution with Cu^+ and (f) without Cu^+ . (g–l) Extinction spectra of AuNDs-(97:3), AuNDs-**Alk-N3**-(93:3:2:2), and AuNDs-**Alk-N3**-(87:3:5:5) after removing the click solution and redispersion in HEPES buffer (10 mM, pH 8.0). (g, h) AuNDs-(97:3) after incubation in click solution: (g) with Cu^+ and (h) without Cu^+ . (i, j) AuNDs-**Alk-N3**-(93:3:2:2) after incubation in click solution: (i) with Cu^+ and (j) without Cu^+ . (k, l) AuNDs-**Alk-N3**-(87:3:5:5) after incubation in click solution: (k) with Cu^+ and (l) without Cu^+ .

2.4 Conclusions

AuNDs were successfully modified with a thermo-responsive ligand (**C1**) and a negatively charge ligand (**COOH**) via ligand exchange reaction. The modified AuNDs exhibited reversible assembly and disassembly upon heating and cooling. The addition of 5% **Alk** or 5% **N3** significantly altered the assembly temperature due to the ligand hydrophobicity and reduced the reversibility of the assembly/disassembly behavior. Subsequently, a click reaction between 2% **Alk**- and 2% **N3**-modified AuNDs was carried out by heating the system to 70 °C. STEM imaging confirmed the formation of small oligomeric structures such as dimers, trimers, and tetramers, likely resulting from the click reaction. To promote the formation of larger assemblies, both the reaction temperature and Cu^+ concentration were increased. However, the results remained inconclusive, as AuNDs incubated in fresh HEPES buffer

without **Alk**- and **N3**-modified AuNDs also exhibited assembled structures. These findings suggest that Cu^+ may have influenced the charge state of the **COOH** ligand, leading to insufficient electrostatic repulsion to facilitate disassembly.

The formation of cross-links between AuNDs-**Alk**-(93:5:2) and the **N3**-PEG-**N3** linker via the click reaction could not be clearly confirmed through analysis of their extinction spectra and assembly structures. This is likely due to strong adhesion—possibly involving hydrophobic interactions and van der Waals forces—which inhibited the dissociation of AuNDs after assembly. The observed assembly appears to be promoted by the **N3**-PEG-**N3** linker and is likely unrelated to the assembly induced by dehydration of the ethylene glycol segments upon heating. Furthermore, the bulkiness of the polymer linker (**N3**-PEG-**N3**) may have restricted the azide groups from effectively accessing the alkyne groups on the AuNDs, thereby reducing the efficiency of the click reaction.

To address these issues, AuNDs were modified with both **Alk** and **N3** ligands to achieve a successful click reaction. Unfortunately, click reactions between AuNDs-**Alk**-**N3** could not be clearly confirmed, as both click solutions with Cu^+ and without Cu^+ suppressed the disassembly behavior. This made it difficult to distinguish whether the observed assemblies were caused by hydrophobic interactions or by covalent linkages formed via the click reaction. The assembly was not only driven by dehydration of the ethylene glycol segments upon heating but also by the presence of salts—particularly Cu^+ —in the click solution. Cu^+ ions reduce the electrostatic repulsion between **COOH** groups and can form strong ionic bonds with deprotonated **COOH** groups. To investigate the contributing factors of the assembly more clearly, experiments were conducted by incubating the system at 30 °C (below the thermal assembly temperature, than T_A) to exclude heat-induced effects. As a result, the introduction of **Alk** and **N3** ligands increased hydrophobicity, which enhanced hydrogen bonding, while strong ionic interactions between Cu^+ and the deprotonated **COOH** groups stabilized the assembled structures. This stabilization made it more difficult to dissociate the assemblies, thereby hindering clear confirmation of covalent bond formation via the click reaction.

Taken together, the approach utilizing organic ligands combining thermo-responsive and click-reactive functions was ineffective for achieving controlled assembly and disassembly of AuNDs. This limitation is attributed to the strong face-to-face interactions driven by hydrophobic effects, van der Waals forces, hydrogen bonding, and ionic interactions.

Chapter 3

Preparation of DNA-modified AuNDs for Controlled Assembly

3.1 Introduction

In **Chapter 2**, controlled assembly of gold nanodiscs (AuNDs) was investigated utilizing organic ligands, combining thermo-responsive ligand with click-reactive functions. However, this approach was ineffective to control reversible assembly and disassembly, in particular dissociation of AuNDs. This limitation is attributed to strong face-to-face interactions driven by hydrophobic effects, van der Waals forces, hydrogen bonding, and ionic interactions. To address this issue, I select an alternative approach that exploits DNA hybridization as the driving force for assembly. Owing to its polyanionic nature, DNA enables reversible assembly and disassembly of AuNDs, as the electrostatic repulsion can overcome even the strong multivalent interactions associated with their broad face-to-face contact. Furthermore, the number of DNA bases can be readily adjusted, providing precise control over the assembly and dissociation temperatures. In this chapter, single-stranded DNA (ssDNA) is used to modify AuNDs surfaces through several methods.

DNA contains of a phosphate backbone and four types of nucleobases, as an example is a simple sequence of 5'-ATCG-3' (**Figure 3. 1a**)⁶⁴. The bases are neutral between pH 5 and 8. When pH is decreased to ~3, adenine (A) and cytosine (C) are protonated, meanwhile guanine (G) is only protonated at pH lower than 2. Each phosphate has negative charge ($pK_a < 2$) which makes DNA is highly negative charged. Gold surface can adsorb DNA with the order of adsorption energy $A > C > G > T \gg \text{phosphate}$ ⁶⁵. DNA hybridization occurs when two complementary ssDNA molecules bind together to form a double-stranded DNA (dsDNA) helix. This process relies on the rules where A pairs with T via two hydrogen bonds, and G pairs with C via three hydrogen bonds (**Figure 3. 1b**)⁶⁶. The distance between two adjacent bases is 0.338 nm⁶⁷. Hybridization is affected by DNA sequences, temperature, and salt concentration. The use of DNA as a ligand enables to precisely control the assembled structures¹³ of gold nanoparticles with accurate and alterable interparticle distance^{14–16} via Watson-Crick base-pairing interactions (dA-dT and dC-dG). Therefore, DNA has been widely used to dictate the assembly of gold nanospheres (AuNPs)^{14,16,68–72}, gold nanorods (AuNRs)^{8,28,73–75}, gold nanocubes (AuNCs)^{30,76}, gold nanotriangles (AuNTs)^{9,25,77}, and AuNDs^{17,24}. However, DNA strands immobilized on Au nanoparticle surfaces not only hybridize via normal base pairing interactions which involve complementary strands but also facilitate a slipping interaction which can additionally stabilize the assembly structures via non-Watson-Crick base pairing interactions which are less complementary than the normal hybridization. The curvatures and DNA lengths strongly affected the slipping interactions⁷⁸.

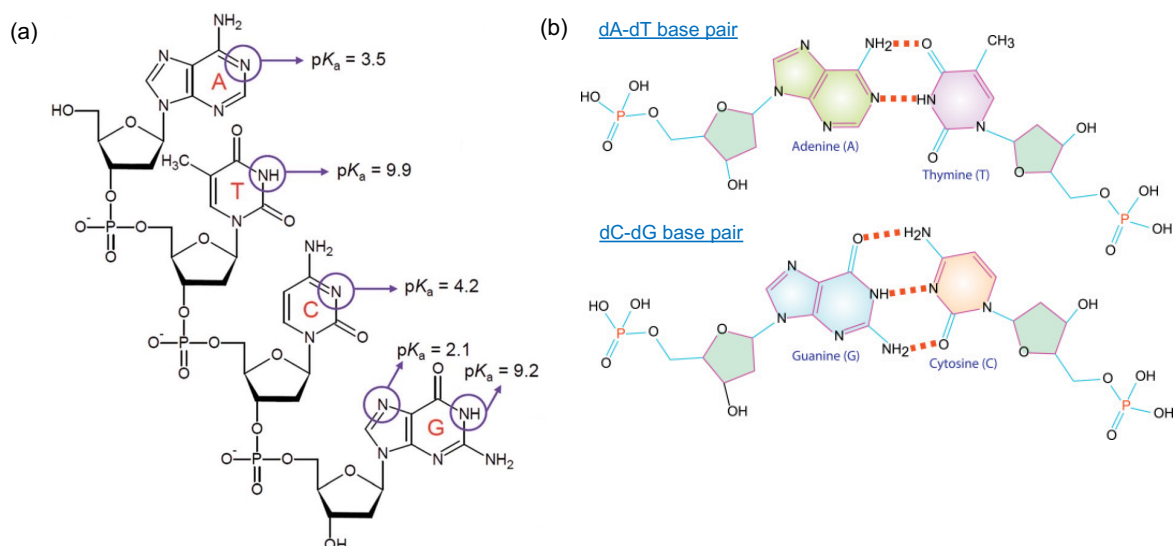


Figure 3. 1. (a) The structure of DNA with four bases and pK_a value of each base⁶⁴. (b) Specific base-pairing occurs in DNA hybridization⁶⁶.

Here, AuNDs will be modified with DNA ligands using several methods to optimize DNA immobilization: (i) a click reaction between DBCO-DNA and azide-modified AuNDs, (ii) salt aging, and (iii) butanol dehydration.

3.2 Materials and Experiments

3.2.1 Materials

Hydrophilic ligands, 11-mercaptoundecanol hexaethyleneglycol ether (**OH-EG6**) and 11-mercaptoundecanol dodecaethyleneglycol (**OH-EG12**) were purchased from ProChimia Surfaces. Sodium chloride (NaCl), sodium acetate (CH_3COONa), disodium hydrogen phosphate (Na_2HPO_4), sodium dodecyl sulphate (SDS), boric acid, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), acetic acid (CH_3COOH), and 1-butanol were purchased from Wako Pure Chemical Industries Ltd. (Japan). Ethylenediaminetetraacetic acid ($\text{EDTA} \cdot 2\text{Na}$) and ultrapure tris were purchased from Dojin Chemical Research Institute, Inc and Thermo Fisher Scientific, respectively. Dibenzocyclooctyne (DBCO)-DNA, DBCO-5'-CGTAAGTCGATGGCGTTCGTAC-3' was purchased from Nihon Gene Research Laboratory Inc. Linker 1 (5'-CGCAGTAGC GCAGTAGCG CAGTAGCGC AGTAGCGTAAGTCGATGG CGTTCGTAC-3') and linker 2 (5'-CTACTGCGC TACTGCGCT ACTGCGCTA CTGCGCGTA AGTCGATGG CGTTCGTAC-3') were purchased from Eurofins Genomics Inc. Different lengths and sequences of HS-DNA were obtained from Integrated DNA Technologies which would be purified by single-use gravity column (PD MiniTrap G-10, Cytiva). Other commercial reagents were used without further purification. Ultrapure water was used to prepare all solutions for

the experiments (Milli-Q reference system).

3.2.2 Instruments for Characterizations

Relative fluorescence intensity of dye was measured by NanoDrop 3300 Fluorospectrometer. Other instruments are same as apparatus used in **Chapter 2**.

3.2.3 Modifying AuNDs with DBCO-DNA via Click Reaction

To address the solubility problem in **Chapter 2**, I select DBCO-DNA to conjugate with **N3**-AuND to increase the repulsion forces between nanoparticles. Firstly, AuNDs were modified with **COOH**, **N3**, and hydrophilic (**OH-EG6** or **OH-EG12**) ligands. Specifically, 1 mL of CTAB-stabilized AuNDs (CTAB concentration: 10 mM) was centrifuged (9,400x g, 8 min, 30 °C) to remove excess CTAB. Then, 900 μ L of supernatant was removed before changing the solution to 700 μ L of H₂O. Next, ligands (**OH**, **COOH**, and **N3**) were reduced by TCEP at 30 °C for 1 h (the ratio of ligand to TCEP equals to 1:1 molar equivalent). Then, 100 μ L of the reduced ligand solution (5 mM) and 100 μ L of 2 mM CTAB were added to 800 μ L of AuNDs solution, followed by mixing with vortex mixer before incubation at 30 °C for 15 hours to promote ligand exchange reaction. The second step modification was conducted with the same ligand concentration and incubated at 30 °C for 24 h. Next, the modified AuNDs were washed with 10 mM HEPES buffer (pH 8.0) followed by 10 mM NaOH to stabilize AuNDs. After centrifugation, 900 μ L supernatant was removed and the same volume of HEPES or NaOH was added to the precipitated AuNDs to be dispersed. Then, AuNDs were continuously washed by HEPES with the same procedure twice to completely remove the excess ligands. The modified AuNDs were dispersed in HEPES and stored at 4 °C for further experiments. Then, salt aging or freeze-thaw method was used to conjugate DBCO-DNA and **N3**-modified AuNDs through click reaction.

Salt aging method

5 μ L of 100 μ M DBCO-DNA solution was brought into 800 μ L of 0.36 nM AuNDs-**EG12**-(92:3:5) or AuNDs-**EG12**-(85:10:5) in 10 mM HEPES (pH 8.0). Water was added up to 1 mL to retain HEPES concentration at 10 mM. Then, solution was incubated at 30 or 60 °C for 1 h to proceed click reaction. Next, salt screening was conducted to bring solutions into 20-, 40-, 60-, 80-, and 100-mM final concentration of NaCl every 30 min to promote click reaction and increase DNA density by shielding charge repulsion. After reaching 100 mM NaCl, samples were incubated overnight at 30 or 60 °C to optimize click reaction. Next, unreacted DBCO-DNA was removed by three times centrifugation. The precipitation was dispersed in 10 mM HEPES (pH 8.0) and stored at 4 °C before characterization.

Freeze-thaw method

5 μL of 100 μM DBCO-DNA dilution was brought into concentrated AuNDs-**EG12**-(92:3:5), AuNDs-**EG12**-(85:10:5), or AuNDs-**EG6**-(92:3:5) with the addition of 15 mM final concentration of NaCl before freezing at $-18\text{ }^{\circ}\text{C}$ for 2 h. Then, the mixture was thawed at $25\text{ }^{\circ}\text{C}$ for 1 h. To optimize DNA density, freeze-thaw was repeated up to 6 cycles with 2 times overnight. Next, unreacted DBCO-DNA was removed by three times centrifugation. The precipitation was dispersed in 10 mM HEPES (pH 8.0) and stored at $4\text{ }^{\circ}\text{C}$ before characterization.

Multimerization of DNA-modified AuNDs driven by hybridization with DNA linkers

After conjugating AuNDs-**EG12**-(85:10:5) with DBCO-DNA via click reaction in freeze-thaw method, AuNDs-**EG12**-DNA-(85:10)-5 was separated into two containers with the same volume. Then, each sample was hybridized with the linker 1 or linker 2. Specifically, 474 μL of AuNDs-**EG12**-DNA-(85:10)-5 (extinction value around 1.02) in 10 mM HEPES (pH 8.0) was mixed with 5 μL of 50 μM linker 1 or linker 2 and 21 μL of 2.5 M NaCl under maintaining pH and concentration of HEPES. The total volume of the mixed solution was 500 μL . Then, sample was heated at $80\text{ }^{\circ}\text{C}$ for 10 min to dehybridize all the DNA connections and then cooled to the room temperature. After overnight incubation at room temperature, colloidal solution was centrifuged two times to remove unconjugated linkers. The precipitation was dispersed in 10 mM HEPES (pH 8.0). Next, the same amount of AuNDs-**EG12**-DNA-(85:10:5)-linker 1 and linker 2 were mixed in 100 mM NaCl to proceed DNA hybridization. After overnight incubation at room temperature, the extinction spectra were investigated upon heating to confirm the hybridization.

3.2.4 Modifying AuNDs with HS-DNA via Ligand Exchange Reaction

Prior to modification, HS-DNA (detail sequences in **Table 3. 1**) was reduced by TCEP with the ratio of HS-DNA and TCEP equals to 1:100 molar equivalent. Specifically, 1 μL of 0.5 M TCEP was added to 50 μL of 100 μM HS-DNA, followed by incubation at $30\text{ }^{\circ}\text{C}$ for 6 h to cleave S-S bonds. Then, TCEP in 22- and 11-nt DNA were removed by ethanol precipitation method. Meanwhile, desalting column (PD MiniTrap G-10) was used to purify 6-nt DNA. To promote precipitation of isolation DNA with ethanol, 4 μL of 3M acetic acid (CH_3COONa) was added together with the addition of 200 μL of ice ethanol. After 1 h incubation at $-80\text{ }^{\circ}\text{C}$, sample was centrifuged at $10\text{ }^{\circ}\text{C}$ and 1200 rpm for 35 min. Next, 150 μL of supernatant was discarded. After the addition of 500 μL of ice ethanol, sample was centrifuged by the same conditions. Lastly, supernatant was removed as much as possible, and

the precipitation was dried at room temperature overnight. Before using for modification, dried DNA was dissolved in H₂O to achieve a desired concentration.

Theoretical maximum length of DNA ligand

DNA ligand used for surface modification composed of three regions: a 5' thiol modification which connected the DNA to the gold nanoparticle surface, a chain linker (C6) to separate the thiol moiety and the oligonucleotide, and the sequences for hybridization (**Figure 3. 2a**). The sequences were designed to link two AuNDs together through fully complementary base pair interaction (**Figure 3. 2b**). DNA ligand does not have unpaired spacer region between C6 and the base pairings. DNA length (**Table 3. 1** and **Table 3. 2**) was obtained by the addition of the length of C6 linker and the length of bases with the assumption that DNA was completely elongated on AuNDs surface. Consequently, ligand was linear with the extended length of C6 linker about 1.2 nm.⁷⁹ Meanwhile, the length of bases was calculated by multiplying the number of bases with 0.34 nm. As a result, 22-base-DNA has a maximum length about 8.7 nm. Melting temperature (T_m) of free DNA was calculated with Oligo Calculator (<https://www.ngri.co.jp/tools/0217oligocalc.htm>) in 50 mM salt concentration. T_m is a temperature at which half of DNA duplexes become single-stranded. Since T_m was affected by salt concentration, T_m of free DNA was further investigated in the solution with the same salt concentration as solution for assembled AuNDs. T_m was determined from the midpoint of the melting curve which was obtained by slowly heating dsDNA and plotting its absorbance at 258 nm as a function of temperature.

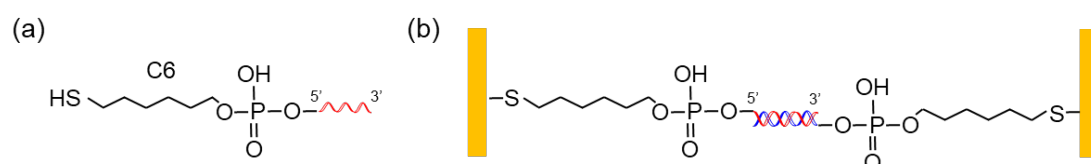


Figure 3. 2. (a) DNA design used for surface modification and (b) assembled AuNDs linked by fully hybridization of DNA sequence.

Table 3. 1. HS-DNA sequences used to modify AuNDs

		DNA sequences	DNA length (nm)	T_m (°C)	M_w
22 nt	(+)-DNA	[ThiolC6] 5'GTACGAACGCCATCGACTTACG3'	8.7	60	6901
	(-)-DNA	[ThiolC6] 5'CGTAAGTCGATGGCGTTCGTAC3'			6963
11 nt	(+)-DNA	[ThiolC6] 5'GTACGAACGCC3'	5	36	3522
	(-)-DNA	[ThiolC6] 5'GGCGTTCGTAC3'			3544
6 nt	(+)-DNA	[ThiolC6] 5'ACTTAC3'	3.2	16	1947
	(-)-DNA	[ThiolC6] 5'GTAAGT3'			2027

Salt aging

To modify AuNDs with HS-DNA via salt aging, two methods were conducted with minor change (using different phosphate solution).

[Method 1]: 1 mL of CTAB-stabilized AuNDs (concentration of CTAB: 10 mM and AuNDs: 0.3 nM) was centrifuged (9,400x g, 8 min, 30 °C) to remove excess CTAB. Then, 900 μ L of supernatant was removed before changing the solution to 739 μ L of H₂O. Next, 50 μ L of 100 μ M HS-DNA (22 nt-(+)-DNA or 22 nt-(-)-DNA) and 100 μ L of 2 mM CTAB were added to stabilize AuNDs during ligand exchange reaction. After incubation at 30 °C for 3 h, the colloidal solution was brought into 0.01% SDS and 10 mM phosphate buffer (KPO₄, pH 7.0). The total volume was 1 mL. After 1 h incubation at 30 °C, NaCl was gradually added (40-, 79-, 173-, 287-, 395-, and 500-mM final concentration of NaCl) every 30 min to the mixture to enhance DNA grafting density. After reaching 500 mM final concentration of NaCl in 2.5 h, sample was incubated at 30 °C overnight. Then, unreacted DNA was removed by 3 times centrifugation. The precipitation was dispersed in 0.01% SDS and stored at 30 °C for further experiments.

[Method 2]: 1 mL of CTAB-stabilized AuNDs (concentration of CTAB: 10 mM and AuNDs: 0.3 nM) was centrifuged (9,400x g, 8 min, 30 °C) to remove excess CTAB. Then, 900 μ L of supernatant was removed before adding 100 μ L of 2 mM CTAB to stabilize AuNDs during ligand exchange reaction and specific volumes of HS-DNA and H₂O (volume of the additional water was adjusting by the concentration of DNA stock solution). The ratio of AuNDs to DNA is 1:16,670 mole equivalent. After incubation at 30 °C for 3 h, the colloidal solution was brought into 0.01% SDS and 10 mM Na₂HPO₄. Total volume of the colloidal solution was 1 mL. After 1 h incubation at 30 °C, NaCl was gradually added (50-, 100-, 200-, 300-, 400-, and 500-mM final concentration of NaCl) every 30 min to enhance the DNA grafting density. After reaching 500 mM final concentration of NaCl, sample was incubated at 30 °C for 24 h. Then, unreacted DNA was removed by 3 times centrifugation. The precipitation was dispersed in 0.01% SDS and stored at 30 °C for further experiments.

Butanol dehydration

Butanol has been used in modifying AuNPs with high DNA grafting density in previous report⁸⁰. This method is a simple, efficient, and instant process which relies on removing water from the mixture of DNA and AuNPs with a butanol phase. In this experiment, various concentrations of CTAB-capped AuNDs were modified with various DNA lengths and sequences (**Table 3. 2**) via Au-thiol (-HS) interaction. CTAB is required to prevent AuNDs

from aggregation, whereas CTAB interacts with negatively charged DNA through electrostatic interaction, which can disturb the immobilization of DNA on AuNDs surfaces⁸¹. The advantage of using dehydration in butanol is its ability to remove CTAB on gold surfaces. Prior to modification, HS-DNA was reduced by TCEP with the ratio of HS-DNA and TCEP equals to 1:100 molar equivalent to cleavage dithiol bonds. After incubating at 25 °C for 20 h, HS-DNA was purified with desalting column (PD MiniTrap G-10) to discard TCEP and salts. Meanwhile, CTAB-stabilized AuNDs was centrifuged several times (9,400x g, 8 min, 30 °C) to remove excess CTAB. Final concentrations of CTAB were 3.2 or 0.32 mM, named as high and low CTAB, respectively. Then, purified HS-DNA was added to 32 µL of a desired AuNDs concentrations (high AuNDs: 0.3 nM, low AuNDs: 0.06 nM) in the presence of 1 mM TCEP. The ratio of AuNDs to HS-DNA was 1:16,670 (named as 100% modification). Total volume of the mixed solution was 100 µL. After quick vortex, 900 µL of 1-butanol was immediately added followed by vigorous stirring for few seconds. Butanol eliminates water from HS-DNA and AuNDs, increasing local concentration and promoting a close contact between DNA and AuNDs to proceed DNA immobilization. Then, 200 µL of 0.5xTBE (44.5 mM Tris, 1 mM EDTA, 44.5 mM boric acid, pH 8.0) was quickly added to rehydrate DNA-modified AuNDs. Liquid phase separation directly occurred after vortex mixing, presenting greenish colloidal solution in the bottom layer, suggesting DNA-modified AuNDs were dispersed in the water phase of two immiscible liquids. Then, unreacted DNA was removed by 3 times centrifugation (9,400x g, 40 min, 30 °C). The precipitation of AuNDs was dispersed in 10 mM phosphate buffer (pH 7.0) containing 0.01% SDS and stored at room temperature for further experiments.

Table 3. 2. HS-DNA sequences used to modify AuNDs

		DNA sequences	DNA Length (nm)	T_m (°C)	M_w
20 nt	R20-(+)-DNA	[ThiolC6] 5'-ACGAACGCCATGGGACTTAC ^{3'}	8.0	66	6439
	R20-(-)-DNA	[ThiolC6] 5'-GTAAGTCCCATGGCGTTCGT ^{3'}			6452
12 nt	Palindrome	[ThiolC6] 5'-TTTCTCGAGAAA ^{3'}	5.3	32	3973
11 nt	R11-(+)-DNA	[ThiolC6] 5'-ACGAACGCCAT ^{3'}	4.9	34	3638.6
	R11-(-)-DNA	[ThiolC6] 5'-ATGGCGTTCGT ^{3'}			3691.7
11 nt	dA11	[ThiolC6] 5'-AAAAAAAAAAAA ^{3'}	4.9	22	3580
	dT11	[ThiolC6] 5'-TTTTTTTTTTTT ^{3'}			3480
8 nt	R8-(+)-DNA	[ThiolC6] 5'-TTTGTGGT ^{3'}	3.9	22	2775
	R8-(-)-DNA	[ThiolC6] 5'-ACCACAAA ^{3'}			2700

3.2.5 Calculation of DNA Grafting Density on AuND Surface

Immobilized DNA on AuND surface was calculated by two methods: (i) hybridization

between immobilized DNA and its complementary with fluorescence dye and (ii) modifying AuNDs with HS-DNA-dye. In the first method, 1 mL of 0.07 nM (–)-DNA-AuND was mixed with 25 μ L of 200 μ M DNA-dye (3'-AGG GTA CCG CAA GCA-5') in the buffer solution contained of 150 mM NaCl. After heating at 80 °C for 15 min, the colloidal solution was cooled down to 25 °C and kept overnight to proceed hybridization between (–)-DNA and DNA-dye. Then, unhybridized DNA-dye was removed by three times centrifugation (9,400x g, 8 min, 25 °C). The precipitated AuNDs was dispersed in 67 μ L of buffer contained of 150 mM NaCl. Next, 2.5 μ L of 0.7 M DDT was added into 67 μ L of colloidal solution following with incubation at 65 °C for 20 h to replace (–)-DNA on AuNDs surfaces. Then, AuNDs were precipitated by centrifugation (9,400x g, 8 min, 25 °C). Finally, 2 μ L of supernatant was taken and measured its fluorescence. The corresponding DNA concentration was calculated according to the standard curve. In the second method, AuNDs were directly modified with HS-DNA-dye (HS-5'-GTA AGT CCC ATG GCG TTC GT3'-Cy3) via butanol dehydration method. Then, HS-DNA-dye was replaced by DTT via ligand exchange reaction. Therefore, 5 μ L of 0.7 M DDT was added into 100 μ L of 1.2 nM DNA-Cy3-AuND followed with incubation at 65 °C for 20 h. Next, AuNDs were precipitated by centrifugation (9,400x g, 8 min, 25 °C). Fluorescence intensity was obtained by measuring 2 μ L of supernatant. Immobilized DNA was calculated by comparing the relative fluorescence intensity with the standard curve.

3.3 Results and Discussions

3.3.1 Surface Modification of AuNDs with DBCO-DNA via Click Reaction

In **Chapter 2**, C1-modified AuNDs exhibited thermo-responsiveness property which inhibited to clearly confirm the assembly and disassembly induced by dehydration and rehydration of alkanethiol ligands. Assembly and disassembly of AuNDs via DNA hybridization and dehybridization was expected to avoid irreversible aggregation with hydrophobic interaction, which enables to control the assembly. Therefore, AuNDs were modified with hydrophilic ligand (OH) (**Figure 3. 3**). OH ligand would reduce the hydrophobicity, leading to easily disperse AuNDs in the colloidal solution.

Firstly, AuNDs modified with 100% OH-EG6 ligand, named as AuNDs-EG6-(100) exhibits low extinction intensity (**Figure 3. 4a**, blue) due to the low dispersibility. The magnified spectrum showed a broad extinction spectrum with the peak top around 725 nm (**Figure 3. 4b**, blue) indicating that most AuNDs were agglomerated. The addition of 3% COOH, named as AuNDs-EG6-(97:3), did not significantly improve AuNDs dispersibility (**Figure 3. 4a-b**, orange) although COOH provides electrostatic repulsion forces between

particles. The addition of 5% **N3** reduced dispersibility of AuNDs-**EG6**-(92:3:5) (**Figure 3. 4a-b**, purple) due to the hydrophobicity of **N3** ligand. These results correlate to the larger hydrodynamic diameter (**Figure 3. 4c**). In contrast, increasing **COOH** concentration to 10% significantly enhanced the extinction intensity of AuNDs-**EG6**-(90:10) with a LSPR peak at approximately 850 nm (**Figure 3. 4a**, yellow). The well dispersion of AuNDs was supported by DLS measurements which reveals two peaks around 21 and 122 nm (**Figure 3. 4c**, yellow), corresponding to the rotational and translational diffusion modes. Higher **COOH** content contributes to a large negative value of zeta potential (**Figure 3. 4d**, yellow) due to the higher negative charge which increases AuNDs dispersibility. The addition of 5% **N3** reduced dispersibility of AuNDs-**EG6**-(85:10:5) and slightly increased the hydrodynamic diameter (**Figure 3. 4a,c**, green).

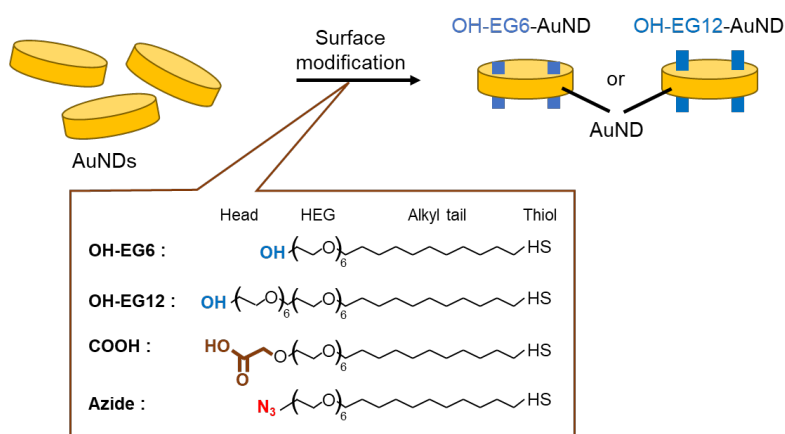


Figure 3. 3. Schematic illustration of ligand-modified AuNDs. AuND surface was modified with hydrophilic ligand to prevent stacking-induced hydrophobic interactions.

When **OH-EG6** was replaced to more hydrophilic ligand (**OH-EG12**) with a longer ethylene glycol group, small peak of AuNDs-**EG12**-(100) appeared at 850 nm (**Figure 3. 4e**) same as well dispersed AuNDs, indicating that **OH-EG12** provides better dispersibility than **OH-EG6**, although the extinction intensity was still low due to the low dispersibility. The addition of 3% **COOH** significantly increased the dispersibility of AuNDs-**EG12**-(97:3) (**Figure 3. 4e**, purple) which corresponds to the smaller hydrodynamic diameter (**Figure 3. 4g**, purple) and a larger negative value of zeta potential (**Figure 3. 4h**, purple) because the thicker layer of 12-mer ethylene glycol group on AuNDs surface effectively inhibits distance-dependent interaction. The addition of 5% **N3** reduced the negative value of zeta potential of AuNDs-**EG12**-(92:3:5) (**Figure 3. 4h**, brown), suggesting **OH** also contributes to the negative charge on AuNDs surface, while the extinction spectrum (**Figure 3. 4e**, brown) and hydrodynamic diameters (**Figure 3. 4g**, brown) did not change, maintaining the dispersibility.

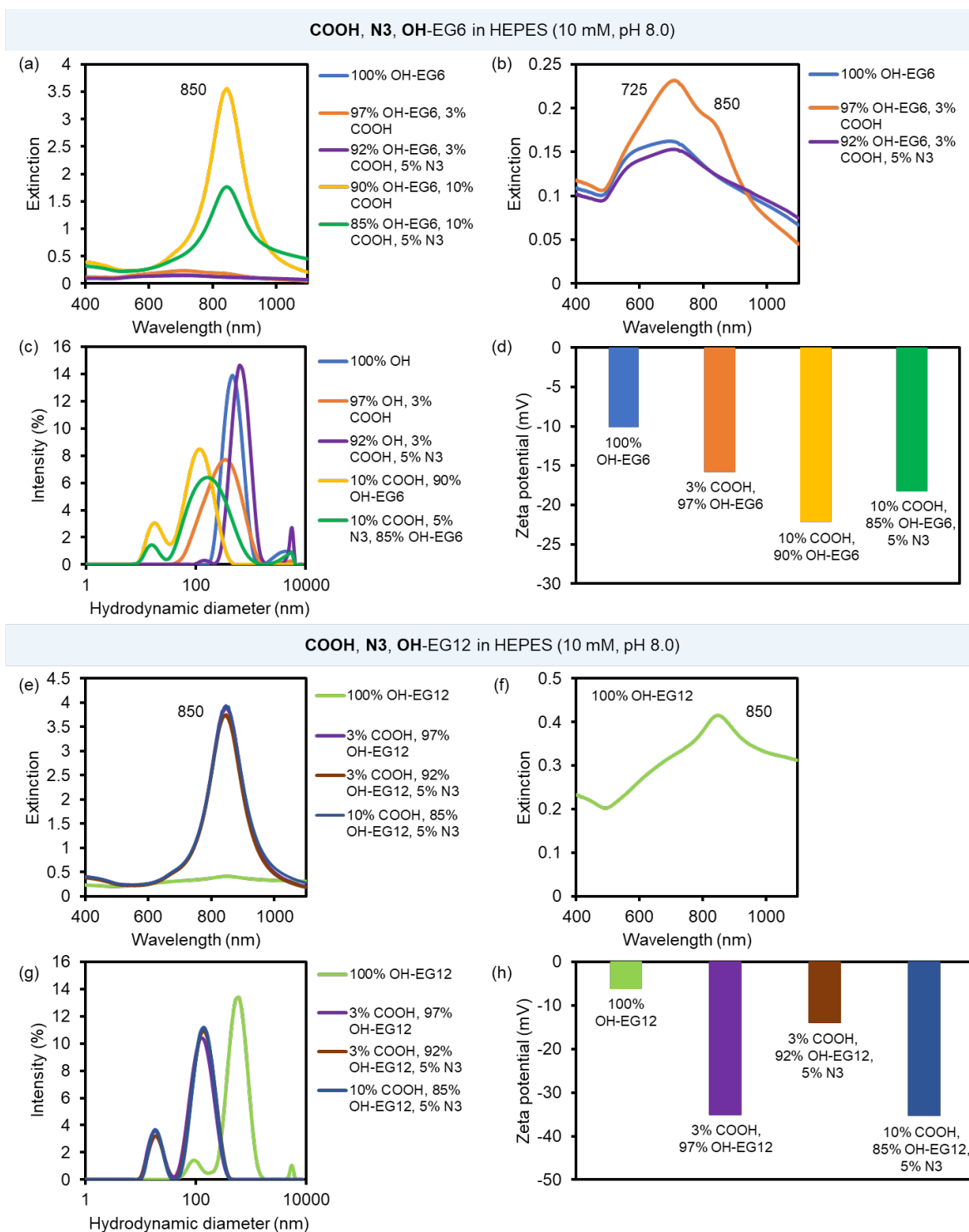


Figure 3. 4. Characterization of **OH**-modified AuNDs dispersed in 10 mM HEPES buffer (pH 8.0). (a-d) Characterization of EG6-based AuNDs: (a) extinction spectra, (b) magnification of the extinction spectra, (c) hydrodynamic diameter, and (d) zeta potentials of AuNDs-EG6. (e-h) Characterization of EG12-based AuNDs: (e) extinction spectra, (f) magnification of the extinction spectra, (g) hydrodynamic diameter, and (h) zeta potentials of AuNDs-EG12. The ratios of **OH**-, **COOH**-, and **N3**-ligands are represented in the figure.

The addition of 10% **COOH** increased a negative value of zeta potential of AuNDs-EG12-(85:10:5) (**Figure 3. 4h**, blue) maintaining the dispersibility (**Figure 3. 4e,g**, blue). Since

N3-functionalized AuNDs nanoparticles tended to aggregate and lack colloidal stability due to the neutral and hydrophobic properties, enough electrostatic forces between nanoparticles are required to disperse **N3**-modified AuNDs in the solution. Thus, hydrophilic **OH** and deprotonation of **COOH** groups in the basic solution (pH 8.0) provided enough electrostatic repulsion forces to maintain the stability of 5% **N3**-modified AuNDs. These results indicate that agglomeration occurs if repulsive force (electrostatic force) is smaller than attractive force (van der Waals force). The length of ligands changes the interparticle distance which affects the distance-dependent interactions. Decreasing the ligand length increases the van der Waals interaction, thereby reducing the stability of nanoparticles⁸².

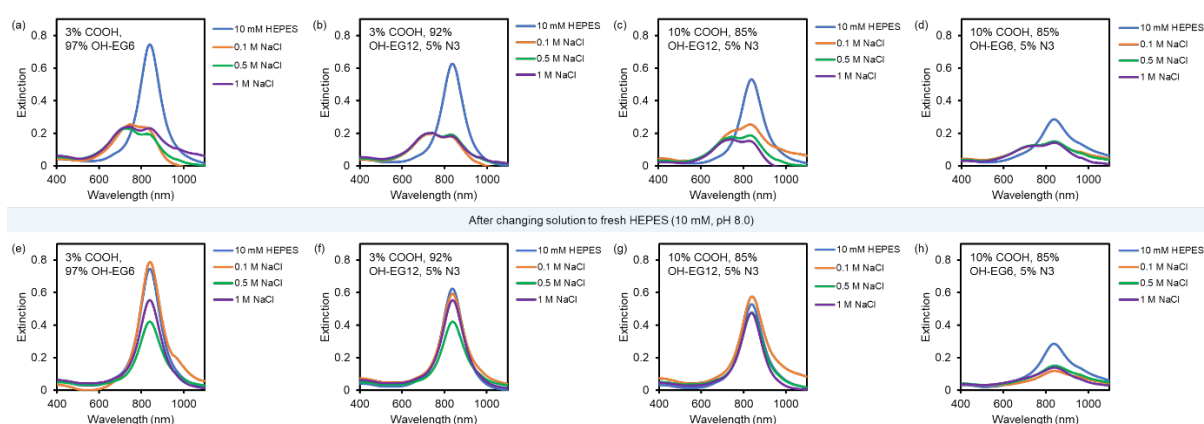


Figure 3. 5. Effect of NaCl on **OH**-modified AuNDs. (a-d) Assembly of (a) AuNDs-EG12-(97:3), (b) AuNDs-EG12-(92:3:5), (c) AuNDs-EG12-(85:10:5), and (d) AuNDs-EG6-(85:10:5) in solutions of 0.1 (orange), 0.5 (green), and 1 M (purple) NaCl with 1x HEPES, respectively. (e-h) Reversibility of (e) AuNDs-EG12-(97:3), (f) AuNDs-EG12-(92:3:5), (g) AuNDs-EG12-(85:10:5), and (h) AuNDs-EG6-(85:10:5) after removing NaCl and changing solution to 1x HEPES (10 mM, pH 8.0).

Then, the effect of NaCl on **OH**-modified AuNDs was investigated in 0.1, 0.5, and 1 M final concentration of NaCl with directly measuring their extinction spectra. In all cases, spectra exhibited reduction of extinction intensity at 850 nm and the appearance of a new plasmon peak at 730 nm (**Figure 3. 5a-d**). These results indicate ions from NaCl promoted the formation of face-to-face assembly of AuNDs⁷ shielding the electrostatic repulsion, while some AuNDs were still dispersed in the solution. Spectra between AuNDs-EG12-(97:3) (**Figure 3. 5a**) and AuNDs-EG12-(92:3:5) (**Figure 3. 5b**) did not show significant difference, suggesting that **N3** does not contribute to the assembly formation. On the other hand, AuNDs-EG12-(85:10:5) in 0.1 M NaCl (**Figure 3. 5c**, orange), showed the lower assembly formation than AuNDs-EG12-(92:3:5) (**Figure 3. 5b**), indicating that 10% **COOH** slightly inhibits the assembly due to the higher electrostatic repulsion. With the same **N3** concentration, AuNDs-EG6-(85:10:5) (**Figure 3. 5d**) showed lower stability than AuNDs-EG12-(85:10:5) (**Figure 3.**

5c), indicating length of the ethylene glycol group in the ligands also contributes to the assembly formation affecting distance-dependent interaction. After removing the salt and changing solution to 10 mM HEPES buffer (pH 8.0), all spectra of AuNDs-**EG12** are recovered (**Figure 3. 5e-h**) because deprotonation of **COOH** under basic condition promotes electrostatic repulsion to redisperse AuNDs. Nevertheless, some extinction intensities were not fully recovered because of losing upon washing. In conclusion, AuNDs-**EG12** were preferable for further DNA modification because of their reversibility and non-thermo-responsive properties.

Then, DNA was immobilized on AuNDs modified with **EG12**-, **COOH**-, and **N3**-ligands through click reaction (**Figure 3. 6a**). Specifically, dibenzocyclooctyne (DBCO)-modified DNA was reacted with and covalently immobilized onto **N3**-ligands on AuNDs via copper-free click reaction. DBCO possesses an alkyne group in a strained cyclic ring which proceed azide-alkyne cycloaddition reaction without Cu^+ as a catalyst. In addition, to increase the number of mobilized DNA on AuNDs surfaces, (i) salt aging method was applied during the click reaction with gradually increasing salt concentration, which reduced electrostatic repulsion by shielding the charges. In addition, (ii) freeze-thaw method was investigated to increase DNA density on AuNDs, in which growing ice crystals expel AuNDs, NaCl, and DBCO-DNA, increase the local concentration, and promote the immobilization reaction. DBCO-DNA was covalently attached to the **N3** groups on AuNDs surfaces to produce DNA-modified AuNDs.

Salt aging method

Firstly, click reaction was combined with salt aging method to minimize electrostatic interaction and steric hindrance of DNA strands to increase the number of attached DNA on AuNDs surfaces. Before DNA conjugation, extinction spectra of AuNDs-**EG12**-(92:3:5) and AuNDs-**EG12**-(85:10:5) in 100 mM NaCl spontaneously decreased and exhibited blue-shift (**Figure 3. 6b,c**), suggesting that the assembly formation of AuNDs owing to the low negative charges. After DNA modification with click reaction at 60 °C, AuNDs-**EG12**-DNA-(92:3:5) and AuNDs-**EG12**-DNA-(85:10:5) were more stable in 100 mM NaCl (**Figure 3. 6d,e**) due to the higher negative charge of DNA, indicating that DNA was successfully modified on the AuNDs. Conversely, under click reaction at 30 °C, extinction spectra of both AuNDs spontaneously decreased and exhibited blue-shift in 100 mM NaCl similar to AuNDs before DNA modification (**Figure 3. 6f,g**) suggesting that enough DNA was not immobilized. These results reveal that higher temperature is required to promote the click reaction, increasing the probability of collision between **N3** and DBCO groups. Besides, **COOH** density on AuNDs

surfaces also affected click reaction. AuNDs-EG12-DNA-(92:3:5) (**Figure 3. 5d**) was more stable than AuNDs-EG12-DNA-(85:10:5) (**Figure 3. 6e**) in 100 mM NaCl for 2 h due to the higher DNA density. These results suggested that higher COOH coverage on AuNDs surfaces inhibited DBCO-DNA to reach N3 due to the higher electrostatic repulsion, resulting in lower DNA modification.

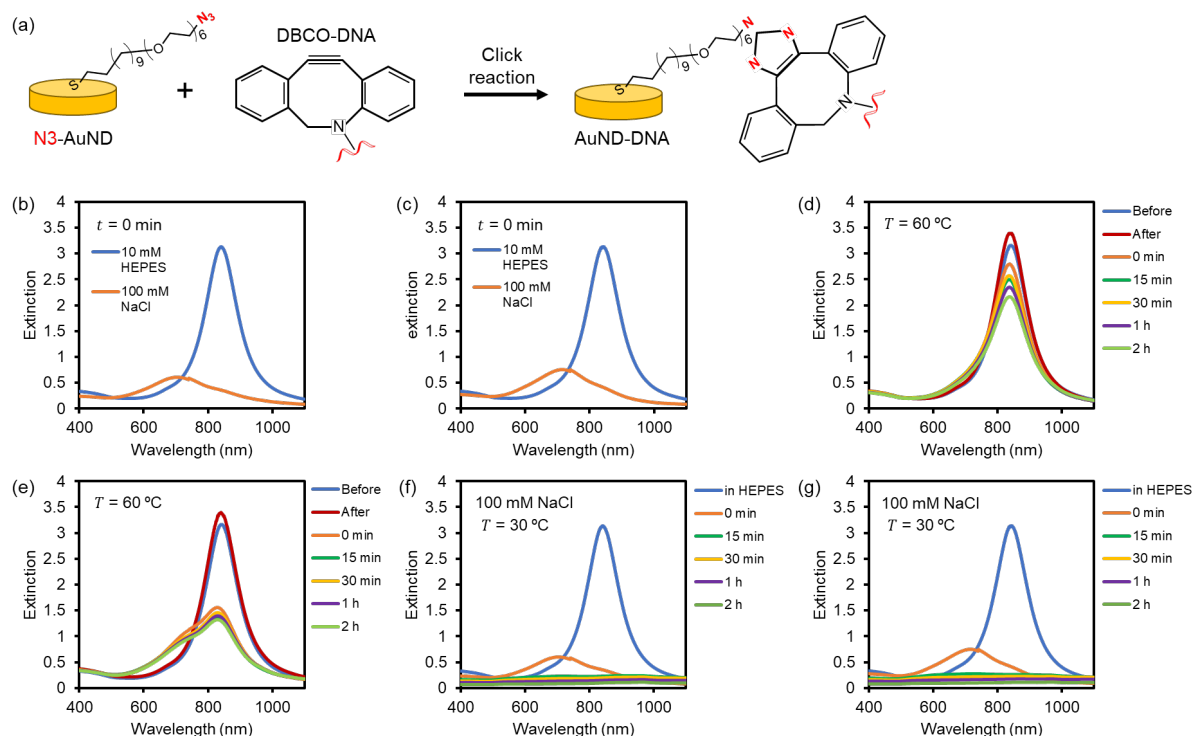


Figure 3. 6. (a) Schematic illustration of click reaction between N3-modified AuNDs and DBCO-DNA. Extinction spectra of AuNDs modified with DNA via click reaction and salt aging method. (b, c) Comparison of dispersibility of (b) AuNDs-EG12-(92:3:5) and (c) AuNDs-EG12-(85:10:5) in 10 mM HEPES (pH 8.0) and 100 mM NaCl before DNA modification. (d-g) Monitoring stability of (d, f) AuNDs-EG12-DNA-(92:3:5) and (e, g) AuNDs-EG12-DNA-(85:10:5) from 0 min until 2 h in 100 mM NaCl after click reaction at (d, e) 60 °C or (f, g) 30 °C, respectively.

Freeze-thaw method

Secondly, click reaction was combined with freeze-thaw method to promote click reaction between DBCO-DNA and N3-modified AuNDs. Compared to AuNDs-EG12-DNA-(92:3:5) with salt aging method at 60 °C (**Figure 3. 6d**), freeze-thaw method showed the lower stability in 100 mM NaCl (**Figure 3. 7a**) after DNA modification, indicating lower DNA immobilization. This result suggests that AuNDs-EG12-(92:3:5) is unstable in the highly concentrated NaCl upon freezing and aggregated. Conversely, higher COOH coverage on AuNDs surfaces improved the stability, resulting in higher DNA density of AuNDs-EG12-(85:10:5) with freeze-thaw method (**Figure 3. 7b**) than salt aging method at 60 °C (**Figure 3.**

7c). Besides, length of an ethylene glycol group in **OH** ligands also affected the stability of AuNDs in high NaCl concentration. AuNDs-**EG6**-DNA-(92:3:5) (**Figure 3. 6c**) showed much lower DNA density than AuNDs-**EG12**-DNA-(92:3:5) (**Figure 3. 7a**) because shorter **OH** ligand does not suppress distance-dependent interaction between AuNDs which result in aggregation of AuNDs and reduction of the efficiency of click reaction.

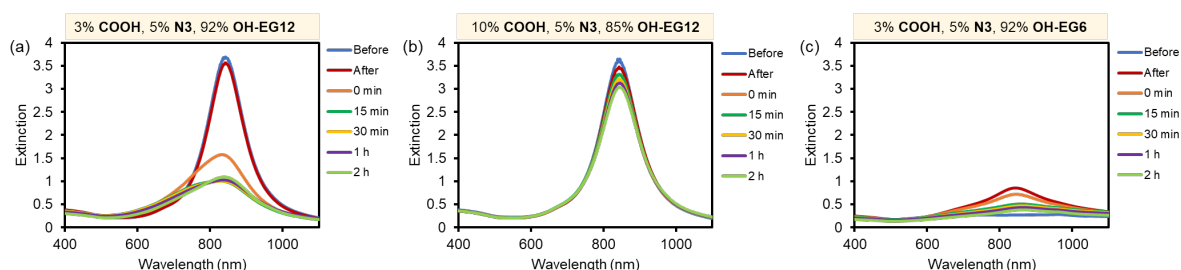


Figure 3. 7. Extinction spectra of AuNDs modified with DNA via click reaction and freeze-thaw method. Monitoring the stability of (a) AuNDs-**EG12**-DNA-(92:3:5), (b) AuNDs-**EG12**-DNA-(85:10:5), and (c) AuNDs-**EG6**-DNA-(92:3:5) from 0 min until 2 h in 100 mM NaCl after click reaction.

Multimerization of DNA-modified AuNDs driven by hybridization with DNA linkers

DNA linkers enable assembly of nanoparticles in nanoscale precision with specific hybridization and defined spacer lengths by designing the sequences and base numbers. Here, AuNDs-**EG12**-DNA-(85:10:5) with freeze-thaw method was conjugated to linker 1 and linker 2 via hybridization and mixed to crosslink AuNDs through DNA hybridization (**Figure 3. 8a**). After conjugating to linkers, AuNDs-**EG12**-DNA-(85:10:5) retained the extinction intensities (**Figure 3. 8b**), suggesting that AuNDs were not lost upon manipulation. After mixing AuNDs-**EG12**-DNA-(85:10:5)-linker 1 and linker 2 in 100 mM NaCl and incubation overnight, the extinction intensity was reduced without plasmonic shifting (**Figure 3. 8b**). To confirm the reducing intensity was originated from the assembly induced by hybridization, the colloidal solution was heating from 25 to 80 °C to promote dehybridization. As the result, the spectrum did not show significant increase of the intensity (**Figure 3. 8c**), indicating that decreasing intensity after mixing was correlated to the aggregation induced by NaCl, not by DNA hybridization. TEM images of the individual state of AuNDs-**EG12**-DNA-(85:10:5)-linker 1 and linker 2 (**Figure 3. 8d,e**) showed the same clusters as the mixed solution (**Figure 3. 8f**), supporting that multimerization via hybridization did not occur. Probably, low DNA density (only 5%) on AuNDs surfaces is not enough to drive assembly via hybridization. To increase DNA coverage, AuNDs would be directly modified with HS-DNA through ligand exchange reaction. Flexible linker with 32 complementary base pairings also reduced the hybridization efficiency, inhibiting AuNDs assembly. In term of plasmonic properties, assembly with large

interparticle gap distance induced a weak plasmonic coupling, leading to insignificant plasmonic shift which inhibited to clearly confirm the assembly formation.

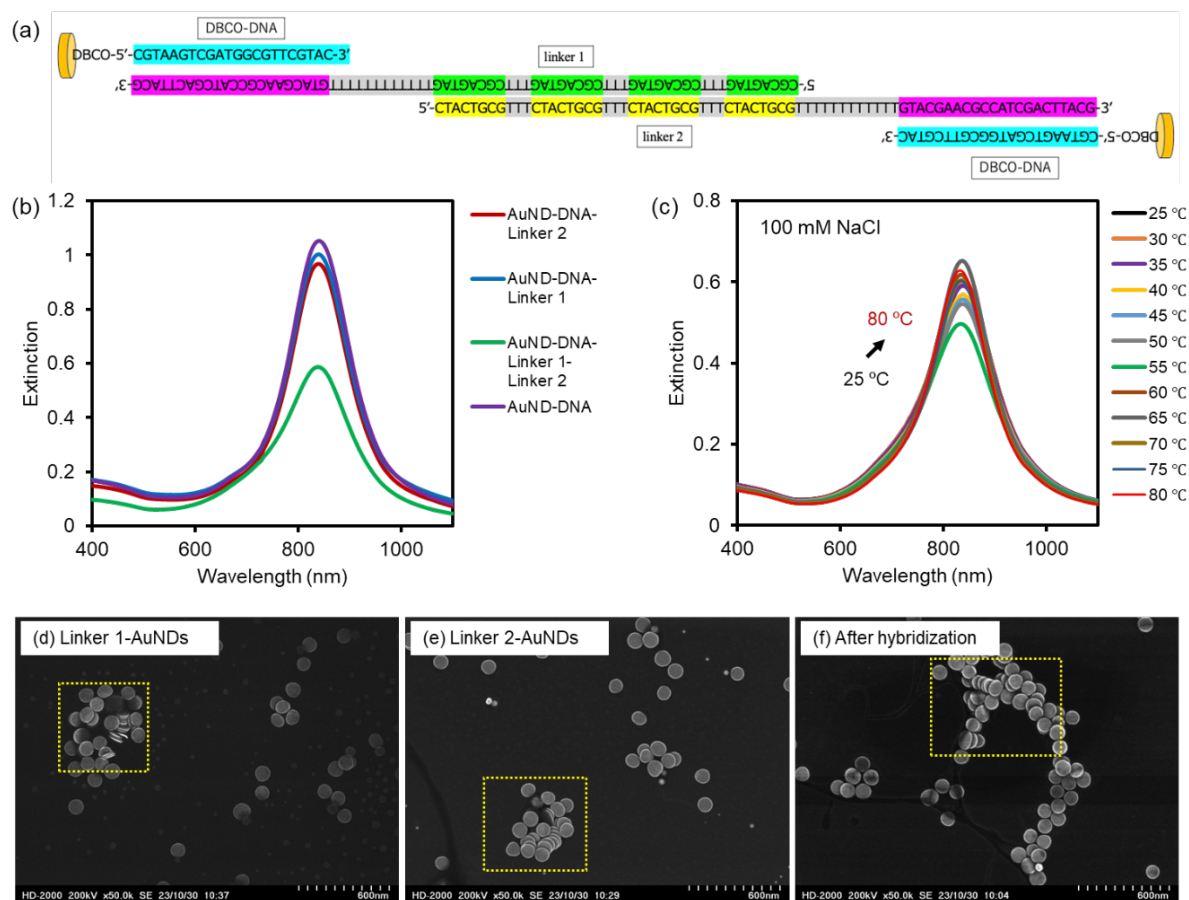


Figure 3. 8. (a) Cross linking of DNA-modified AuNDs (click reaction) through DNA hybridization assisted with linker DNA 1 and 2. (b) Extinction spectra of AuNDs-EG12-DNA-(85:10:5) before (black) and after conjugation to linker 1 (blue) and linker 2 (red) dispersed in 10 mM HEPES buffer (pH 8.0) and the mixed solution of AuNDs-EG12-DNA-(85:10:5)-linker 1 and linker 2 in 100 mM NaCl (green). (c) Extinction spectra of the mixed solution of AuNDs-EG12-DNA-(85:10:5)-linker 1 and linker 2 in 100 mM NaCl upon heating from 25 to 80 °C. (d-f) TEM images of (d) AuNDs-EG12-DNA-(85:10:5)-linker 1, (e) AuNDs-EG12-DNA-(85:10:5)-linker 2, and (f) the mixture of AuNDs-EG12-DNA-(85:10:5)-linker 1 and linker 2 in 100 mM NaCl.

3.3.2 Surface Modification of AuNDs with HS-DNA via Salt Aging

(A) Salt Aging --- Method I

AuNDs were functionalized with HS-DNA contained of 22 bases (22 base-(+)-DNA or 22 base-(-)-DNA) through Au-S bonds by replacing CTAB on AuNDs surfaces. CTAB is required to prevent AuNDs from aggregation, whereas CTAB interacts with negatively charged DNA through electrostatic interaction, which can disturb the immobilization of DNA on AuNDs surfaces⁸¹. As a compromised condition, the ligand exchange reaction was proceeded in 0.2 mM CTAB. After removing the unbound nucleotides, salt was gradually added to the

dispersed AuNDs in 0.01% SDS to reach 70-, 139-, 205-, 270-, 333-, 394-, 454-, and 512-mM final concentration of NaCl to investigate the stability of DNA-modified AuNDs and confirm DNA immobilization. Compared to the non-modified AuNDs (**Figure 3. 9a**), 22 base-(+)-DNA-AuND and 22 base-(-)-DNA-AuND show a better stability (**Figure 3. 9b-c**) in higher NaCl concentration, indicating that AuNDs are successfully modified with HS-DNA via ligand exchange reaction under salt aging. Lower stability of 22 base-(+)-DNA-AuND (**Figure 3. 9d**) than that of 22 base-(-)-DNA-AuND (**Figure 3. 9e**) suggested that grafting density of (+)-DNA on AuNDs surfaces was lower than that of (-)-DNA. On the other hand, stability of 22 base-(+)-DNA-AuND was improved in solution containing of 0.01% SDS and 10 mM phosphate buffer (pH 7.0) (**Figure 3. 9f**) than in 0.01% SDS (**Figure 3. 9d**).

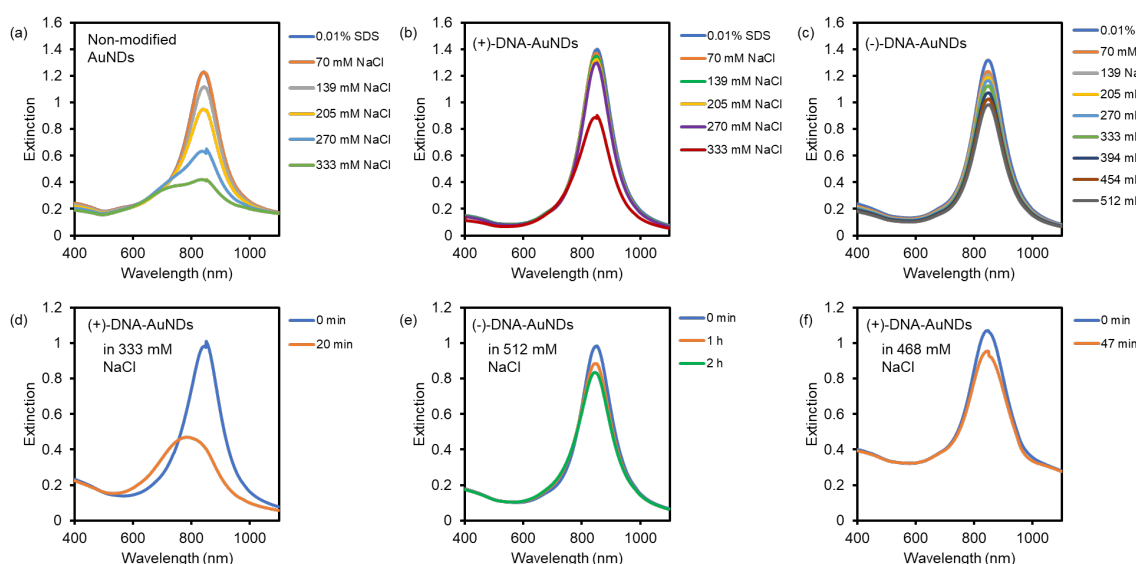


Figure 3. 9. Stability of 22 nt DNA-modified AuNDs in 0.01% SDS and salt solution. (a-b) Extinction spectra of (a) non-modified AuNDs, (b) AuNDs-22 nt-(+)-DNA, and (c) AuNDs-22 nt-(-)-DNA in various NaCl concentrations. (d) Extinction spectra of AuNDs-22 nt-(+)-DNA in 333 mM NaCl from 0 to 20 min. (e) Extinction spectra of AuNDs-22 nt-(-)-DNA in 512 mM NaCl from 0 to 2 h. (f) Extinction spectra of AuNDs-22 nt-(+)-DNA in 10 mM phosphate buffer (pH 7.0) with 468 mM NaCl from 0 to 47 min. SDS concentration was 0.01% for all measurements.

(i) Assembly and disassembly of DNA-modified AuNDs driven by hybridization

To assemble AuNDs, the same ratio of 22 base-(+)-DNA-AuND and 22 base-(-)-DNA-AuND were mixed in the solution containing 0.01% SDS, 10 mM phosphate buffer (pH 7.0), and 505 mM NaCl. The mixture of 1:1 ratio was incubated at 25 °C in UV spectrophotometer with recording their time-dependent extinction spectra. After 30 min, the extinction intensity was reduced more than a half with broadening plasmonic peak (**Figure 3. 10a**), suggesting that AuNDs formed large assembly driven by DNA hybridization. Longer incubation time up to 1

h only reduced small extinction intensity, suggesting that hybridization quickly occurred after mixing of the AuNDs and was saturated after 30 min. Then, the mixture solution was heated at 70 °C (higher than melting temperature, 60 °C) for 10 and 40 min (**Figure 3. 10b**) to clarify that assembly was caused by hybridization rather than salt-induced assembly. The unaltered extinction spectra indicated that heating at 70 °C up to 40 min did not promote dehybridization of duplex DNA. In contrast, the extinction intensity was returned close to the initial intensity when temperature was increased to 80 °C (**Figure 3. 10b**), indicating assembly and disassembly of AuNDs were attributed to DNA hybridization and dehybridization, respectively.

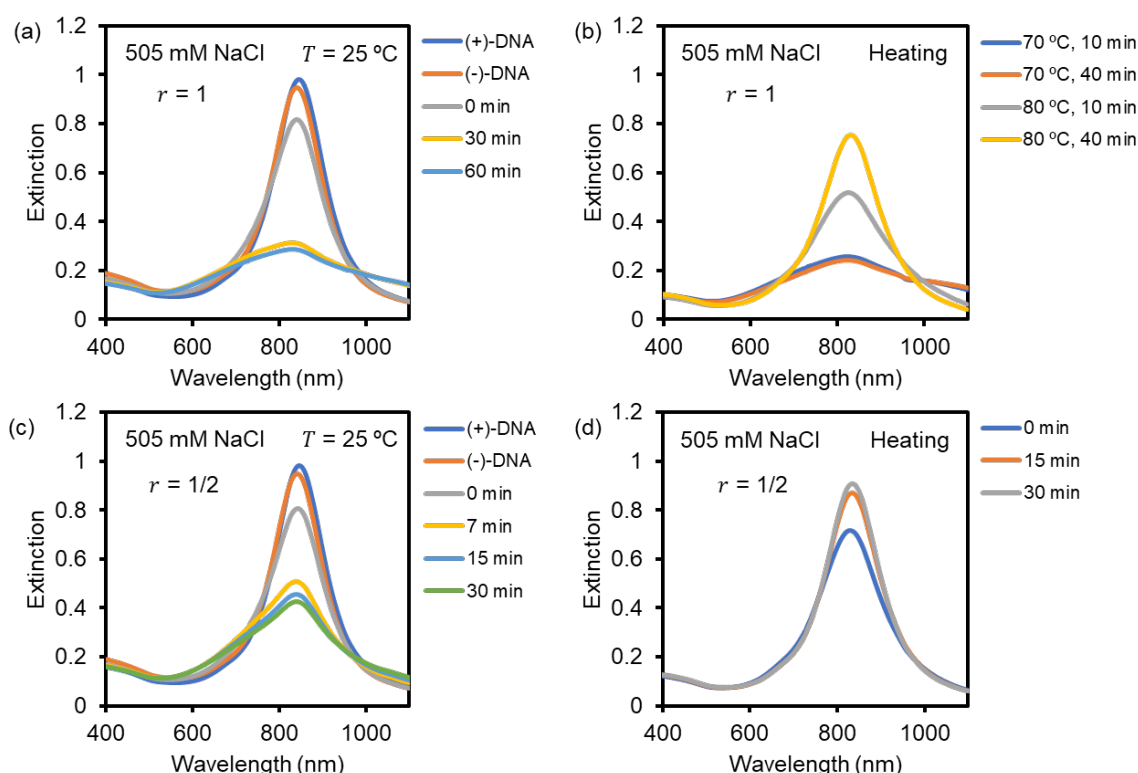


Figure 3. 10. Assembly and disassembly of AuNDs induced by DNA hybridization and dehybridization. Extinction spectra of (a-b) 1:1 and (c-d) 2:1 ratio of 22 base-(+)-DNA-AuND to 22 base-(-)-DNA-AuND in 505 mM NaCl. Hybridization and dehybridization were proceeded at 25 and 80 °C, respectively under time-dependent investigation of the extinction spectra. Melting temperature (T_m) of free 22 base-DNA in 50 mM NaCl is 60 °C.

When the ratio of 22 base-(+)-DNA-AuND and 22 base-(-)-DNA-AuND equals to 2:1, a small shoulder peak around 700 nm appeared after 7 min incubation at 25 °C (**Figure 3. 10c**). Possibly, number of oligomers were smaller than the 1:1 ratio. This result suggests that number of assemblies can be controlled by adjusting the mixing molar ratio of DNA-modified AuNDs. Besides, decrease rate of the extinction intensity was significantly fast (in 7 min), indicating that assembly driven by DNA hybridization is quick. Unfortunately, these results did not clearly confirm that AuNDs assembly was driven by hybridization between (+)-DNA and (-)-DNA

because they include self-hybridization sequence (“GTAC”), which can contribute to the assembly of AuNDs. To confirm this issue, further experiments are required: (1) using alternative sequences of DNA which do not include self-hybridization sequences, or (2) investigating the extinction spectrum of 22 base-(+)-DNA-AuND and 22 base-(-)-DNA-AuND in 505 mM NaCl as a control experiments. Even if the intensity decreases, confirming that the intensity is not increased with heating (up to 80 °C).

(ii) *Assembly/disassembly of DNA-modified AuNDs driven by blunt-end stacking*

Full-complementary dsDNA forms blunt-end stacking between outermost base pairs when the diffusional motion is restricted. Blunt-end stacking facilitates attractive interaction between dsDNA layers with hydrophobic interaction caused by a dehydration of DNA in high salt concentrations⁸³. In this section, assembly of DNA-modified AuNDs driven by blunt-end stacking was investigated by adding the complementary DNA in high NaCl concentration. Accordingly, free fully complementary DNA (22 base) was mixed with 22 base-(+)-DNA-AuND in 0.01% SDS and 10 mM phosphate buffer (pH 7.0). Then, NaCl was gradually added into the mixture to proceed the hybridization at 25 °C and form “dsDNA-functionalized AuNDs”. Compared to 22 base-(+)-DNA-AuND (“ssDNA-functionalized AuNDs”, **Figure 3. 11a**), the extinction spectra of dsDNA-functionalized AuNDs exhibited a larger decrease of the extinction intensity (**Figure 3. 11b**) with the increase of NaCl concentrations. Besides, dsDNA-functionalized AuNDs showed a plasmonic shifting from 851 to 806 nm when NaCl concentration increased from 270 to 505 mM (**Figure 3. 11d,f**), probably due to face-to-face assembly. Conversely, ssDNA-functionalized AuNDs did not exhibit plasmonic shifting (**Figure 3. 11c,e**). In the intermediate NaCl concentration, dsDNA-functionalized AuNDs showed blue-shifting after longer incubation (47 min, **Figure 3. 11f**), supporting that the reduction of extinction intensity was induced by the complementary dsDNA terminal. To investigate disassembly of blunt-end stacking induced by DNA dehybridization, the mixed sample was heated at 70 °C (higher than T_m in 50 mM NaCl, 60 °C) for 10 min. As the result, only small return of the spectrum in plasmonic shifting and intensity was observed (**Figure 3. 11g**). This result indicates that assembled structure of DNA-modified AuNDs via blunt-end stacking was not disassembled reversibly with dehybridization, whose cause is needed further investigation. In the next section, AuND assembly solely induced by hybridization was investigated.

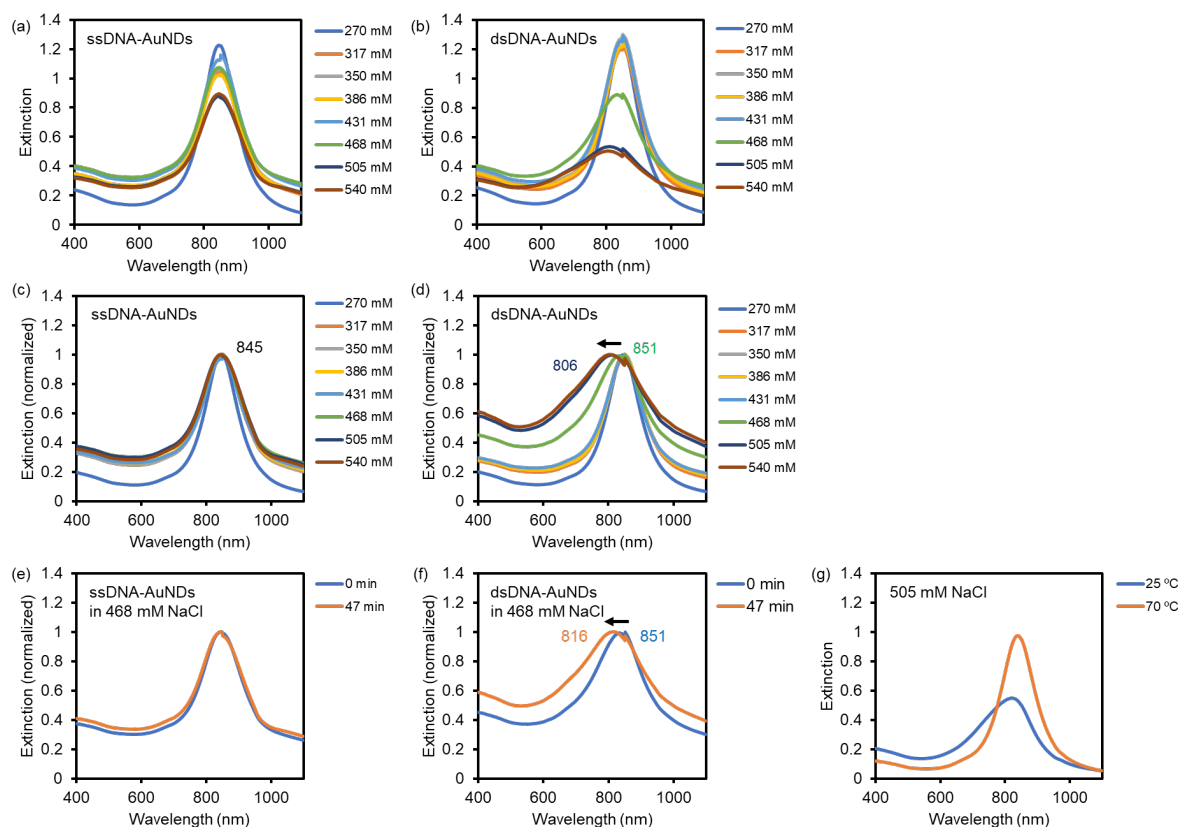


Figure 3. 11. Assembly of AuNDs induced by blunt-end stacking. Extinction spectra of (a, c) ssDNA-modified and (b, d) dsDNA-functionalized AuNDs with the increase of NaCl concentration. (c, d) Normalized extinction spectra. (e, f) Time-dependent measurements of (e) ssDNA-modified and (f) dsDNA-functionalized AuNDs in 468 mM NaCl. (g) Extinction spectra of dsDNA-functionalized AuNDs at 25 and 70 °C.

(B) Salt Aging --- Method II

In previous section, AuNDs were modified with HS-DNA of 22 base-(+)-DNA and 22 base-(-)-DNA through Au-S bonds and assembly of the DNA-modified AuNDs was demonstrated via DNA hybridization. To further investigate the assembly driven by DNA hybridization, AuNDs modified with different lengths of DNA (22 base, 11base, and 6 base) followed with evaluating their stability in salt solutions. There is no significant difference between method I and method II, except for the phosphate solution. Extinction spectra of 22 nt- and 11 nt-DNA-modified AuNDs exhibited the same plasmon peak as CTAB-stabilized AuNDs (**Figure 3. 12a,b**), suggesting that DNA-modified AuNDs was well dispersed in 0.01% SDS, while slight broadening of the spectra was observed probably due to different refractive index. Conversely, 6 nt-(-)-DNA-modified AuNDs showed broadening of the plasmonic peak and shifting from 829 to 821 nm (**Figure 3. 12c**) suggesting that AuNDs partially agglomerate due to the lower stability. These results were in line with the stability of AuNDs modified with **OH-EG6** and **OH-EG12** in **Section 3.3.1** which indicates that ligand lengths on AuND

surfaces affects the interparticle interaction. Shorter DNA reduced the interparticle distance, increasing the length-dependent force which decreased nanoparticle stability.

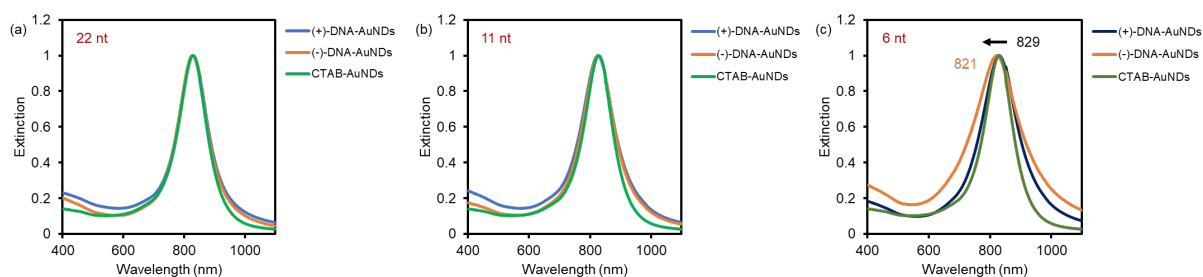


Figure 3. 12. Extinction spectra of (a) 22 nt-, (b) 11 nt-, and (c) 6 nt-DNA-modified AuNDs in 0.01% SDS. Two DNA sequences ((+)-DNA, blue; (-)-DNA, orange) were modified individually. Spectrum of CTAB-stabilized AuNDs is also shown (green).

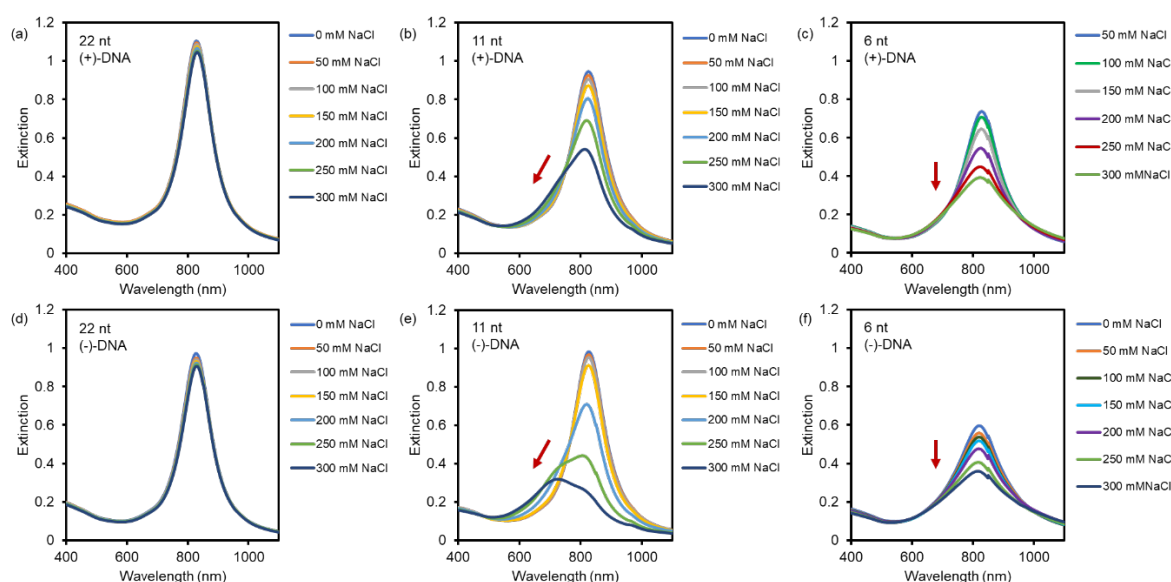


Figure 3. 13. Extinction spectra of (a, d) 22 nt-, (b, e) 11 nt-, and (c, f) 6 nt-DNA-modified AuNDs in various NaCl concentrations. Two DNA sequences ((+)-DNA (a, c), (-)-DNA (d-f)) were modified individually. All solutions contained 0.01% SDS and 10 mM Na_2HPO_4 .

Next, the stability of DNA-modified AuNDs were investigated upon gradually increasing NaCl concentration in 0.01% SDS and 10 mM Na_2HPO_4 solution. As the results, 22 nt-DNA-modified AuNDs showed a better stability (**Figure 3. 13a,d**) than 11 and 6 nt-DNA-modified AuNDs (**Figure 3. 13b,e** and **Figure 3. 13c,f**, respectively) especially in 300 mM NaCl due to stronger electrostatic repulsion because longer DNA has larger numbers of phosphate anions and is more negatively charged. Stability of AuNDs in the salt solution was decreased when DNA length was reduced to 11 nt based on decrease of its extinction intensity (**Figure 3. 13b,e**). Specifically, the intensities of both 11 nt-DNA-modified AuNDs started decreasing from 200 mM NaCl with exhibiting a new plasmon peak around 700 nm due to AuNDs agglomeration (**Figure 3. 13**). This agglomeration may be promoted by self-

hybridization of individual DNA strands because they include self-hybridization sequence (“GTAC”).

Effect of DNA length on the assembly and disassembly of AuNDs

Previously, assembly of AuNDs driven by DNA hybridization were investigated by mixing AuNDs modified with 22 nt-(+)-DNA and 22 nt-(-)-DNA. Extinction spectra of the mixture showed reduction of intensity and broadening of the plasmonic peak, indicating that AuNDs formed large assembly driven by DNA hybridization. However, the spectra only showed small blue-shifting probably due to weak plasmonic coupling (**Figure 3. 10a,c**). These results suggested that the length of DNA (22 nt) may be too long to strengthen the plasmonic coupling of assembled AuNDs and the spectral shifting. Accordingly, AuNDs were modified with various lengths of DNA to investigate the effect of DNA length on the assembly and disassembly properties.

22 nt-DNA-modified AuNDs:

In individual state, 22 nt-(+)-DNA- and 22 nt-(-)-DNA-modified AuNDs were stably dispersed in 300 mM NaCl after 1 h incubation at 25 °C without changing the plasmonic spectra (**Figure 3. 14a,b**). Next, the (+)-DNA- and (-)-DNA-modified AuNDs were mixed with different ratios (50:50, 60:40, and 67:33) in 300 mM NaCl, 0.01% SDS, and 10 mM Na₂HPO₄ solution. Time-dependence of the extinction spectra were monitored at 25 °C from 0 to 60 min. All spectra showed a decrease of the extinction intensity without plasmonic shifting with increasing incubation time (**Figure 3. 14c-e**), indicating that hybridization of (+)-DNA and (-)-DNA promoted AuNDs assembly. No plasmonic shifting was probably attributed to large interparticle nanogap (~9.9 nm) between the assembled AuNDs, only inducing weak plasmonic coupling. Importantly, different ratios of (+)-DNA- and (-)-DNA-modified AuNDs exhibited different degree of the reducing extinction intensity, suggesting that AuNDs formed different number of oligomers. Possibly, 50:50 ratio produced larger assembly than 60:40 and 67:33 ratios based on the probability of DNA hybridization.

To confirm that the assembly was caused by hybridization, samples were heated at 80 °C (higher than T_m of free DNA in 50 mM NaCl, 60 °C) after 1 h incubation at 25 °C to promote dehybridization of duplex DNA. The extinction intensity of 50:50 and 60:40 ratios slightly increased (**Figure 3. 14f-g**), but the spectra were not fully recovered after heating for 30 min. Probably, fully matched 22 nt-DNA took a longer time or higher temperature to completely dehybridize due to the stability of duplex structure. Besides, number of oligomers also affected the rate of disassembly, which is supported by the result that 67:33 ratio was almost returned

to the initial state upon heating for 10 min (**Figure 3. 14h**) because it only formed small assembly structure (**Figure 3. 14e**). Therefore, it was necessary to keep the same concentration in each experiment since the reaction rate and assembly sizes greatly depended on DNA-AuND concentration.

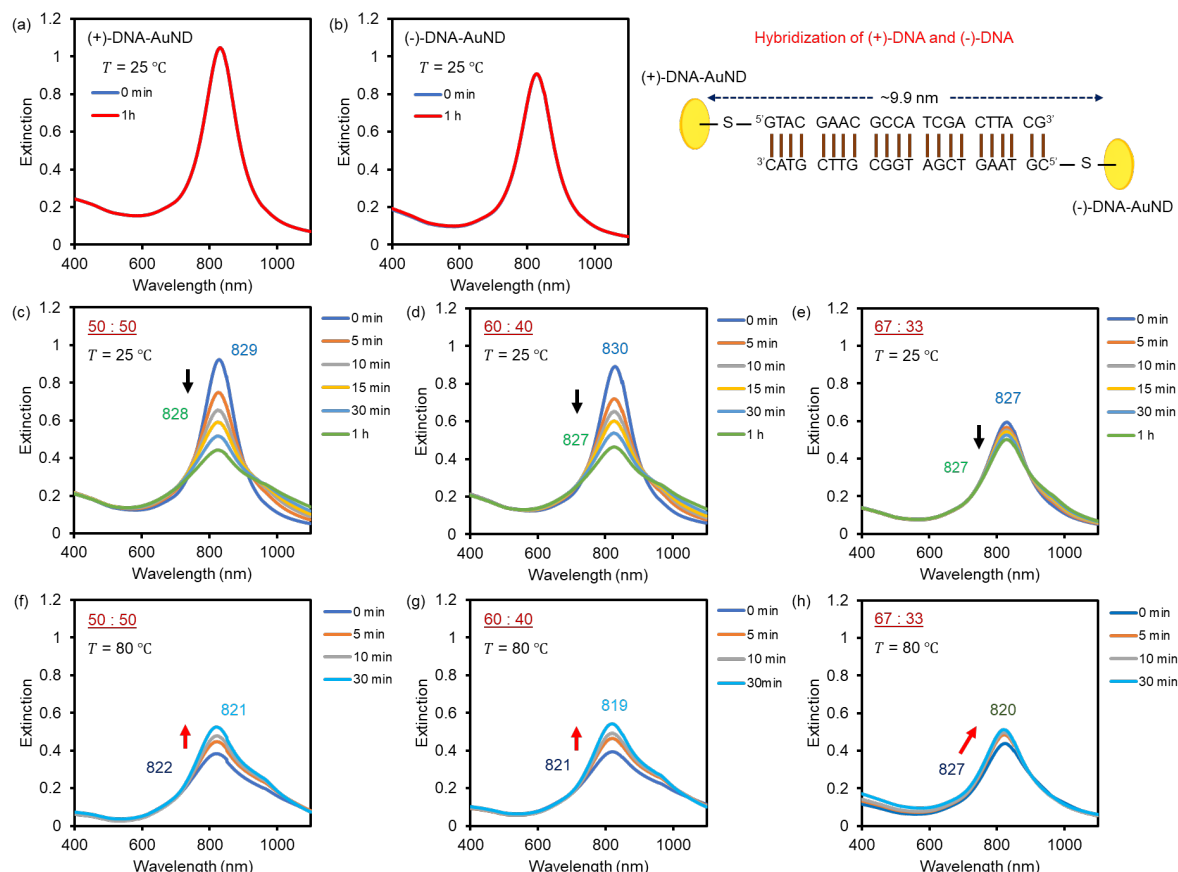


Figure 3. 14. Assembly and disassembly of 22 nt-DNA-modified AuNDs driven by hybridization and dehybridization. (a, b) Extinction spectra of (a) (+)-DNA- and (b) (-)-DNA-modified AuNDs in 1 h after dispersion at 25 °C. Inset: schematic diagram of 22 nt-DNA hybridization. (c-h) Time-dependent extinction spectra of mixture of (+)-DNA- and (-)-DNA-modified AuNDs at different ratios: (c, f) 50:50, (d, g) 60:40, and (e, h) 67:33 at (c-e) 25 °C and (f-h) 80 °C. All solutions contained 300 mM NaCl, 0.01% SDS, and 10 mM Na₂HPO₄.

11 nt-DNA-modified AuNDs:

Then, the DNA bases were reduced to 11 nucleotides to enhance the plasmonic coupling of assembled AuNDs driven by DNA hybridization. In individual state, 11 nt-(+)-DNA- and 11 nt-(-)-DNA-modified AuNDs were stably dispersed in 150 mM NaCl after 1 h incubation at 25 °C although slight decrease of extinction intensity was observed with a little blue-shifting (2-3 nm, **Figure 3. 15a,b**). Then, the (+)-DNA- and (-)-DNA-modified AuNDs were mixed at different ratios (50:50, 60:40, and 67:33) in 150 mM NaCl, 0.01% SDS, and 10 mM Na₂HPO₄ solution. The extinction spectra were monitored at 25 °C from 0 to 60 min. The plasmonic peak

of 50:50 ratio shifted from 818 nm at 0 min to 796 nm at 5 min (**Figure 3. 15c**), suggesting that hybridization quickly happened after mixing the AuNDs, forming oligomers. Subsequently, the plasmonic peak gradually reduced the intensity and shifted to the shorter wavelength (up to 751 nm in 1 h, **Figure 3. 15c**) with increasing incubation time, indicating that AuNDs were gradually assembled to polymers driven by DNA hybridization via face-to-face interaction. Compared to assembly of AuNDs modified with hydrophobic ligands in the previous result⁷, the blue-shift was smaller due to the larger interparticle gap of DNA linkers. On the other hand, imbalanced ratios (60:40 and 67:33) of (+)-DNA- and (-)-DNA-modified AuNDs showed smaller blue-shift of the plasmonic peaks and lower decrease in intensity (**Figure 3. 15c-e**). In particular, the mixture of 67:33 ratio showed a lower decrease in intensity and sharper plasmonic peak at 818 nm, followed by shifting to 808 nm after 1 h incubation with small shoulder peak around 700 nm (**Figure 3. 15e**). These results suggest that majority of AuNDs formed small oligomers such as dimer or trimer.

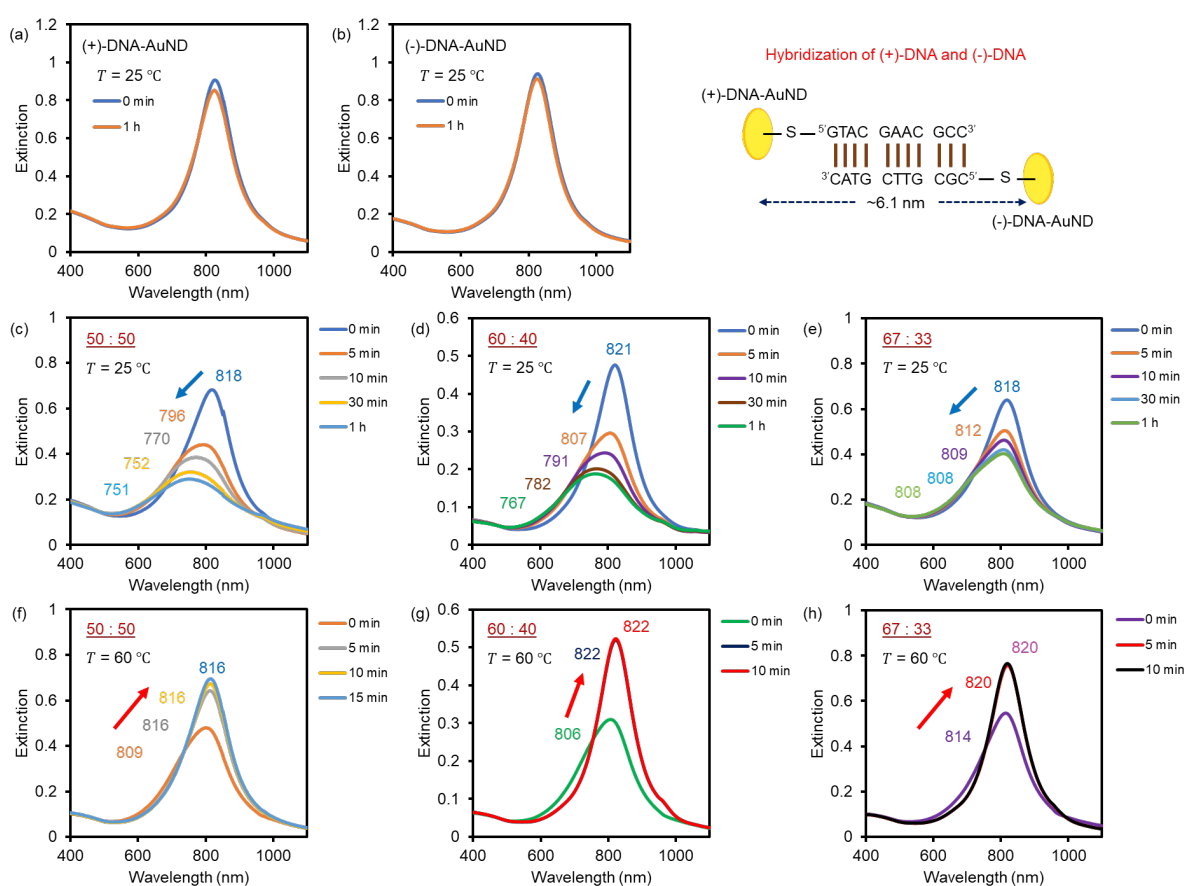


Figure 3. 15. Assembly and disassembly properties of 11 nt-DNA-modified AuNDs driven by hybridization and dehybridization. (a, b) Extinction spectra of (a) (+)-DNA- and (b) (-)-DNA-modified AuNDs in 1 h after dispersion at 25 °C. Inset: schematic diagram of 11 nt-DNA hybridization. (c-h) Time-dependent extinction spectra of mixture of (+)-DNA- and (-)-DNA-

modified AuNDs at different ratios: (c, f) 50:50, (d, g) 60:40, and (e, h) 67:33 at 25 °C and 60 °C, respectively. All solutions contained 150 mM NaCl, 0.01% SDS, and 10 mM Na₂HPO₄.

To demonstrate disassembly of AuNDs driven by dehybridization of DNA duplex, all samples were heated at 60 °C (higher than the melting temperature of free DNA in 50 mM NaCl, $T_m = 41$ °C). All extinction spectra were fully recovered in 10 min (**Figure 3. 15f-h**), revealing that dehybridization quickly occurred upon heating. Time-dependent analysis of hybridization/dehybridization (**Figure 3. 16**) revealed that 11 nt-DNA-modified AuNDs showed faster assembly/disassembly and fuller reversibility compared to 22 nt-DNA-modified AuNDs, suggesting that shorter DNA duplex shows superior thermo-responsivity.

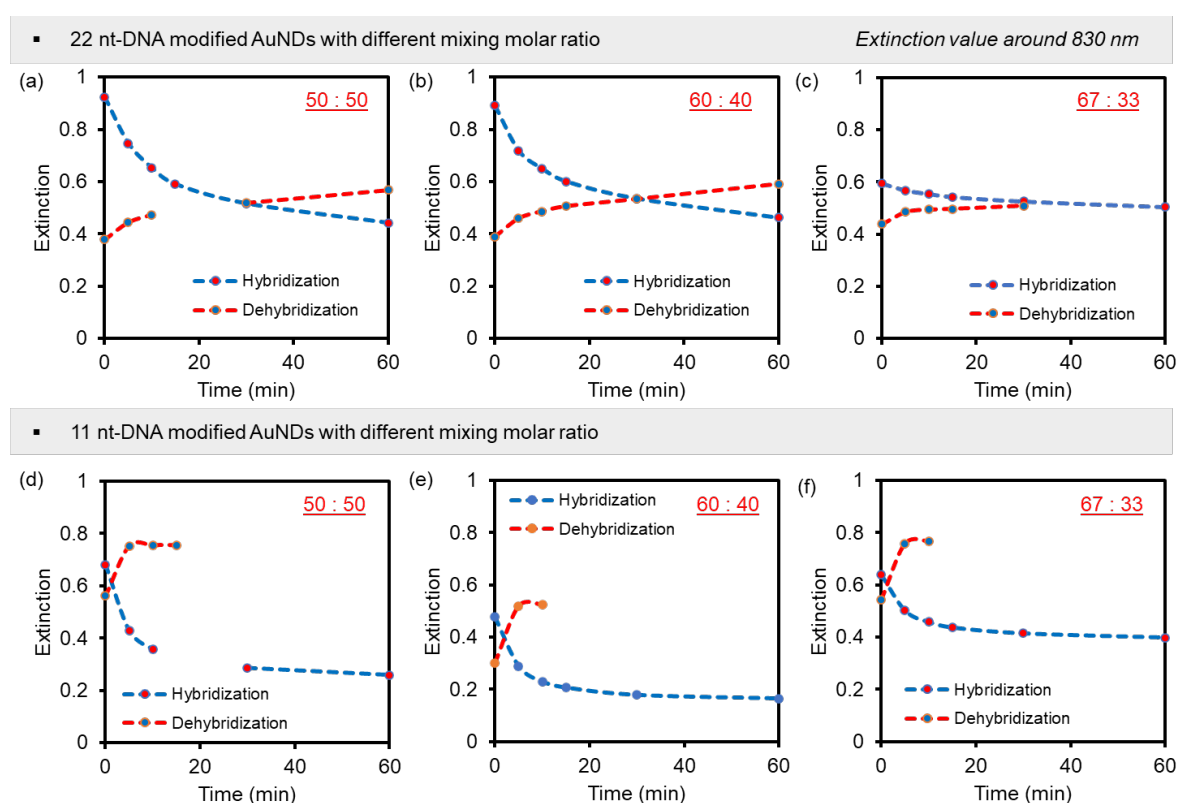


Figure 3. 16. Assembly/disassembly rates of AuNDs driven by hybridization/dehybridization. (a-c) 22 nt-DNA-modified AuNDs mixed at different ratios of (+)-DNA to (-)-DNA: (a) 50:50, (b) 60:40, and (c) 67:33. Temperatures: assembly, 25 °C; disassembly, 80 °C. (d-f) 11 nt-DNA-modified AuNDs mixed at different ratios of (+)-DNA to (-)-DNA: (d) 50:50, (e) 60:40, and (f) 67:33. Temperatures: assembly, 25 °C; disassembly, 60 °C.

6 nt-DNA-modified AuNDs:

The DNA bases were further reduced to 6 nucleotides aiming to increase plasmonic coupling between assembled AuNDs. Compared to spectra of individual 6 nt-(+)-DNA- and 6 nt-(-)-DNA-modified AuNDs (**Figure 3. 17a,b**), mixture of (+)-DNA- and (-)-DNA-modified AuNDs at 50:50 ratio did not show reduction of intensity and blue-shift of the plasmonic peak

after incubation for 1 h in 75 mM NaCl (**Figure 3. 17c**). This result suggested that hybridization did not occur. To improve the plasmonic coupling, higher NaCl concentration or longer incubation time were required to decrease the electrostatic repulsion between DNA strands. Accordingly, mixture of 50:50 ratio was brought into 100, 150, and 200 mM NaCl solution (**Figure 3. 17d-f**) to promote hybridization. Consequently, extinction intensities were slightly decreased in 150 and 200 mM NaCl solution at 25 °C in 5-30 min (**Figure 3. 17e,f**). Possibly, the reduction of intensity was not induced by hybridization, but aggregation with high NaCl concentrations. Further examination is needed to obtain a clear conclusion after improvement of DNA-modification method.

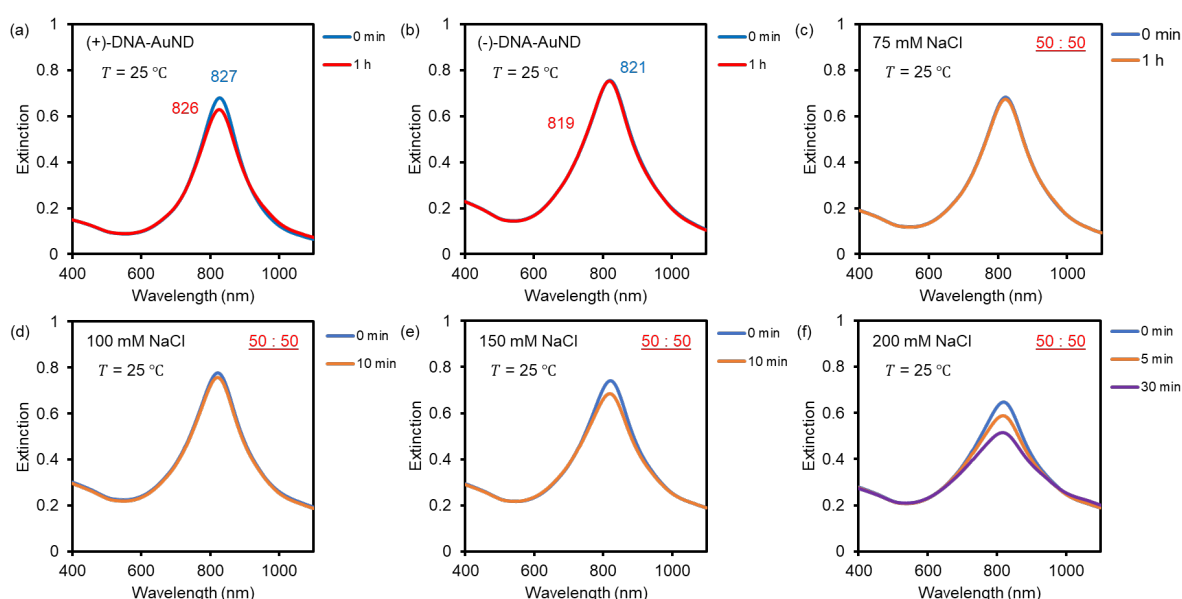


Figure 3. 17. Assembly of 6-nt DNA-modified AuNDs driven by hybridization. (a, b) Extinction spectra of (a) DNA 1- and (b) DNA 2-modified AuNDs in 75 mM NaCl after 1 h incubation at 25 °C. (c-f) Time-dependent extinction spectra of mixture of DNA 1- and DNA 2-modified AuNDs at 50:50 ratio in various NaCl concentrations: (c) 75, (d) 100, (e) 150, and (f) 200 mM NaCl at 25 °C with different incubation time. All solutions contained 0.01% SDS and 10 mM Na₂HPO₄.

3.3.3 Surface Modification of AuNDs with HS-DNA via Butanol Dehydration

Previously, AuNDs were successfully modified with DNA via salt aging, but the method was tedious and time-consuming process. Besides, NaCl for shielding the electrostatic repulsion between the phosphate groups induced irreversible aggregation of AuNDs, reducing DNA surface coverage. Therefore, instant dehydration method was used to quickly immobilize HS-DNA on AuNDs surfaces with various CTAB and AuNDs concentrations. Effect of CTAB and AuNDs concentrations on DNA grafting density was investigated. Firstly, high concentration of AuNDs (0.3 nM) was functionalized with random complementary sequences of 11 base-(+)-DNA and 11 base-(-)-DNA through Au-S bonds by replacing high concentration

of CTAB (3.2 mM) on AuNDs surfaces. After removing the unbound nucleotides, 11 base-DNA-AuND were dispersed in 0.5xTBE or phosphate buffer (pH 7.0) containing 0.01% SDS. Compared to AuNDs dispersed in 2 mM CTAB (**Figure 3. 18a**, blue line), 11 base-DNA-AuND of high CTAB and high AuNDs are slightly blue-shifting (**Figure 3. 18a**) due to the different refractive index. Lower extinction intensity of 11 base-(+)-DNA- than 11 base-(-)-DNA-AuNDs (**Figure 3. 18a**) correlated to the lower dispersibility of 11 base-(-)-DNA-AuNDs in 0.5xTBE owing to AuNDs aggregation induced by low DNA coverage on the large flat surface⁸⁴ due to the strong inter-strand repulsion between close DNA strands⁸⁵. Increasing the extinction intensity of 11 base-(+)-DNA-AuND and 11 base-(-)-DNA-AuND after changing their solutions to the mixture of phosphate buffer (pH 7.0) containing 0.01% SDS, named as buffer solution (**Figure 3. 18b**), suggested that anionic surfactant was required to improve the dispersibility. Hydrocarbon tails of SDS were adsorbed on the exposed gold surfaces⁸⁶, increasing the negative charges which improved the repulsion forces between nanoparticles.

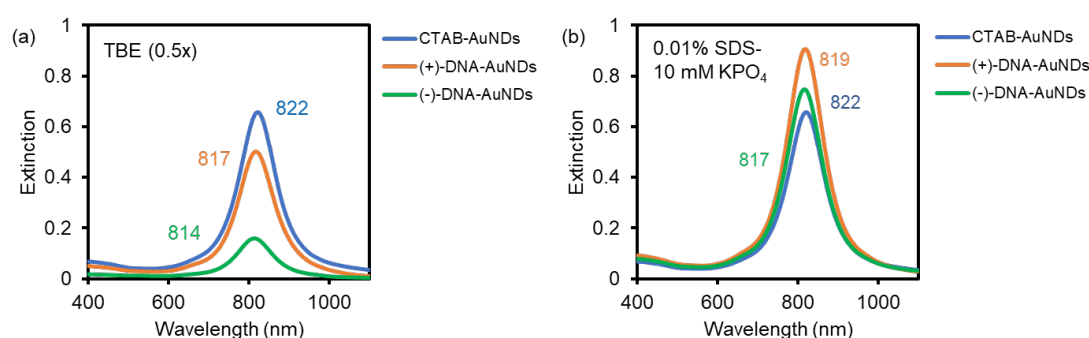


Figure 3. 18. Dispersibility of 11 base-DNA-AuND (high CTAB, high AuNDs) in different solutions. AuNDs were dispersed in (a) 0.5xTBE and (b) buffer solution contained of 0.01% SDS and 10 mM KPO₄ (pH 7.0). All spectra were measured at 25 °C. Lower extinction intensity of CTAB-stabilized AuNDs correlated to the higher dilution factor.

CTAB is required to prevent AuNDs from aggregation during modification, whereas CTAB interacts with negatively charged DNA through electrostatic interaction, which can disturb the immobilization of DNA on AuNDs surfaces⁸¹. Therefore, optimum concentration is required to maintain AuNDs stability and highly immobilize DNA on AuNDs surfaces. To obtain the optimum condition, DNA immobilization was carried out with the presence of different AuNDs and CTAB concentrations. Then, salt was gradually added to the dispersed 11 nt-DNA-modified AuNDs in buffer solution to reach 50-, 100-, 150-, 200-, 250-, and 300-mM final concentration of NaCl to investigate their stability (**Figure 3. 19a-h**) and confirm DNA immobilization. Higher reducing extinction intensity of 11 nt-(-)-DNA- than 11 nt-(+)-DNA-AuNDs for all conditions after 1 h incubation (**Figure 3. 19b,d,f,h**) indicated that lower grafting density of 11 nt-(-)-DNA on AuNDs surfaces.

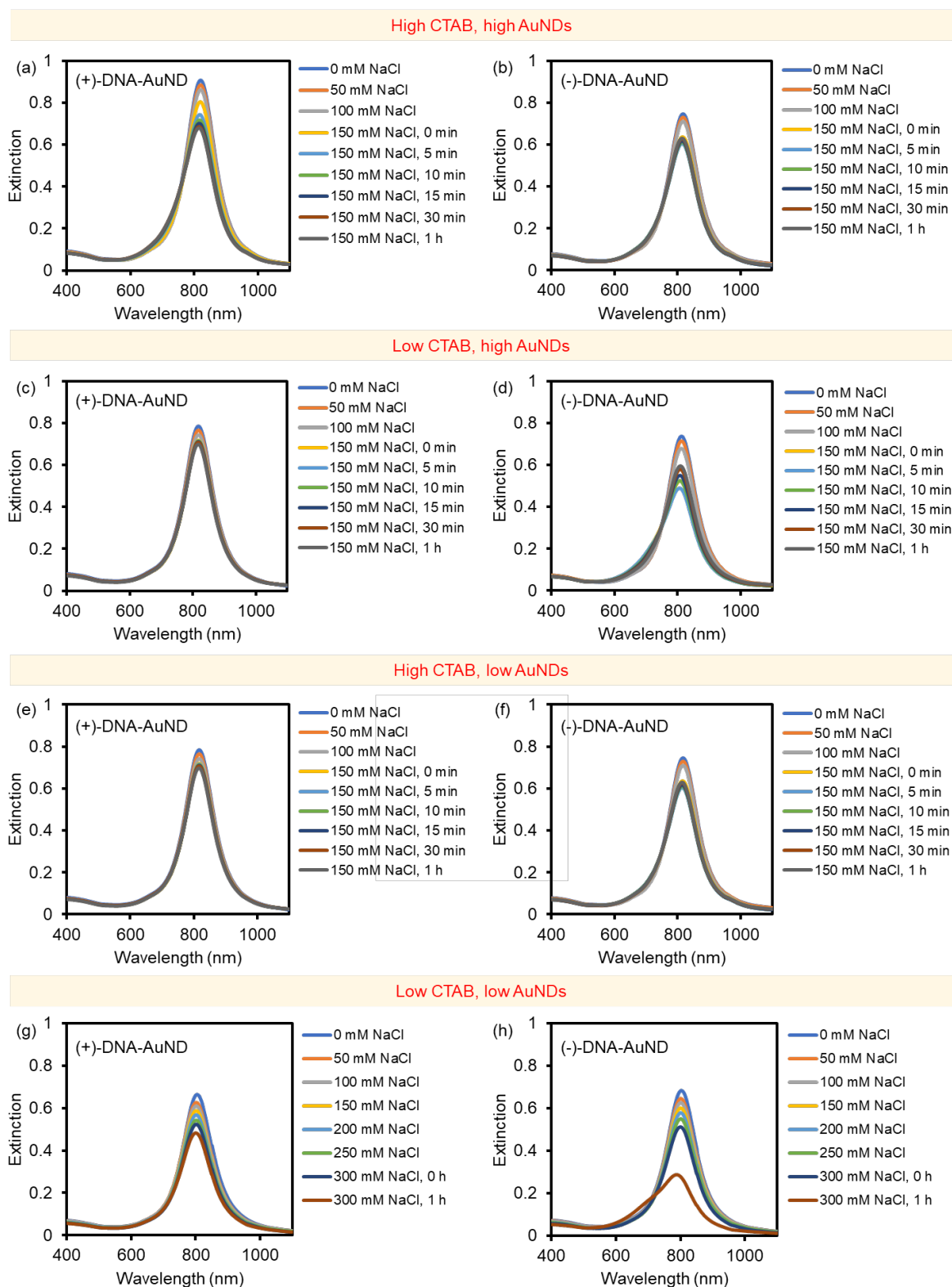


Figure 3. 19. Stability of 11 base-DNA-modified AuNDs with different CTAB and AuNDs concentrations in various NaCl concentrations. (a-b) Extinction spectra of (a) (+)-DNA- and (b) (-)-DNA-AuNDs (high CTAB, high AuNDs). (c-d) Extinction spectra of (c) (+)-DNA- and (d) (-)-DNA-AuNDs (low CTAB, high AuNDs). (e-f) Extinction spectra of (e) (+)-DNA- and (f) (-)-DNA-AuNDs (high CTAB, low AuNDs). (g-h) Extinction spectra of (g) (+)-DNA- and (h) (-)-DNA-AuNDs (low CTAB, low AuNDs). All solutions contained of 0.01% SDS-10 mM

KPO₄ (pH 7.0). All spectra were measured at 25 °C. CTAB concentrations: high (3.2 mM), low (0.32 mM). AuNDs concentrations: high (0.3 nM), low (0.06 nM).

Generally, DNA grafting density enhanced with reducing CTAB and AuNDs concentrations, corresponding to the better stability of 11 nt-DNA-AuNDs in higher salt concentration (**Figure 3. 19g,h**) due to the stronger repulsion forces between negative charges of phosphate groups. Therefore, AuNDs would be modified with low CTAB (0.32 mM) and low AuNDs (0.06 nM) concentrations for the next experiments.

Sequences also affected grafting density on AuNDs surfaces due to the different binding affinity between Au atoms and DNA bases. Stability of AuNDs modified with 100% dA11 and 100% dT11 in 150 mM NaCl (**Figure 3. 20a,d**) exhibited large discrepancy due to the contrast DNA coverage. Higher binding affinity of dA induced strong interaction between oligonucleotides and gold surface, reducing surface coverage because absorbed DNA on AuNDs surfaces inhibited other oligonucleotides to attach⁶⁵. Then, AuND to HS-DNA ratio was reduced to 1:8,335 (50% modification, **Figure 3. 20b,e**) and 1:1,667 (10% modification, **Figure 3. 20c,f**) to obtain similar grafting density between dA11- and dT11-AuNDs. Similar reducing extinction intensity of 50% dA11- and 10% dT11-AuNDs in 150 mM NaCl after 1 h incubation (**Figure 3. 20b,f**) indicated a balance DNA coverage of 50% dA11 and 10% dT11 on AuNDs surfaces. However, it proved challenging to produce similar grafting density of repetitive sequences on AuNDs surfaces. Subsequently, AuNDs would be modified with random sequences for further experiments.

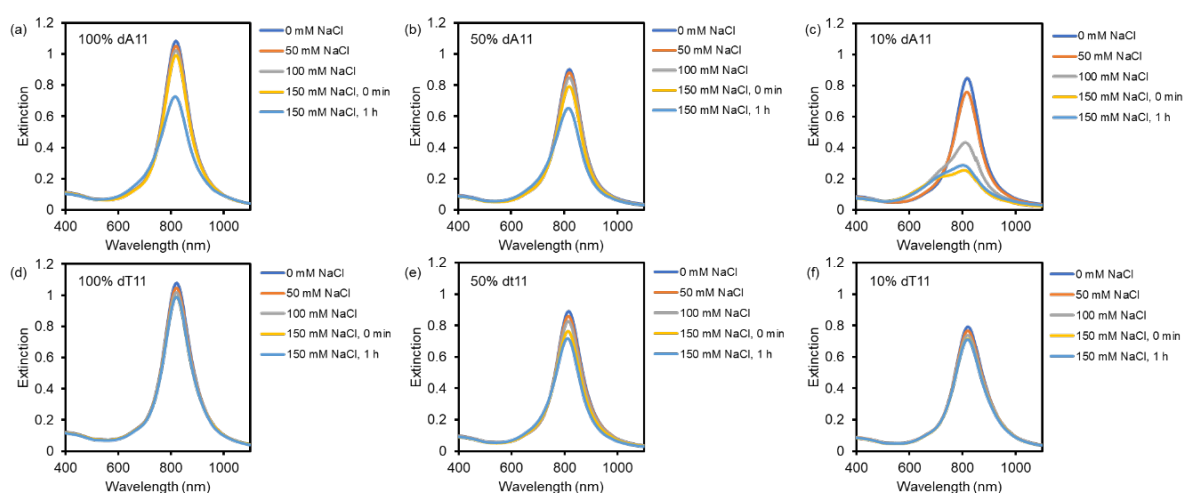


Figure 3. 20. Stability of dA11- or dT11-modified AuNDs with different ratios of AuND to HS-DNA. (a-c) Extinction spectra of dA11-AuNDs: (a) 100%, (b) 50%, and (c) 10%. (d-f) Extinction spectra of dT11-AuNDs: (d) 100%, (e) 50%, and (f) 10%. The ratio of AuND to HS-DNA was 1:16,670 (100%); 1:8,335 (50%); and 1:1,667 (10%). All solutions contained of 0.01% SDS-10 mM KPO₄ (pH 7.0). All spectra were measured at 25 °C.

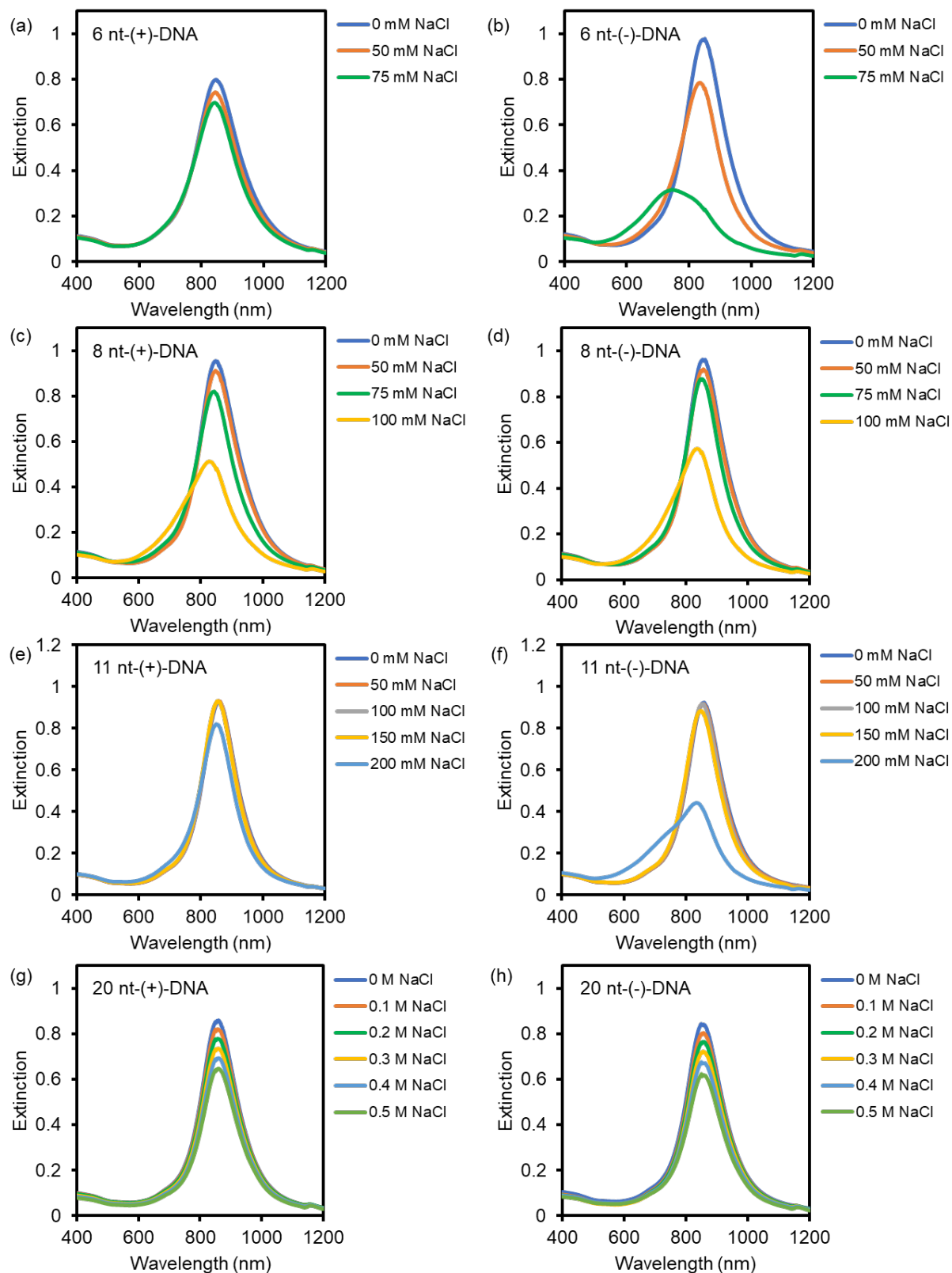


Figure 3. 21. Stability of AuNDs modified with different DNA lengths. (a-b) Extinction spectra of 6 nt-DNA-AuNDs: (a) 6 nt-(+)-DNA and (b) 6 nt-(-)-DNA . (c-d) Extinction spectra of 8 nt-DNA-AuNDs: (c) 8 nt-(+)-DNA and (d) 8 nt-(-)-DNA. (e-f) Extinction spectra of 11 nt-DNA-AuNDs: (e) 11 nt-(+)-DNA and (f) 11 nt-(-)-DNA. (g-h) Extinction spectra of 20 nt-DNA-AuNDs: (g) 20 nt-(+)-DNA and (h) 20 nt-(-)-DNA. Ratio of AuND to HS-DNA was 1:16,670.

All solutions contained of 0.01% SDS-10 mM KPO₄ (pH 7.0). All spectra were measured at 25 °C.

Stability of DNA-modified AuNDs in salt solution correlated to the DNA lengths, corresponding to the negative charges of the phosphate groups. Length of DNA ligands on AuND surfaces also affected the interparticle interactions. Therefore, 6 nt- and 8 nt-DNA-AuND (**Figure 3. 21a-d**) exhibited lower stability than 11 nt-DNA-AuND (**Figure 3. 21e,f**) due to the lower repulsion forces of the shorter DNA. Stability in salt solution was significantly improved when AuNDs modified with 20 nt-DNA (**Figure 3. 21g,h**) due to the higher repulsion forces of longer DNA. Besides, longer DNA reduced length-dependent attraction forces due to the larger particle distance.

Hydrodynamic diameters also support the dispersibility and stability of DNA-modified AuNDs in salt solution. Even though all DNA-modified AuNDs showed the same dispersibility in phosphate buffer (**Figure 3. 22a-d**), the stability in NaCl was length-dependent (**Figure 3. 22e-h**) due to the different repulsion forces between strands on AuNDs surfaces. Larger hydrodynamic diameter of 6 nt-(+)-DNA and 6 nt-(-)-DNA after 3.5 h incubation in 75 mM NaCl (**Figure 3. 22e**) than their dispersed conditions without NaCl (**Figure 3. 22a**) correlated to the salt induced aggregation due to the low negative charges of short DNA. Longer DNA showed better AuNDs stability in higher NaCl concentrations (**Figure 3. 22f-h**).

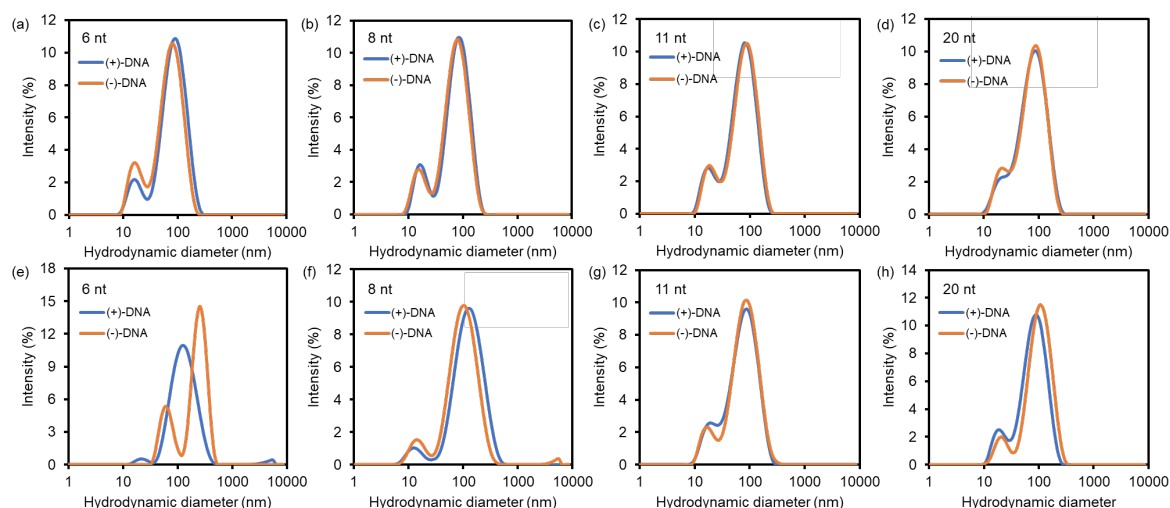


Figure 3. 22. Dispersibility and stability of AuNDs modified with different DNA lengths in salt solutions. (a-d) Hydrodynamic diameter of DNA-modified AuNDs dispersed in 0.01% SDS-10 mM KPO₄ (pH 7.0): (a) 6 nt-DNA-AuNDs, (b) 8 nt-DNA-AuNDs, (c) 11 nt-DNA-AuNDs, and (d) 20 nt-DNA-AuNDs. (e-h) Hydrodynamic diameter of DNA-modified AuNDs in various NaCl concentrations after 3.5 h incubation: (e) 6 nt-DNA-AuNDs in 75 mM NaCl, (f) 8 nt-DNA-AuNDs in 100 mM NaCl, (g) 11 nt-DNA-AuNDs in 150 mM NaCl, and (h) 20 nt-DNA-AuNDs in 0.5 M NaCl. All solutions contained of 0.01% SDS-10 mM KPO₄ (pH 7.0). All measurements were conducted at 25 °C.

3.3.4 Calculation of Immobilized DNA on AuNDs Surface

Immobilized DNA was calculated for AuNDs modified via butanol dehydration method. First, DNA grafting density was obtained by measuring the relative fluorescence intensity of hybridized 15 base-(+)-DNA-Cy3 with 11 base(-)-DNA-AuND on AuNDs surfaces. The measured fluorescence intensity of Cy3 dye is 112.6 RFU. By comparing this fluorescence intensity with the calibration curve (**Figure 3. 23**), the concentration of 11 base(-)-DNA in 2 μL of sample solution is 0.093 μM . Number of DNA in each AuND was obtained by dividing the concentration of DNA to AuND concentration (0.964 μM). Based on this method, number of DNA attached to AuND surface is 96 strands. AuND is same as cylinder with 10 and 90 nm of height and diameter, respectively. By applying the formula of surface area for cylinder, $A = 2\pi r(r + t)$, the area of AuND is 15,543 nm^2 . The DNA grafting density was calculated from dividing the number of DNA by AuND surface area. Consequently, DNA grafting density on the surface was 0.006 strands/ nm^2 , much lower than the established report⁸⁷. Possibly, this method was incorrect due to the incomplete hybridization between 11-base(-)-DNA and 15 base-(+)-DNA-Cy3. Therefore, more reliable method was applied to determine the number of DNA immobilization.

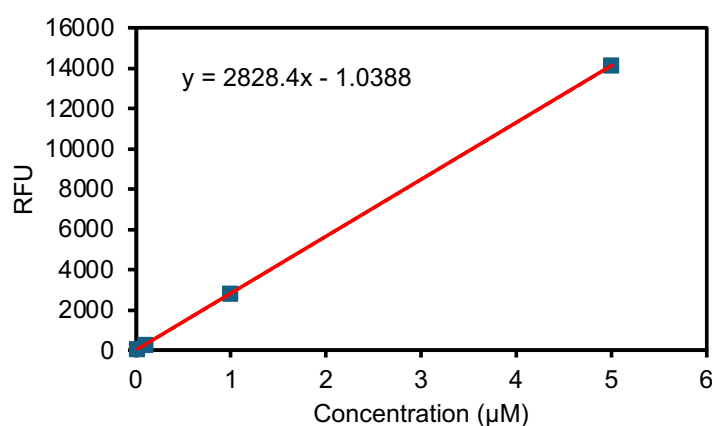


Figure 3. 23. Relative fluorescence intensity of 15 base-(+)-DNA-Cy3 as a function of concentration in the buffer solution contained of 150 mM NaCl and 25 mM DDT.

Second, AuNDs were modified with 20 base(-)-DNA-Cy3 via butanol dehydration followed with displacement of 20 base(-)-DNA-Cy3 with DTT. The fluorescence intensity of 20 base(-)-DNA-Cy3 was 217 RFU. By converting this value with corresponding calibration curve (**Figure 3. 24**), 20 base(-)-DNA-Cy3 concentration was to be 231.6 nM. Consequently, number of DNA on each AuND was 203 strands. The DNA grafting density on AuNDs surface was 0.013 strands/ nm^2 , four times lower than the value reported in the previous report⁸⁷. Low DNA grafting density correlated to the large flat surface with low curvature which reduced the

distance between DNA strands, enhancing the interstrand repulsion forces^{88,89}.

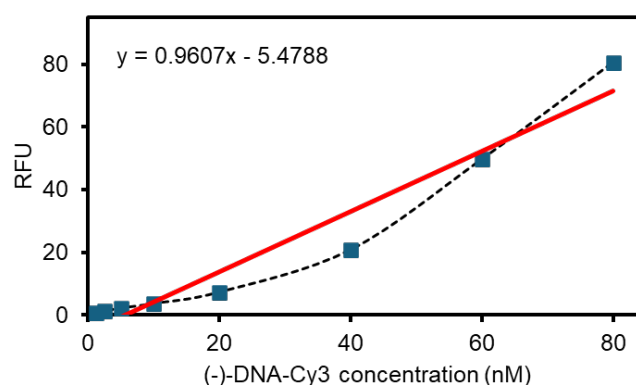


Figure 3. 24. Relative fluorescence intensity of 20 base-(-)-DNA-Cy3 as a function of concentration in the buffer solution contained of 25 mM DDT.

3.4. Conclusions

In this chapter, AuNDs were modified with DNA ligands using three different methods—(i) click reaction between DBCO-DNA and azide-functionalized AuNDs, (ii) salt aging, and (iii) butanol dehydration—to obtain DNA-immobilized AuNDs with high surface coverage.

AuNDs have been successfully modified with **OH**, **COOH**, and **N3** ligands to conjugate with DBCO-DNA through click reaction. However, low DNA density (5% or less) was insufficient to promote DNA hybridization-driven AuNDs assembly. Besides, flexible linker with 32 base pairings possibly reduced the hybridization efficiency and increased the base pair slipping effect, inhibiting thermally stable assembly of AuNDs. regarding the plasmonic properties, assembly with large interparticle gap distance (>25 nm) induced weak plasmonic coupling, leading to insignificant plasmonic shift which inhibited to clearly confirm the assembly formation.

Therefore, AuNDs were directly modified with short DNA ligands (<10 nm) via ligand exchange reaction with salt aging method to enhance DNA coverage on AuNDs surfaces. Assembly and disassembly properties of AuNDs were affected by DNA length, correlating to the interparticle gap distance and stability of DNA duplex after hybridization. Importantly, assembled AuNDs can be controlled by adjusting the mixing molar ratios of (+)-DNA-AuND to (-)-DNA-AuND which opens the possibility to alter the assembly sizes for tunable plasmonic coupling. However, salt aging method was tedious and time-consuming process to immobilize DNA on AuND surfaces. Salt for shielding the electrostatic repulsion between the phosphate groups also induced irreversible aggregation, reducing the stability of AuNDs and DNA surface coverage.

To overcome these issues, an instant immobilization method based on butanol dehydration was employed to rapidly attach HS-DNA onto AuND surfaces without salt-induced aggregation. Various HS-DNA ligands with different lengths and sequences (6, 8, 11, and 20 bases) were successfully immobilized via Au-S bond under dehydration state. The stability of DNA-AuND in different salt concentrations was length-dependent because longer DNA ligand induced stronger repulsion forces due to the higher negative charges of the phosphate groups. The grafting density of 20 base DNA was 0.013 strands/nm², four times lower than the value in the previous report. Considering the successful immobilization of DNA on AuNDs, the butanol dehydration method was adopted as a simple and effective strategy for preparing DNA-modified AuNDs to control assembly, as described in the next chapter.

Chapter 4

Controlled Assembly of AuNDs via DNA Hybridization

4.1. Introduction

In **Chapter 3**, various lengths and sequences of HS-ssDNA (6, 8, 11, and 20 bases) were successfully immobilized on gold nanodiscs (AuNDs) surfaces via Au-S bonds using butanol dehydration method to optimize DNA immobilization. In this chapter, assembly of DNA-modified AuNDs was driven by hybridization of complementary short DNA ligands.

Shapes and DNA lengths greatly affect the assembly structures of gold nanoparticles³⁶. Gold nanospheres (AuNPs) tend to assemble into large and uncontrolled sizes of three-dimensional (3D) aggregates^{14,71}. Meanwhile, gold nanorods (AuNRs) require flexible chains⁷⁵ and specific binding^{27,28} of DNA at the ends or sides to arrange one-dimensional (1D) or two-dimensional (2D) assemblies²⁹. Uncontrolled attachment of DNA on AuNRs surface leads random assemblies^{19,74,87}. Although there have been reports of DNA-directed assembly, it remains difficulties to obtain 1D arrangement and finite assembly without using a template^{14,68} or specific DNA configuration¹⁶ which has limited number of studies and applications due to the high cost and intricate process. Therefore, a simple approach is required for the fabrication of 1D nanostructures with controlled assembly. In addition, AuNPs with high curvature inhibited DNA hybridization from a perfect match because of slipping, reducing the stability of assembly structure⁹⁰. On the other hand, gold nanoplates like gold nanotriangles (AuNTs) and AuNDs preferentially assemble into face-to-face direction owing to the large flat surface^{7,9}. Besides, face-to-face interaction of the low curvature helps nanoparticle-bound DNA to access their complementary on the other surfaces more efficiently due to the large contact area which will increase the number of DNA hybridization. Thus, the use of DNA and gold nanoplates enables to precisely control the assembly structure.

Previously, shape-directed assembly of AuNTs and AuNDs exhibited 1D structures that could not be achieved through the assembly of AuNPs^{13,17,24,25,77}. Face-to-face assembly concentrated DNA that enhanced the association rate. Additionally, flat surface nanoparticles allowed DNA to release the conformational stress upon duplex formation which increased the thermodynamic stability²⁵. However, long DNA (>10 nm) with enough rigidity and flexibility was required to rearrange 1D assembly^{13,17,24,91}. Consequently, the large interparticle distance reduced the strength of the plasmonic coupling which inhibited a clear observation of plasmonic shifting^{9,92} when gold nanoplates assembled into linear chain. Long DNA also decreased the directionality of interparticle interaction due to the large free volume for DNA hybridization³⁶. In addition, the use of spacer not only increased the interparticle distance but also enhanced DNA hybridization via cooperative and multivalent effects,

significantly increased the disassembly temperature of nanoparticle compared to the melting temperature of free DNA^{9,78}. Besides, a single type of DNA-modified gold nanoplates containing self-complimentary could not control the interparticle distance and finite-size assembly^{9,13,25}. Even though plasmonic properties of assembled AuNDs driven by DNA hybridization has been studied in previous report, their assembly sizes were not controlled experimentally^{17,24}. Moreover, reversible self-aggregation was unable to completely exclude in high salt concentration because large nanoparticles easily allowed hybridization even though only one base pair which included in the reducing extinction intensity of assembled nanoparticles⁹³. Therefore, it is necessary to investigate the effect of reversible self-aggregation on the plasmonic shift. Two types of short DNA-modified AuNDs enables to clearly confirm the stability and plasmonic properties.

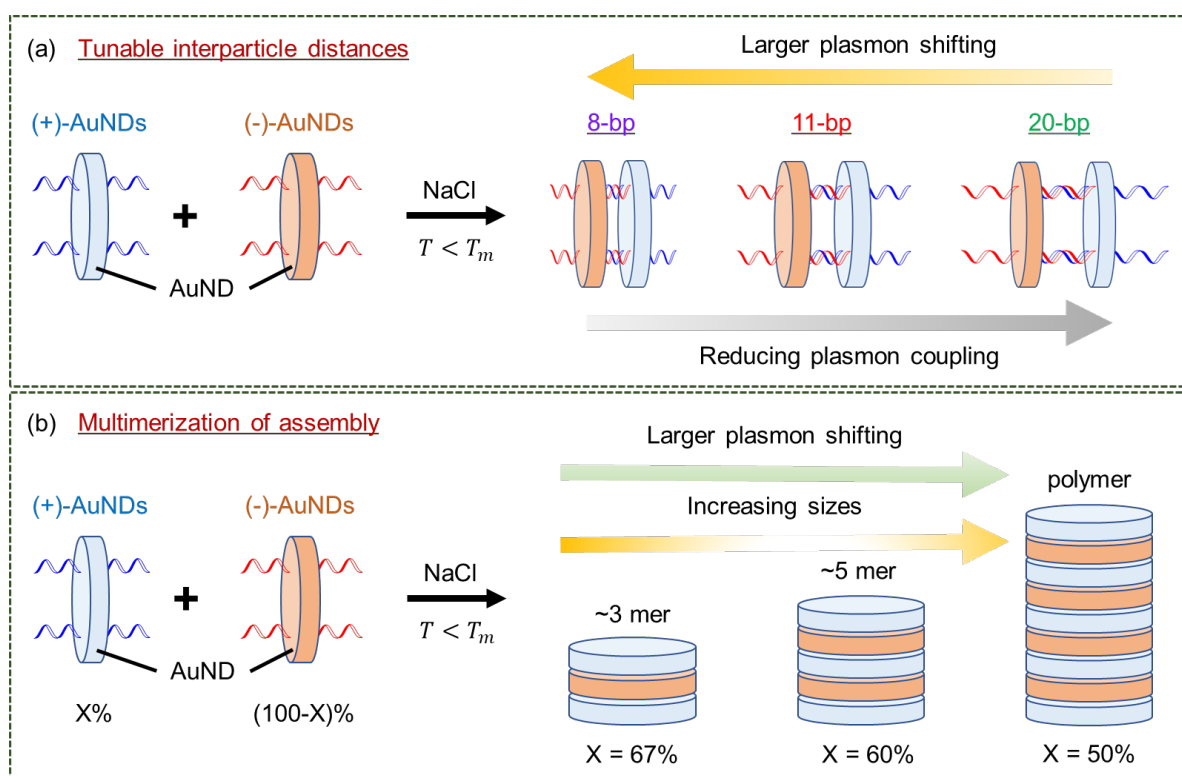


Figure 4. 1. Schematic illustration of controlled AuNDs assembly by DNA hybridization to tune the strength of plasmonic coupling: (a) controlled interparticle distances by tuning DNA lengths and (b) controlled assembly sizes by adjusting the mixing molar ratio of (–)-DNA-AuND to (+)-DNA-AuND.

Here, the assembly of AuNDs was demonstrated, driven by the hybridization of complementary short DNA ligands without the use of linker strands. As a result, the AuNDs assembled into one-dimensional (1D) cylindrical structures through face-to-face interactions. The assembly and disassembly processes were reversible upon repeated heating and cooling cycles. The strength of plasmonic coupling was tuned by adjusting the interparticle gap

distance using various lengths of DNA strands. (**Figure 4. 1a**). Owing to the DNA-directed assembly, the size of the assembled structures could be modulated by varying the salt concentrations. In addition, the degree of multimerization was controlled by changing the mixing molar ratio of AuNDs modified with (+)-DNA and (-)-DNA, following the principles of condensation polymerization (**Figure 4. 1b**). Furthermore, size-based fractionation of the multimers was investigated by gel-electrophoresis and centrifugal separation. The assembled AuNDs were also cross-linked using (i) a psoralen-based crosslinker for DNA duplexes and (ii) dithiol-PEG (SH-PEG-SH) via Au-S bonds, resulting in static assembly structures. Further treatment of these static assemblies has the potential to isolate discrete AuND oligomers exhibiting homogeneous and well-defined plasmonic properties.

4.2. Materials and Experiments

4.2.1. Materials

Dimethyl sulfoxide (DMSO), trisodium citrate dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$), and tannic acid ($C_{14}H_{10}O_9$) were purchased from Wako Pure Chemical Industries Ltd. (Japan). Crosslinker molecule, 8-Methoxypsoralen (8-MOP) was purchased from Tokyo Chemical Industry. SH-PEG-SH ($M_w = 1500$) was purchased from Sigma Aldrich. Dithiothreitol (DDT) was obtained from Merck (Sigma Aldrich Chemie GmbH, München, Germany). Other materials were already described in previous chapters.

4.2.2. Instruments for Characterizations

For cryo-TEM measurements, the dispersion of AuND assemblies was deposited onto a TEM grid, and excess solvent was removed using a filter paper. The sample was then rapidly frozen in liquid nitrogen (N_2). Cryo-TEM measurements were performed using a CRYO ARM™ 300II (JEOL, Japan) operating at an acceleration voltage of 300 kV. TEM images were acquired at 5° intervals over a range of -60° to +60°, and 3D reconstruction from the tilt series was performed using the IMOD software package. For photo reaction, samples were irradiated with UV 365 nm using 4500 mW/cm² LC8 Lightningcure source (Hamamatsu Photonics). Dispersed and assembly structures were obtained using an Ultimate Field Emission Scanning Electron SU-8230 (Hitachi High-Tech, Tokyo, Japan). To investigate the assembly driven by DNA hybridization, samples were prepared by dropping 2 µL solution of concentrated samples on indium tin oxide (ITO) substrate which had been hydrophilized using UV/ozone treatment (UV253MINI) for 30 min irradiation. After 2 min deposition, the droplet was removed by KimWipe to prevent drying effect. SEM measurements were conducted under 10 µA emission current and 5 kV accelerating voltage, respectively. Other instruments are same as apparatus

used for experiments in **Chapter 2** and **Chapter 3**.

4.2.3. AuNDs Assembly Driven by DNA Hybridization

AuNDs were functionalized with complementary short DNA ligands (positive-sense DNA, (+)-DNA; negative-sense DNA, (-)-DNA, **Table 4. 1**) and designated as (+)-DNA-AuND and (-)-DNA-AuND, respectively. To induce AuND assembly via hybridization, (+)-DNA-AuND and (-)-DNA-AuND were mixed at the same (50:50) or various mixing molar ratios (60:40, 67:33, 75:25, and 90:10) in a buffer solution containing of 0.01% SDS and 10 mM phosphate (pH 7.0). Solution was made to have an optical extinction intensity of 0.92 in the total volume of 700 μ L-2.4 mL to provide sufficient sample for characterization. Low concentration of DNA-modified AuNDs ensured to eliminate the entropic effects which might cause the formation of 1D assemblies due to the spontaneous transition from a disordered isotropic phase to the ordered nematic phase^{13,62,63}. Then, the mixture was heated at 80 °C for 15 min. After adding the specific volumes of 2 M NaCl, each solution was cooled to 25 °C at a rate of 1 °C per minute, with isothermal holds of 15 minutes every 5 °C decrease in temperature.

Table 4. 1. HS-DNA sequences used to modify AuNDs and AuNPs

		DNA sequences	DNA Length (nm)	T_m (°C)	M_w
20 nt	R20-(+)-DNA	[ThiolC6] 5'ACGAACGCCATGGGACTTAC3'	8.0	66	6439
	R20-(-)-DNA	[ThiolC6] 5'GTAAGTCCCATGGCGTTTCGT3'			6452
12 nt	Palindrome	[ThiolC6] 5'TTTCTCGAGAAA3'	5.3	32	3973
11 nt	R11-(+)-DNA	[ThiolC6] 5'ACGAACGCCAT3'	4.9	34	3638.6
	R11-(-)-DNA	[ThiolC6] 5'ATGGCGTTTCGT3'			3691.7
11 nt	dA11	[ThiolC6] 5'AAAAAAAAAAAA3'	4.9	22	3580
	dT11	[ThiolC6] 5'TTTTTTTTTTTT3'			3480
8 nt	R8-(+)-DNA	[ThiolC6] 5'TTTGTGGT3'	3.9	22	2775
	R8-(-)-DNA	[ThiolC6] 5'ACCACAAA3'			2700

5.

4.2.4. Numerical Simulation of Extinction Spectra and Near Field Distribution of AuNDs

Finite-difference time-domain (FDTD) simulations were performed with the commercial software (Lumerical solutions) to calculate the extinction spectrum of AuNDs. In the simulation, a disc model with diameter of 90 nm and thickness of 14 nm was used to represent AuND. For the simulation of AuND oligomers, the AuNDs were stacked in a face-to-face geometry with an interparticle gap distance between AuNDs of 5.7 nm. Because AuND is a kind of central symmetric structure, a linear polarized plane wave of normal incidence to

the surface of AuND was chosen as the light source. The surrounding medium of AuND was treated as water. Johnson and Christy's experimental results were used to determine the dielectric function of the gold⁹⁴. A uniform mesh in size of 0.5 nm was set in the simulation. For the simulation of AuND dimers, the interparticle gap distance of AuNDs ranged from 2 to 20 nm.

4.2.5. Synthesis of AuNPs

Colloidal citrate stabilized AuNPs were synthesized using trisodium citrate dihydrate ($\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2\text{H}_2\text{O}$) and tannic acid ($\text{C}_{14}\text{H}_{10}\text{O}_9$) as reducing agents based on previous report.⁹⁵ Specifically, 4 mL of 1% (w/v) trisodium citrate dihydrate and 57 μL of 1% (w/v) tannic acid were added to 16 mL of milli-Q. Then, the mixed solution was heated in water bath at 60 °C. In another place, 294 μL of 0.1 M hydrogen tetrachloroaurate (III) tetrahydrate ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) was added to 80 mL of milli-Q and heated to 60 °C in oil bath. Then, the reducing solution was rapidly added by stirring to hydrogen tetrachloroaurate (III) tetrahydrate solution with increasing temperature up to 120 °C. After the colour of the mixed solution changed to red, the solution was heated at that temperature for 10 minutes. Finally, the as-produced colloidal AuNPs was cooled to room temperature and stored at 4 °C for following experiments.

4.2.6. Modifying AuNPs with HS-DNA via Ligand Exchange Reaction

AuNPs were modified with 11-nt-(+)-DNA and 11-nt-(-)-DNA (Table 4. 1). Before surface modification, 200 μL of 2 nM citrate-capped AuNPs was purified by centrifugation (20,000x g, 20 min, 25 °C). After removing 168 μL of supernatant, the purified 11-nt-DNA was added in the presence of 1 mM TCEP (the ratio of AuNP to thiol-DNA was 1:500). Total volume of the mixed solution was 100 μL . After quick vortex, 900 μL of 1-butanol was immediately added followed by vigorous stirring for few seconds. Then, 200 μL of 0.5xTBE was quickly added to rehydrate DNA-modified AuNPs. Next, unreacted DNA was removed by 3 times centrifugation (20,000x g, 20 min, 25 °C). The precipitated AuNPs were dispersed in 0.5xTBE and stored at 4 °C for further experiments.

4.2.7. Assembly of AuNPs via DNA Hybridization

To proceed AuNPs assembly via hybridization, (+)-DNA-AuNP and (-)-DNA-AuNP were mixed at the same mixing molar ratio (50:50) in 0.5xTBE. Then, a specific volume of 2 M NaCl was subsequently added to the mixed solution to obtain 0.075, 0.1, 0.15, 0.2, 0.6, 0.7, 0.8, 0.9, or 1 M NaCl. After mixing, solution was heated at 80 °C for 15 min. Then, the colloidal solution was gradually cooled to 25 °C followed with 24 h incubation in each salt concentration to promote the assembly.

4.2.8. Reversible Assembly and Disassembly of DNA-modified AuNDs

AuNDs assembly occurred through the hybridization of complementary DNA. Firstly, DNA modified AuNDs and complementary modified AuNDs were mixed with 1:1 ratio in a glass cuvette. Assembly was promoted by gradually cooling down from higher to lower temperature than T_m . The mixed solutions were heated to temperature higher than T_m of free DNA to release all DNA connections. Then, hybridization was promoted by slowly cooling the temperature lower than T_m while simultaneously recording the extinction spectra for every 5 °C. A heating rate of 1 °C/min and a waiting time of 15 min were applied for the measurements. Next, gradual increase of temperature over T_m dissociated assembled AuNDs, confirming that assembly driven by hybridization, not salt-induced aggregation. Cuvette was covered with parafilm to reduce evaporation upon heating and cooling. Hydrodynamic diameters of AuNDs at lower and higher than T_m were measured to clarify the assembly/disassembly driven by hybridization/dehybridization.

4.2.9. Determination of Assembly and Disassembly Temperatures

Assembly (T_a) and disassembly (T_d) temperatures were determined by following previous method²⁴, which defined T_a and T_d as freezing (T_f) and melting (T_m) temperatures. To simplify calculate the baseline subtraction method, I assumed that hybridization of 8 bp-DNA was saturated at temperature lower than 25 °C. This approach did not affect T_f and T_m of oligonucleotides. Besides, dehybridization of 20 bp-DNA continued at temperature higher than 80 °C but the limitation of instrument inhibited to fully observe AuNDs dissociation. I believe that 20 base pairings DNA fully dehybridize at 100 °C, promoting all AuNDs dispersed in solution.

4.2.10. Determination of Interparticle Gap Distance of Assembled AuNDs

Interparticle gap distance was determined by measuring a distance between two particles of assembled AuNDs which was mixed at (33:67) mixing molar ratio. Assembly of 11-bp and 20-bp were promoted in 150 mM and 400 mM NaCl, respectively. For 11-bp DNA-AuNDs, the averaged interparticle distance was obtained by measuring 76 interparticle distances in three different areas with various tilting conditions by converting 1 pixel to 0.203 nm. The averaged interparticle distance of 20-bp DNA-AuNDs was obtained by measuring 72 interparticle distances in nine different areas with various tilting conditions. For 8-bp DNA-AuNDs, the averaged interparticle distance was unidentified due to aggregation.

4.2.11. Suppression of AuND Assembly with Blocking DNA Strands

Free complementary DNA ((-)-DNA or (+)-DNA) firstly hybridizes with (+)-DNA-AuND or (-)-DNA-AuND to block AuNDs assembly. Specifically, 5 nmol of free complementary DNA ((-)-DNA or (+)-DNA) was added into 350 μ L of 0.1 nM (+)-DNA-AuND or (-)-DNA-AuND in the buffer solution containing 150 mM NaCl. After heating at 80 °C for 15 min, the mixed solution was maintained at 25 °C for 1 h to proceed DNA hybridization. Then, 350 μ L of 0.1 nM complementary DNA-AuND was added into the colloidal solution under maintaining NaCl concentration. The extinction spectra and hydrodynamic sizes were measured after 12-hour incubation at 25 °C.

4.2.12. Multimerization of Assembled AuNDs

Assembly sizes could be controlled by two methods: (i) changing the mixing molar ratios of DNA-modified AuNDs or (ii) changing salt concentrations. Firstly, assembly was controlled by adjusting the mixing ratio between 11 base-(+)-DNA- and 11 base-(-)-DNA-AuNDs (50:50; 60:40; and 67:33) with the presence of 150 mM NaCl. Secondly, different assembly sizes were obtained by adding different NaCl concentrations (50, 75, 100, 150, or 200 mM NaCl) into the same molar mixing ratio of 11 base-(+)-DNA- and 11 base-(-)-DNA-AuNDs. Then, solution was brought to 80 °C followed by gradually cooling down to 25 °C while simultaneously recording the extinction spectra for every 5 °C. Additionally, various NaCl concentrations (150, 175, 200, 300, and 400 mM NaCl) were applied to the equimolar mixing ratio of 20 base-(+)-DNA- and 20 base-(-)-DNA-AuNDs to control the assembly sizes. Then, solution was heated followed by gradually cooling down as same as 11 bp-DNA-AuNDs.

4.2.13. Separation of Assembled AuNDs by Gel-electrophoresis

The assembled AuNDs mixed at 50:50 mixing ratio in 200 mM NaCl, (+)-DNA-AuND, and (-)-DNA-AuND were loaded in a 1 wt% agarose gel and run in 0.5xTAE buffer for 1 h. AuNDs moved toward the positive electrode of the electrophoresis instrument under the driving force of electric field. To maintain DNA hybridization, gel was prepared with 0.5xTAE buffer containing 150 mM NaCl and electrophoresis was run in 0.5xTAE buffer with 150 mM NaCl. Unfortunately, electrophoresis apparatus automatically stops in a few seconds.

4.2.14. Separation of Assembled AuNDs by Centrifugal Force

Prior to separation, hybridization of 11 bp-DNA-AuND was promoted in 150 mM NaCl upon gradually cooling down from 80 to 25 °C following by keeping the assembled AuNDs at 25 °C overnight. Then, the mixtures of 60:40 and 67:33 ratios were separated by applying different centrifugal forces (1000g, 500g, 400g, 300g, 200g, and 100g), including the

precipitation of sample with lowest g. Separation was started from higher centrifugal force at 25 °C for 8 min with maintaining NaCl concentration. At higher centrifugation speed, larger oligomers were sedimented at the bottom as a precipitation and the smaller oligomers were suspended in the upper supernatant. After each centrifugation, the supernatant was taken, and the precipitation was resuspended in the buffer with 150 mM NaCl and centrifuged at the lower centrifugal forces. Each supernatant was directly characterized to prevent rearrangement of the assembled nanoparticles. This technique was quick, simple, and successful to separate the broad size distribution of assembled AuNDs into several fractions without losing nanoparticles. The size-based population was analyzed by counting more than 500 particles of each sample.

4.2.15. Crosslinking of Assembled AuNDs

Crosslinking of DNA duplexes with 8-methoxypsoralen (8-MOP)

Thus, 8-MOP was used to crosslink DNA duplexes followed the previous method⁹⁶. Before crosslinking, 150-, 200-, or 250-mM final concentration of NaCl was added into 700 μ L of the mixed 11 base-(+)-DNA-AuND and 11 base-(−)-DNA-AuND in the buffer solution (pH 7.0). Then, samples were heated at 60 °C for 10 min followed with incubating at 25 °C for 3.5 h to proceed AuNDs assembly driven by DNA hybridization. Next, 10 μ L of 40 mM 8-MOP dispersed in DMSO was added into the colloidal mixtures (final concentration: 500 μ M). Photoreaction occurred under 100% intensity of 40- and 60-min UV irradiation. Duration and irradiation modes were varied to optimize the crosslinking. Upon irradiation, samples were surrounded by ice to maintain AuNDs assembly. Then, the yellowish solution was discarded by 3 times centrifugation. The precipitated AuNDs were redispersed in fresh buffer solution (pH 7.0) for characterizations. For SEM observation, solution was changed to H₂O by 2 times centrifugation. Then, 2 μ L of 7-time concentrated AuNDs was dispersed on hydrophilic ITO substrate followed with quickly drying at 60 °C under vacuum.

Crosslinking of assembled AuNDs with SH-PEG-SH

SH-PEG-SH ($M_w = 1500$) was used to covalently link assembled AuNDs. Firstly, different final concentrations of SH-PEG-SH (0.1, 1, 10, 50, or 100 μ M) was brought into 200 μ L of 20 base-(−)-DNA-AuNDs (~0.2 nM) followed by incubating the mixed solutions at 25 °C for 15 h. An excess amount of SH-PEG-SH was required to prevent both thiols connected to the same particles. Next, the colloidal solutions were centrifuged 3 times to remove unreacted SH-PEG-SH. The precipitation of 20 base-(−)-DNA-PEG_x-AuNDs were dispersed in fresh buffer solution (pH 7.0). Assembly was proceeded at 25 °C after mixing the same ratio of 20 base-(−)-DNA-PEG_x-AuNDs and 20 base-(+)-DNA-AuNDs in the presence of 0.5 M NaCl.

After 5 h incubation, the mixed solutions were gradually heated to 80 °C while measuring the extinction spectra for every 5 °C. A heating rate of 1 °C/min and a waiting time of 15 min were applied for the measurements. To clearly confirm the successful crosslinking, NaCl was discarded by 3 times centrifugation. The precipitated AuNDs were dispersed in fresh buffer solution for further characterizations.

4.3. Results and Discussions

4.3.1. Assembly of AuNDs Driven by DNA Hybridization

Firstly, assembly was performed for AuNDs modified with fully matched complementary 11-base DNA ligands ((+)-DNA and (–)-DNA) (**Figure 4. 2a**). The DNA-modified AuNDs ((+)-DNA-AuND and (–)-DNA-AuND) exhibited similar extinction spectra (the peaks at 860 and 859 nm, respectively: **Figure 4. 2b**, blue and orange) and hydrodynamic diameters (18 and 91 nm; **Figure 4. 2c**, blue and orange) to those of unmodified AuNDs (**Figure 2. 3b,c**), indicating good dispersibility of the DNA-modified AuNDs in the buffer solution containing 150 mM NaCl. Subsequently, (+)-DNA-AuND and (–)-DNA-AuND were mixed at the equimolar ratio (50:50, $r = 1$) in the buffer solution with 150 mM NaCl and assembled via hybridization of fully complementary DNA (**Figure 4. 2a**). Notably, hybridization occurs between short DNA ligands without the additional linkers and there is no unpaired spacer region between AuND and the base pairings (**Figure 3. 2b**). Short DNA and low grafting density reduces the steric hindrance, enabling hybridization at low salt concentration. The combination of short DNA ligand and low AuND curvature eliminate the slipping interactions of non-Watson-Crick base pairings which is supported by similar T_D as T_m of free DNA duplexes⁷⁸, detail discussion in the next section. After gradually cooling down from 80 to 25 °C, the AuNDs exhibited a significant blue shift ($\Delta\lambda = 115$ nm) in the extinction spectra (**Figure 4. 2b**, green), attributed to the strong plasmonic coupling resulting from the narrow interparticle distance (theoretically 6.2 nm). The intensity decreased due to the formation of larger assemblies, which reduced the light penetration and plasmonic coupling between AuNDs¹⁷. Additionally, the hydrodynamic diameter of translational diffusion increased from 91 nm to 190 nm, while the smaller diameter (18 nm) associated with rotational diffusion disappeared (**Figure 4. 2c**, green), indicating the formation of large AuND assemblies with reduced rotational diffusion mode.

Furthermore, scanning electron microscope (SEM) image revealed that up to 39 AuND particles were stacked into 1D cylindrical assembly with well-aligned face-to-face interactions (**Figure 4. 2d**). In addition, cryo-TEM imaging (**Figure 4. 2e**), which captures the structure

dispersed in solution, confirmed the cylindrical assembly of AuNDs with an interparticle distance of 5.7 ± 1.0 nm. Due to the limitation of measurement, the multimerization size was reduced by adjusting the mixing molar ratio to 67:33 ($r = 1/2$) to produce shorter 1D assembly, as discussed in detail below. The interparticle distance and AuND thickness were obtained by measuring 76 gap distances and 90 particles, respectively (**Figure 4. 3a-n**). Moreover, the average thickness of AuNDs is 14.0 ± 1.6 nm (**Figure 4. 3o**), slightly larger than the thickness obtained from SEM images (10.3 ± 2.5 nm, **Figure 2.3Figure 2. 3f**) due to tilting position of cryo-TEM images. This highly ordered assembly was attributed to the well-oriented DNA ligands on the large flat surfaces which stabilized face-to-face hybridization both kinetically and thermodynamically, whereas less-oriented DNA ligands on the edge surfaces scarcely contributed to the assembly. It should be noted that the edge is not atomically flat which has higher curvature than AuND surface⁸⁷. Consequently, AuNDs preferentially form face-to-face assembly than side-to-side interactions due to more DNA ligands can hybridize in the low curvature.

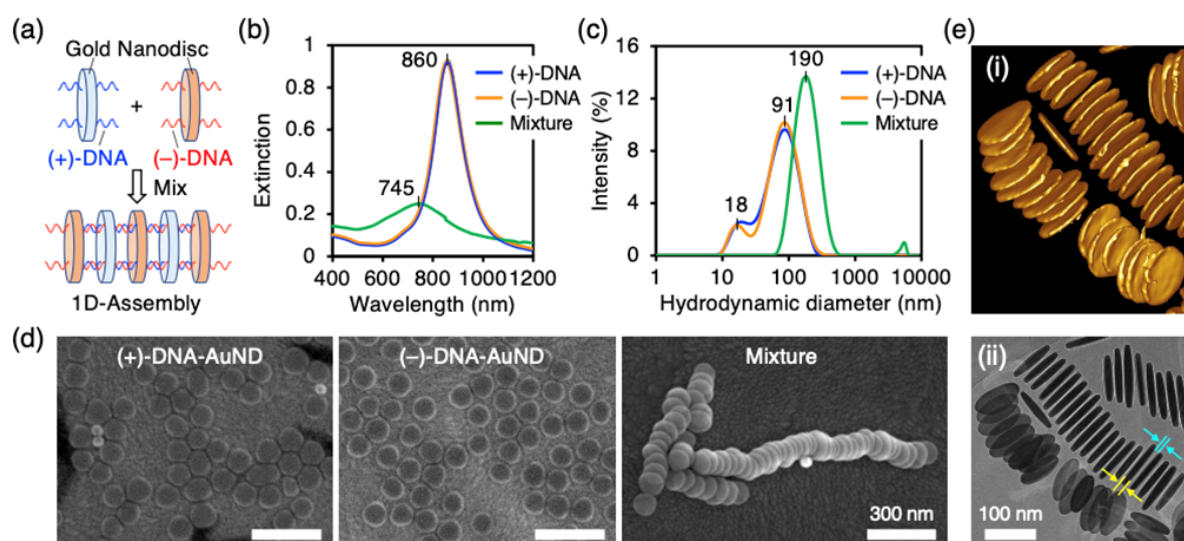


Figure 4. 2. Assembly of AuNDs modified with complementary 11-base DNA ligands ((+)-DNA and (-)-DNA). (a) Schematic illustration of AuNDs assembly driven by DNA hybridization. (b,c) (b) Extinction spectra and (c) hydrodynamic size measurements of (+)-DNA-AuND (blue), (-)-DNA-AuND (orange), and the mixture in an equimolar ratio (50:50) (green) at 25 °C. (d) SEM images of (+)-DNA-AuND, (-)-DNA-AuND, and the equimolar mixture. (e) Cryo-TEM images of AuND assemblies mixed at a 67:33 ratio: (i) a reconstructed 3D model and (ii) a representative snapshot. Arrows indicate the interparticle gap distance (blue) and the thickness of a disc (yellow). Note: Due to measurement limitations for large particles, the size of the multimers was reduced by adjusting the mixing ratio to 67:33. Solution composition: phosphate buffer (pH 7.0) containing 0.01% SDS and 150 mM NaCl.

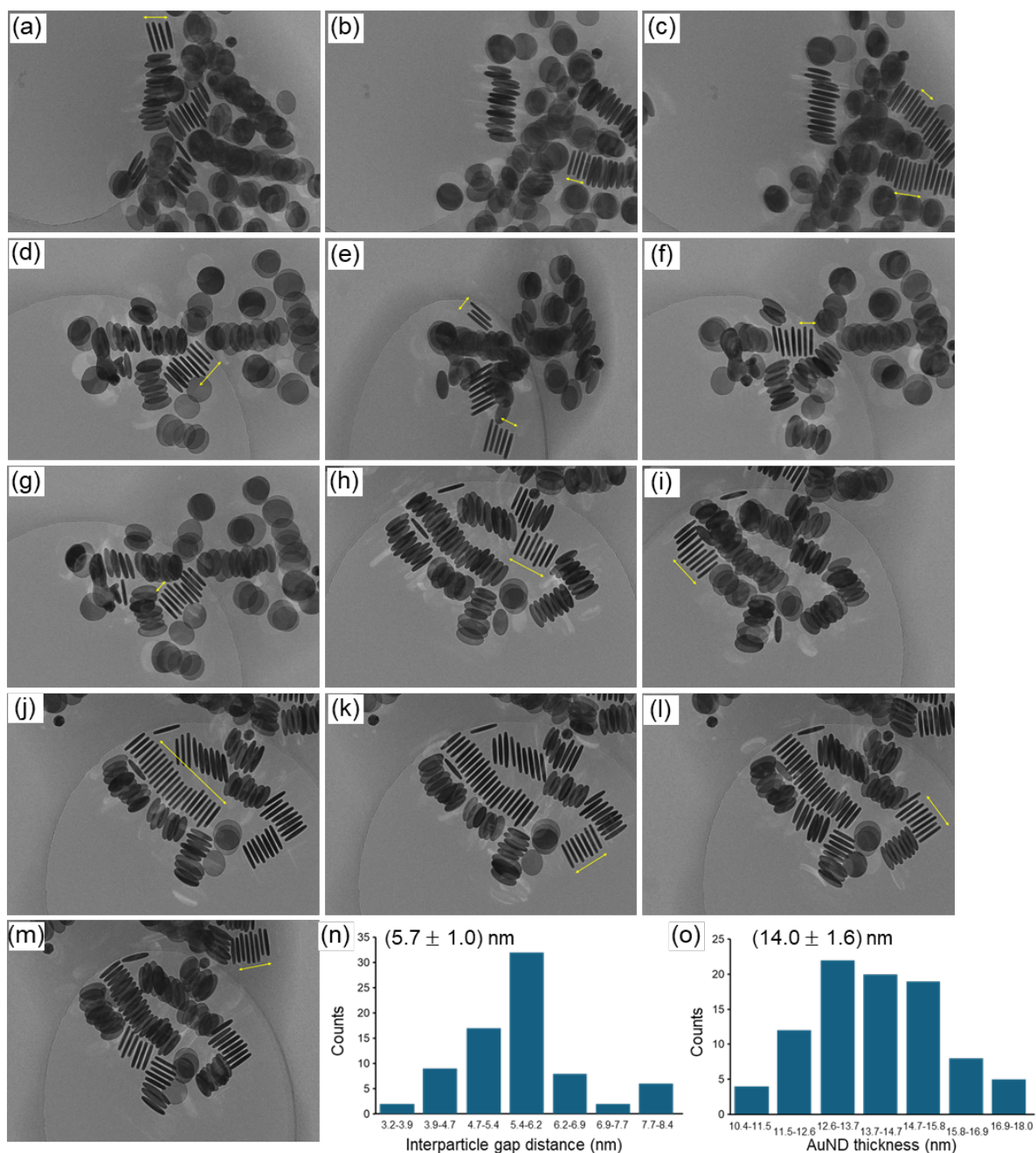


Figure 4. 3. CryoTEM images of assembled 11 bp-DNA-AuND with (67:33) mixing molar ratio in 150 mM NaCl. Images were obtained from three different positions: (a-c) first position, (d-g) second position, and (h-m) third position with various tilting conditions. Distance was measured by ImageJ in pixel unit. Histogram of (n) interparticle gap distance and (o) AuND thickness by calculating 76 particles.

To confirm that the observed blue shifts in the extinction spectra originated from plasmonic coupling within the AuND assemblies and to directly probe localized electric field enhancement, finite-difference time-domain (FDTD) simulations was performed based on the configurations obtained from cryo-TEM observations (**Figure 4. 4**). Simulations of varying degrees of AuND oligomerization (1-5 mers) showed that even a small extent of oligomerization, with a short interparticle gap distance of 5.7 nm, induced substantial

plasmonic shifts in the extinction spectra (**Figure 4. 4a**), consistent with the results of a previous simulation study¹⁷. Additionally, varying the interparticle gap distances within a narrow range (2–20 nm) showed that the plasmonic peaks in the extinction spectra were highly sensitive to small changes in distance (**Figure 4. 4b**), highlighting the potential for precise control over plasmonic coupling. Moreover, a model of a larger multimer (20-mer AuND) exhibited a pronounced blue shift (**Figure 4. 4c**) relative to the monomer, in good agreement with the experimental results (**Figure 4. 2b**).

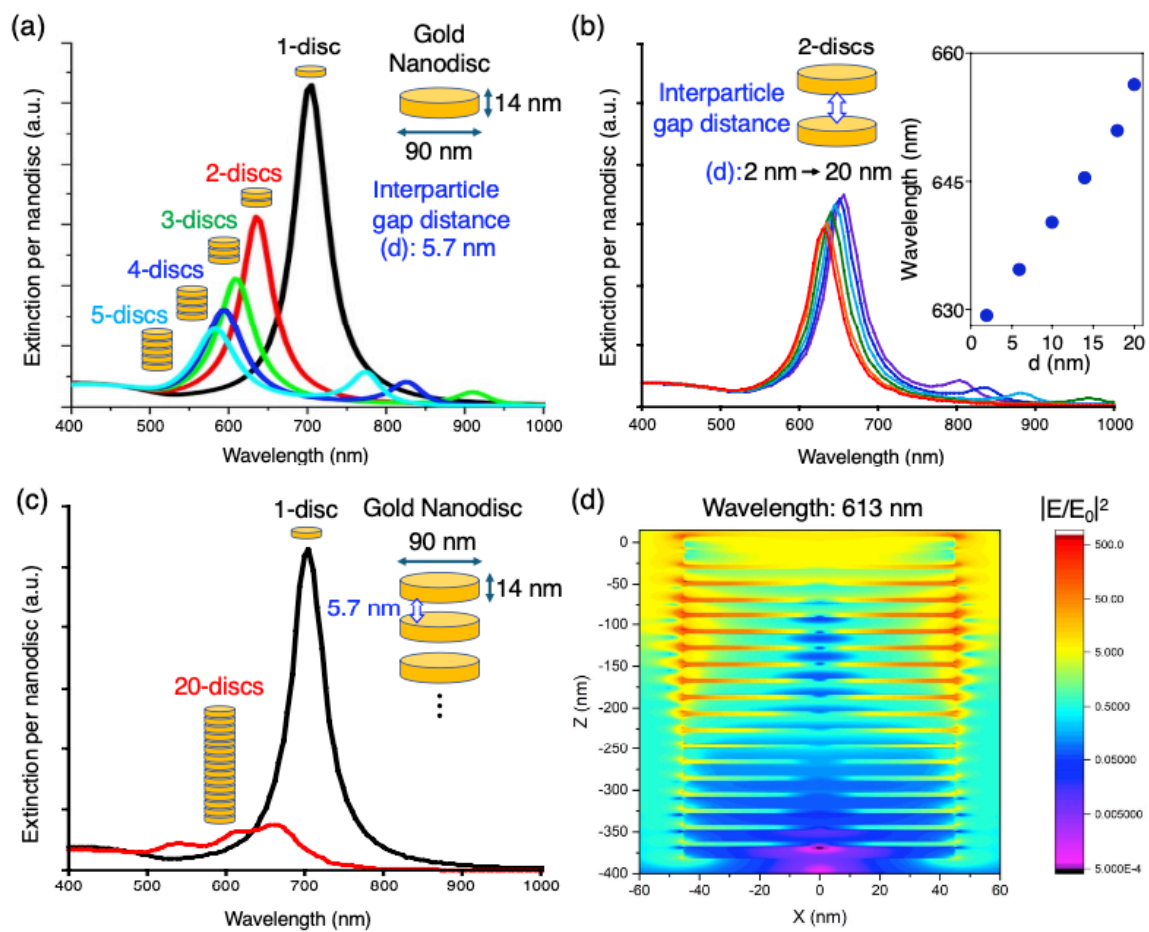


Figure 4. 4. FDTD-simulated extinction spectra for varying (a) degrees of AuND oligomerization (monomer to pentamer) with a fixed interparticle gap of 5.7 nm and (b) interparticle gap distances between AuNDs ranging from 2 to 20 nm. Inset in (b): extinction peak wavelength as a function of interparticle gap distance. (c) Simulated extinction spectra of a monomer AuND (black) and a 20-mer AuND assembly (red). (d) Cross-sectional view of the near-field distribution of the 20-mer AuND assembly at a wavelength of 613 nm, with near-field intensity plotted on a logarithmic scale.

Although discrepancies in the absolute resonance wavelength were observed (e.g., non-assembled monomer: 860 nm in the experiment vs 705 nm in the simulation; highly multimerized assemblies such as the 20-mer: 745 nm in the experiment vs 663 nm in the simulation), these were likely due to idealized assumptions in the model. Specifically: (i) the

dimensions of the AuND model in the FDTD simulations could not perfectly match those of the experimental AuNDs—for example, the thickness and diameter measured from TEM images may be overestimated and underestimated, respectively, unless analyzed from precise orientations and positions; and (ii) the refractive index of the DNA modified on the AuND surface, which causes a red shift in the plasmonic peaks of the AuNDs⁹⁷, was not considered because accurately modeling the DNA configuration in the simulation is challenging. These factors may account for the observed discrepancies. Near-field distribution simulations of the 20-mer AuND assembly further revealed strong localized electric field enhancement (“hot spots”) within the nanogaps between adjacent AuNDs (**Figure 4. 4d**), confirming that coupling occurs at the interparticle gap distances.

To confirm the assembly driven by DNA hybridization, assembly would be blocked by firstly introducing DNA strands which are complementary to DNA ligands. To confirm this suppression, free blocking strands (BS1 or BS2; single-stranded DNA strands complementary to (+)-DNA and (–)-DNA, respectively) were added to (+)-DNA-AuNDs and (–)-DNA-AuNDs, respectively, prior to assembly (**Figure 4. 5a**). After mixing the DNA-modified AuNDs at 50:50, no changes were observed in the plasmonic spectra (**Figure 4. 5b**) or the hydrodynamic diameters (**Figure 4. 5c**) after 24 h incubation. These results confirm that the blocking strands hybridized with (+)-DNA and (–)-DNA on AuND surfaces, thereby inhibiting their assembly.

DNA duplexes dissociate when heated at temperature higher than their melting temperature, breaking the hydrogen bonds between the base pairs. Consequently, the assembly and disassembly of AuNDs can be reversibly repeated through cooling and heating cycles. To confirm this reversibility, the dissociation of assembled DNA-modified AuNDs mixed at 50:50 was investigated by heating the solution from 25 to 60 °C. As the temperature increased, the plasmonic peak of the AuNDs exhibited a red shift from 745 to 846 nm, along with an increase in the extinction intensity (**Figure 4. 5d**), almost returning to the initial peak position of individual DNA-AuNDs, confirming disassembly. Notably, in the hydrodynamic sizes, the rotational diffusion peak reappeared at 18 nm at 60 °C, while the peak for translational diffusion decreased to 106 nm (**Figure 4. 5e**). Furthermore, the reversible assembly and disassembly of AuNDs were demonstrated through multiple thermal cycles between 25 and 60 °C (**Figure 4. 5f**). These results indicate that the assembly and disassembly of AuNDs can be reversibly repeated through thermal cycling. Taken together, these results confirm that assembly was driven by DNA hybridization.

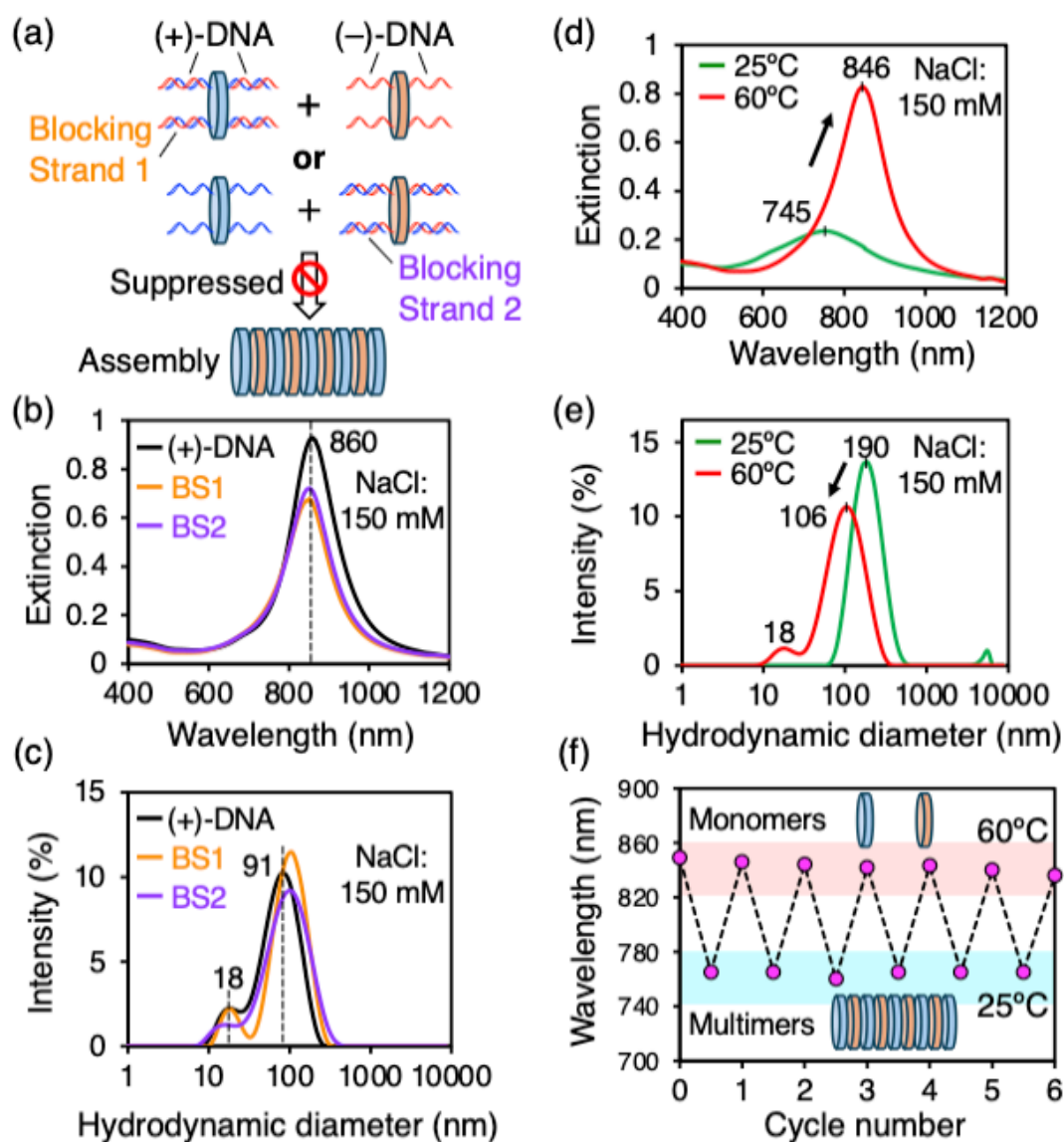


Figure 4. 5. Confirmation of AuNDs assembly driven by DNA hybridization: (a-c) Suppression of AuND assembly by introducing blocking DNA strands: (a) Schematic illustration. (b) Extinction spectra and (c) hydrodynamic size measurements of the AuNDs mixtures hybridized with blocking DNA strands 1 (orange) or 2 (purple). (d-f) Reversible assembly and disassembly of the AuNDs through cooling and heating cycles: (d) Extinction spectra and (e) hydrodynamic size measurements of the AuNDs mixtures at 25°C (green) and 60°C (red). (f) Reversible peak wavelength shifts between 25 and 60°C. Solution composition: 0.01% SDS, 10 mM KPO₄, pH 7.0 containing 150 mM NaCl.

Additionally, AuNDs were modified with 12 base-palindromic sequence to investigate the effect of sequence on the assembly properties of AuNDs driven by DNA hybridization. Surprisingly, the low reducing intensity and small plasmon shifting ($\Delta\lambda = \sim 27$ nm, **Figure 4. 6a**) of AuNDs in 250 mM NaCl suggested that palindromic sequence was insufficient to direct assembly formation. Possibly, palindromic sequence on large AuNDs surfaces enabled hybridization on intraparticle, reducing face-to-face interparticle interactions. Hydrodynamic diameter corresponded to the formation of AuNDs assembly (**Figure 4. 6b**).

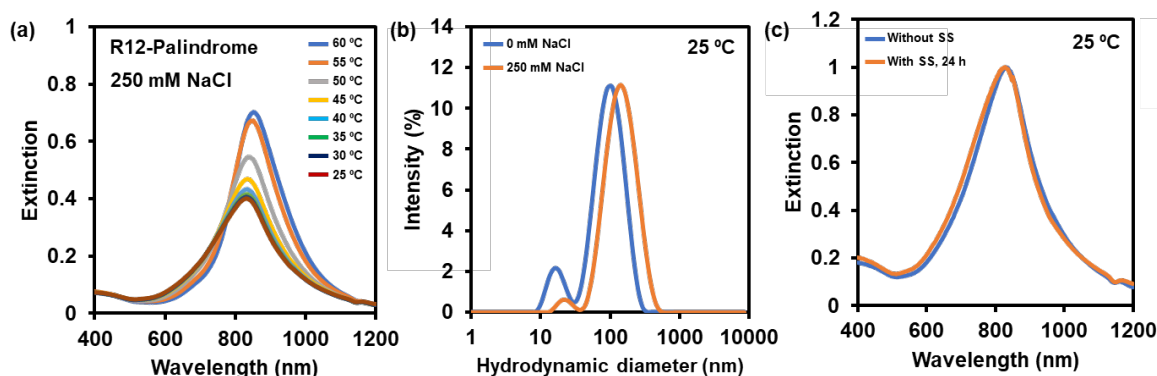


Figure 4. 6. Assembly and disassembly of AuNDs modified with 12 nt-palindromic sequence in 250 mM NaCl. (a) Extinction spectra of AuNDs in 250 mM NaCl upon cooling from 60 to 25 °C. (b) Hydrodynamic diameters of AuNDs in 0 and 250 mM NaCl at 25 °C. (c) Extinction spectra of assembled AND with and without addition of blocking strand in 250 mM NaCl after 24 h incubation at 25 °C. Solution composition: 0.01% SDS, 10 mM KPO₄, pH 7.0.

To confirm the assembly driven by hybridization, 5 times molar equivalent of complimentary blocking strand (BS, 5'AAAGAGCTCTTTT^{3'}) was added into assembled AuNDs to replace self-hybridization, driving the dissociation of assembled AuNDs. Besides, three bases of the frying motion at the end of blocking strand provided entropic repulsion⁸⁷ when blocking strand and palindromic sequences formed DNA duplexes, enhancing AuNDs dispersibility in high salt concentration. After 24 h incubation, the unalterable extinction spectrum indicated that blocking strand could not replace self-hybridization of palindromic sequence. In conclusion, 12 base-palindromic sequence with comparable length to 11 bp-complimentary sequences was not effective to direct face-to-face assembly formation.

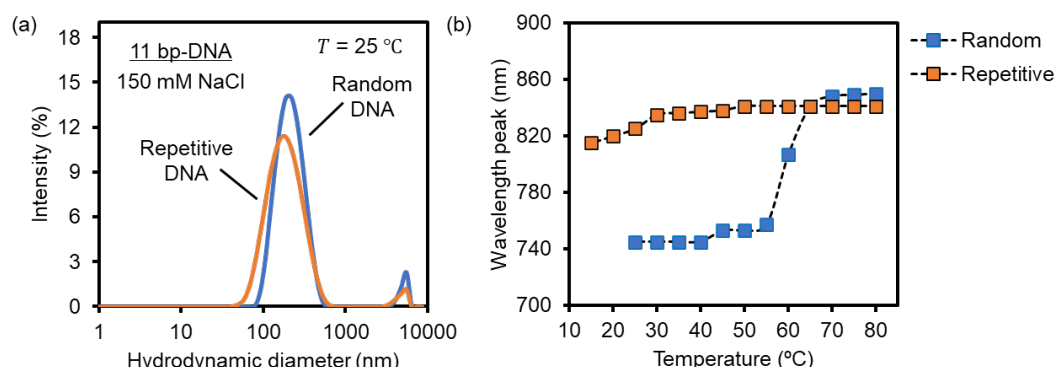


Figure 4. 7. Assembly of AuNDs modified with random and repetitive sequences. (a) Hydrodynamic sizes and (b) LSPR shift of assembled AuNDs driven by hybridization of 11 bp-random DNA (blue) and 11dA-11dT (orange) in buffer solution (pH 7.0) containing of 150 mM NaCl.

Then, assembly formation was observed for random and repetitive sequence of 11-bp DNA. For the comparable assembly sizes (Figure 4. 7a), random sequence exhibited larger plasmon shift than repetitive sequence (Figure 4. 7b) which indicated that DNA sequences

also affected the assembly formation. Repetitive sequence has higher probability to form misalignment of the base pairings which will affect both spatial arrangement and interparticle gap distance. It seems that self-aggregation was included in the assembly size by decreasing the extinction intensity but did not induce a plasmonic shift. Further investigation was required to clarify the origin of plasmonic shift.

4.3.2. Dispersion Stability of DNA-modified AuNDs

Different stability corresponded to the different DNA lengths. Longer DNA provides higher electrostatic repulsion forces which is stable in higher NaCl concentration. Increasing salt concentration reduced the stability, inducing reversible self-aggregation which also contributed to the assembly formation. Therefore, contribution of self-aggregation on the plasmonic properties was investigated upon cooling and heating of AuNDs modified with various DNA lengths in different salt concentrations. Even though reversible self-aggregation of (+)-DNA-AuND and (-)-DNA-AuND were partially observed in few conditions, their contribution was not significant on the plasmonic shift but significantly reduced the extinction intensity which would contribute on the assembly (T_a) and disassembly (T_d) temperatures of assembled AuNDs (**Figure 4. 8-13**). It was unable to completely exclude self-aggregation in high salt concentration because large nanoparticles easily allowed hybridization even though only one base pair⁹³. Importantly, I confirmed that plasmonic shift of assembled AuNDs was dominantly originated from face-to-face assembly driven by DNA hybridization. This is a merit of using short DNA because strong plasmon coupling enabled to clearly observe the plasmonic shift which could not be achieved previously by using long DNA linkers^{17,24}. In addition, low steric hindrance due to the use of short DNA ligands with low grafting density allowed the assembly with low salt concentration as discussed in the next section which would eliminate self-aggregation effect.

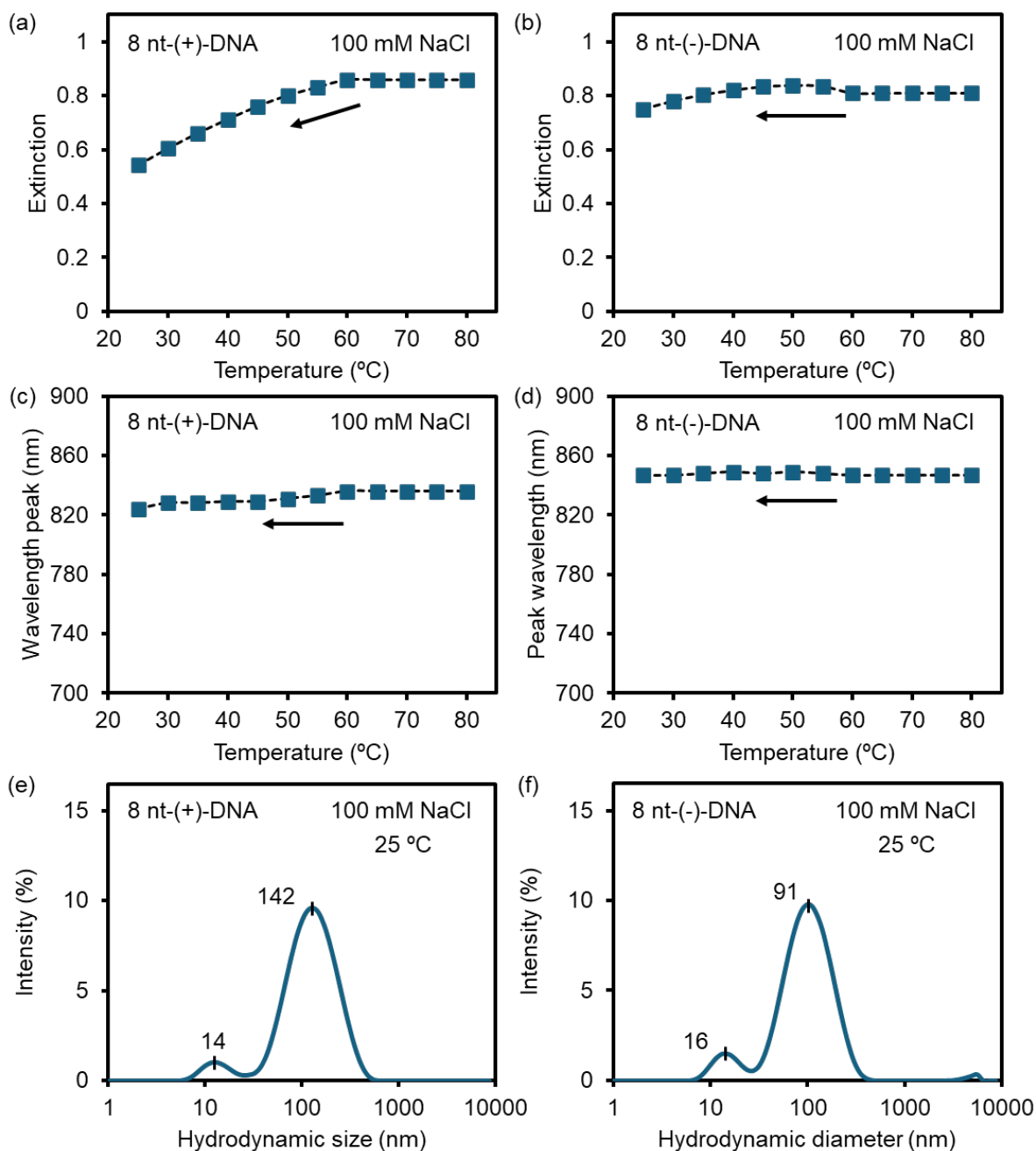


Figure 4. 8. Stability of 8-base-DNA-AuND upon cooling in 100 mM NaCl. (a-b) Extinction intensity of (a) (+)-DNA-AuND and (b) (-)-DNA-AuND. (c-d) Plasmonic shift induced by reversible self-aggregation of (c) (+)-DNA-AuND and (d) (-)-DNA-AuND. (e-f) Hydrodynamic sizes of (e) (+)-DNA-AuND and (f) (-)-DNA-AuND at 25 °C. These results indicated that (-)-DNA-AuND was slightly more stable than (+)-DNA-AuND.

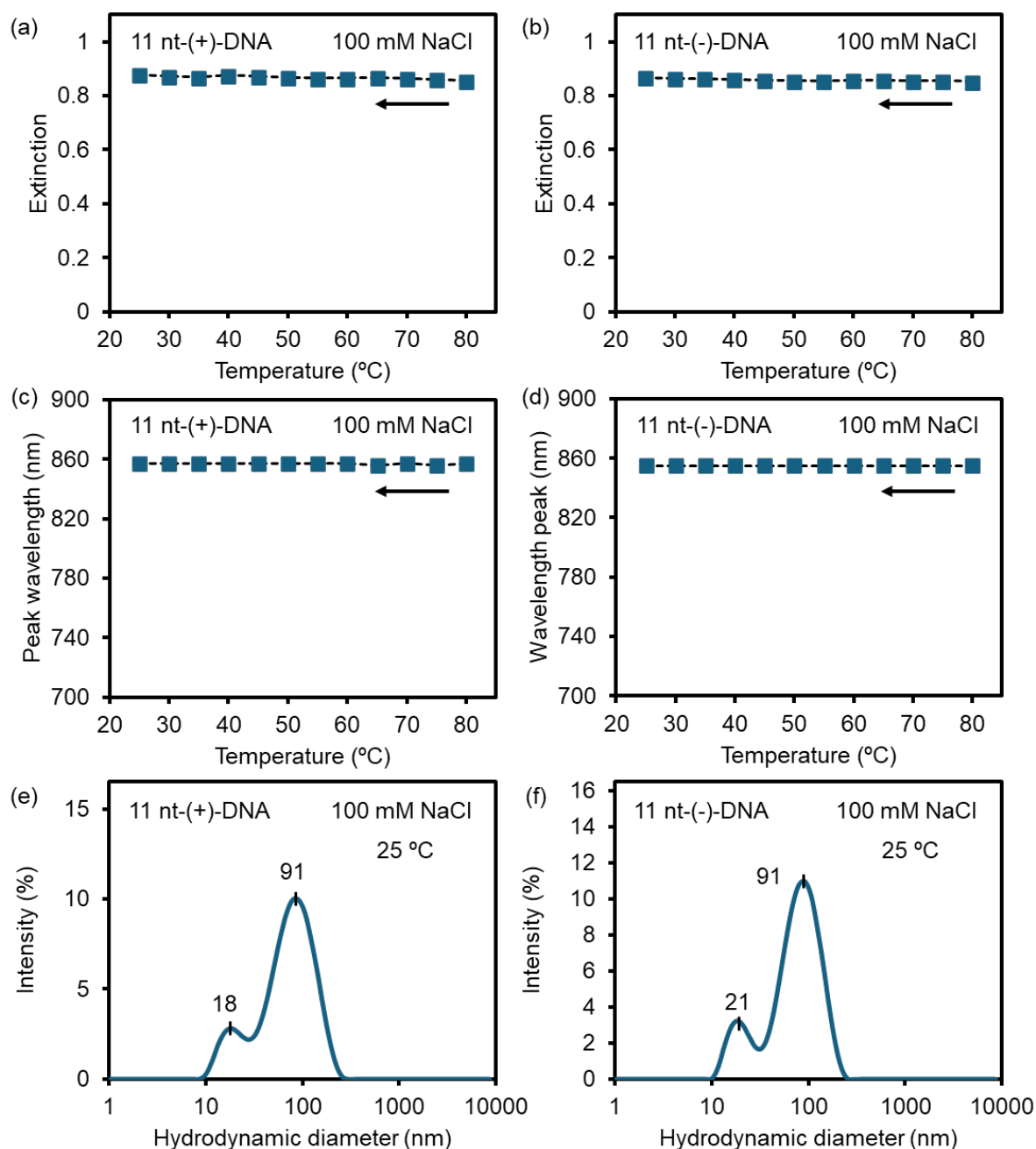


Figure 4. 9. Stability of 11-base-DNA-AuND upon cooling in 100 mM NaCl. (a-b) Extinction intensity of (a) (+)-DNA-AuND and (b) (-)-DNA-AuND. (c-d) Plasmonic shift induced by reversible self-aggregation of (c) (+)-DNA-AuND and (d) (-)-DNA-AuND. (e-f) Hydrodynamic sizes of (e) (+)-DNA-AuND and (f) (-)-DNA-AuND at 25 °C. Both (+)-DNA-AuND and (-)-DNA-AuND exhibited excellent stability without plasmonic shift induced by reversible self-aggregation.

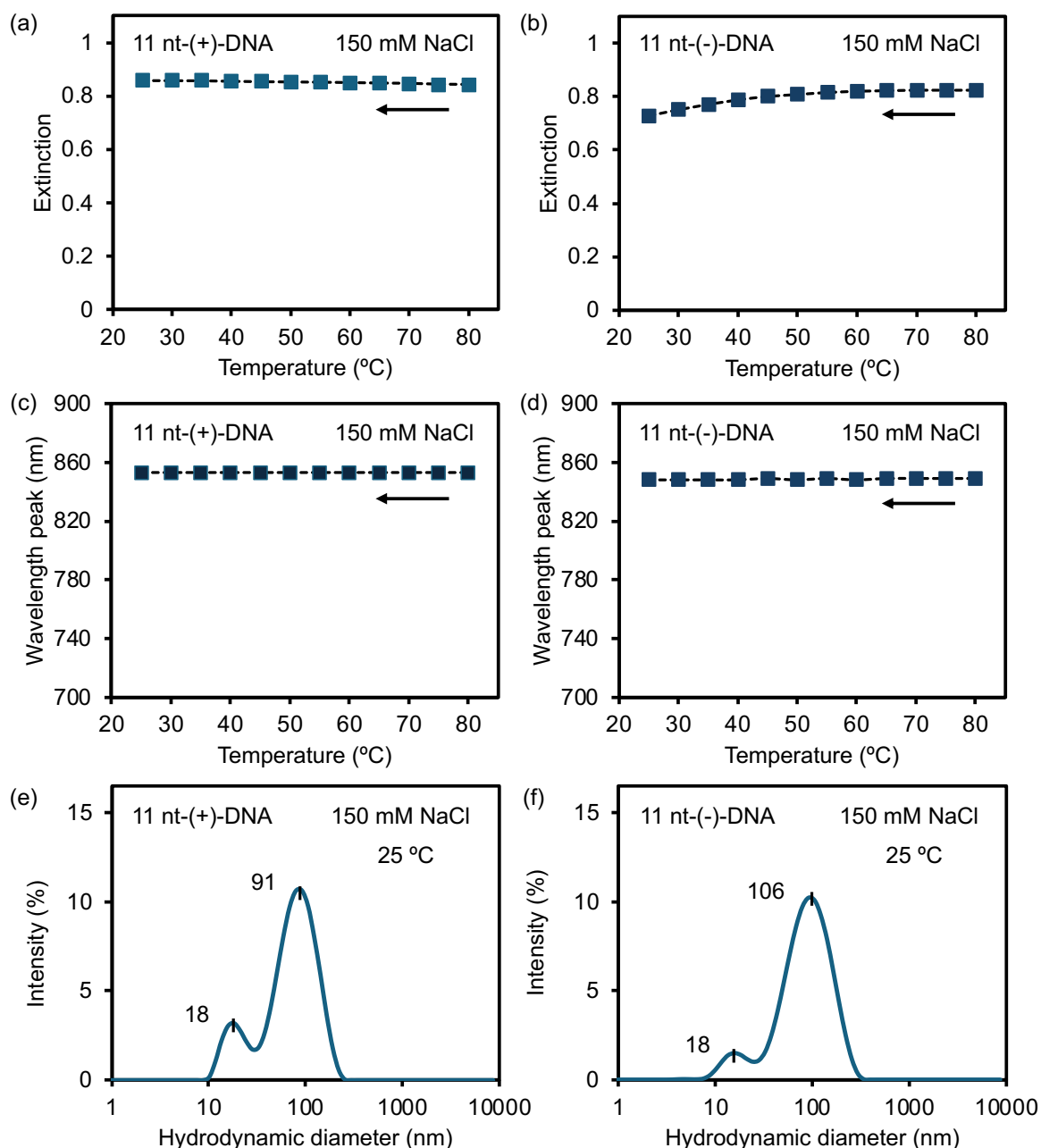


Figure 4. 10. Stability of 11-base-DNA-AuND upon cooling in 150 mM NaCl. (a-b) Extinction intensity of (a) (+)-DNA-AuND and (b) (-)-DNA-AuND. (c-d) Plasmonic shift induced by reversible self-aggregation of (c) (+)-DNA-AuND and (d) (-)-DNA-AuND. (e-f) Hydrodynamic sizes of (e) (+)-DNA-AuND and (f) (-)-DNA-AuND at 25 °C. Both (+)-DNA-AuND and (-)-DNA-AuND exhibited excellent stability without plasmonic shift induced by reversible self-aggregation.

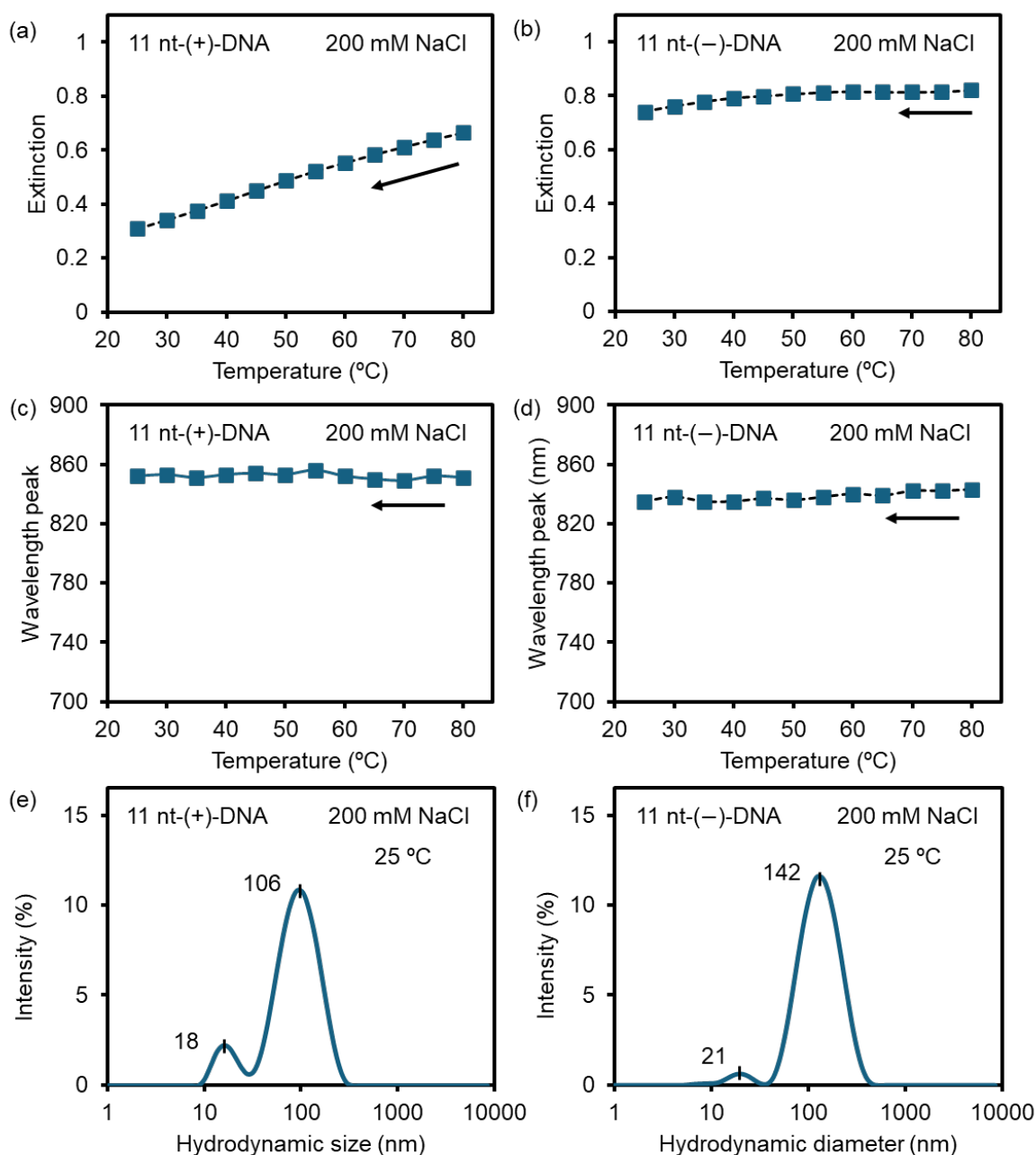


Figure 4.11. Stability of 11-base-DNA-AuND upon cooling in 200 mM NaCl. (a-b) Extinction intensity of (a) (+)-DNA-AuND and (b) (-)-DNA-AuND. (c-d) Wavelength peak of (c) (+)-DNA-AuND and (d) (-)-DNA-AuND. (e-f) Hydrodynamic sizes of (e) (+)-DNA-AuND and (f) (-)-DNA-AuND at 25 °C. Reversible self-aggregation only reduced the extinction intensity without significant plasmonic shift.

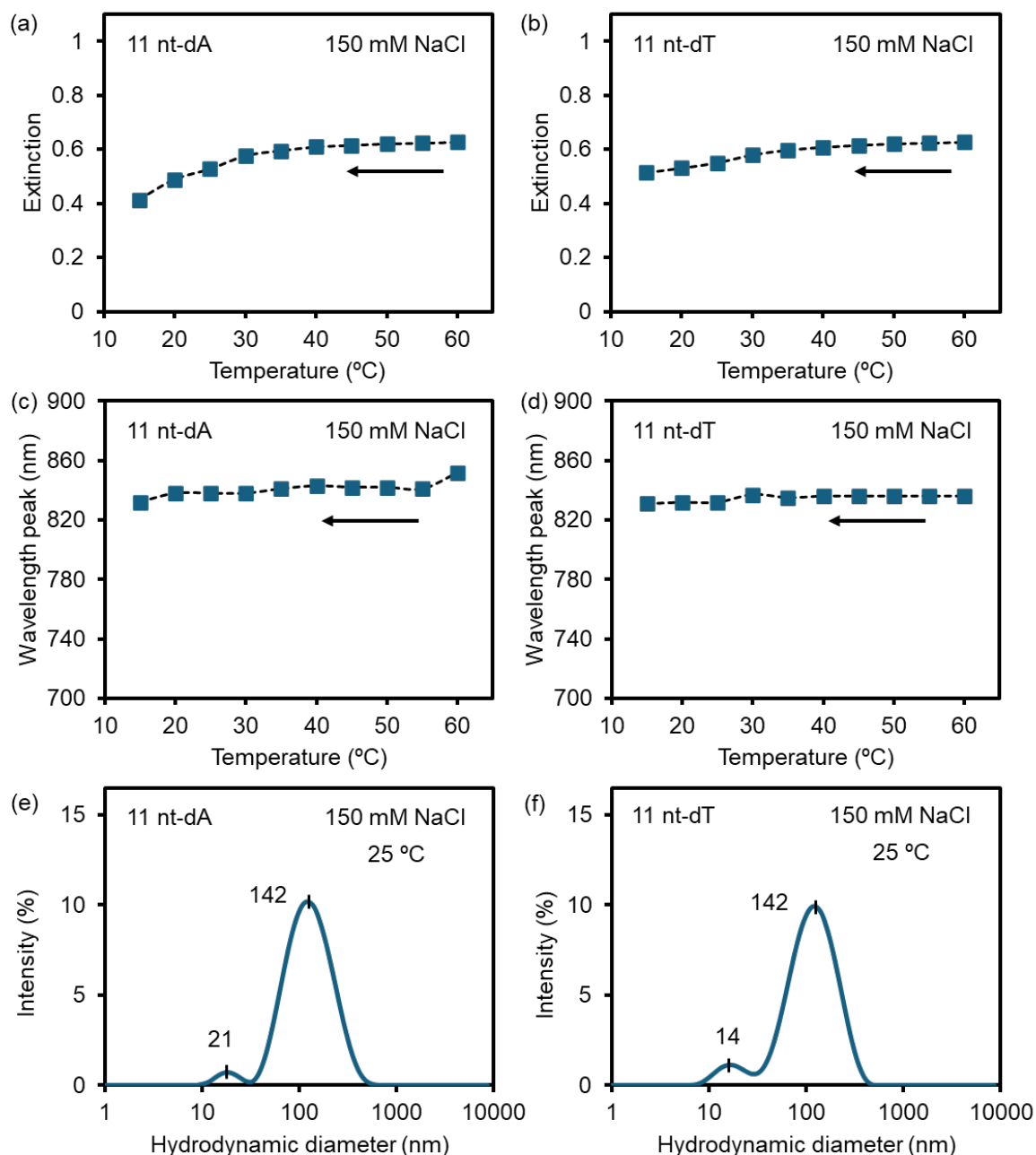


Figure 4. 12. Stability of AuNDs modified with repetitive DNA sequences in 150 mM NaCl. (a) Extinction intensity of dA11 and dT11 upon cooling. (b) Plasmonic shift induced by self-aggregation of dA11 and dT11. (c-d) Hydrodynamic diameters of (c) dA11 and (d) dT11 at 25 °C. Strong interaction between adenine and gold surface induced lower grafting density of dA11⁶⁵, leading to imbalance negatively charge which possibly promoted larger hysteresis of assembled AuNDs with repetitive sequences (dA-dT).

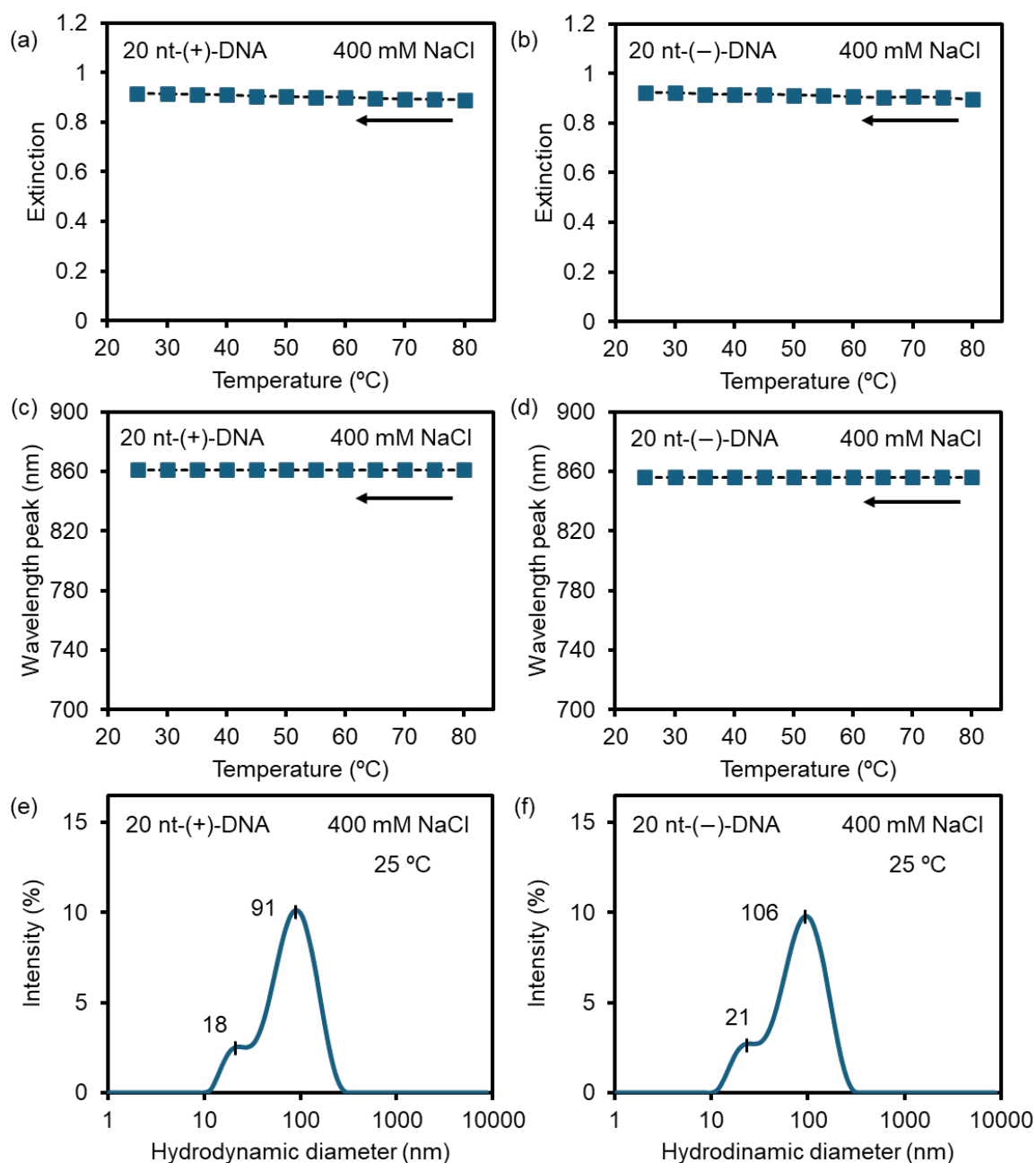


Figure 4. 13. Stability of 20-base-DNA-AuND upon cooling in 400 mM NaCl. (a) Extinction intensity of (+)-DNA-AuND and (–)-DNA-AuND. (b–c) Hydrodynamic sizes of (b) (+)-DNA-AuND and (c) (–)-DNA-AuND at 25 °C. Both (+)-DNA-AuND and (–)-DNA-AuND exhibited excellent stability in 400 mM NaCl due to the strong negatively charged.

4.3.3. Effect of Curvature on DNA Hybridization

Curvatures also affect the hybridization of DNA ligands on nanoparticles surfaces^{25,90,93}. Circular nanoparticles with high curvatures reduce the hybridization efficiency and increase the slipping effect of non-Watson-Crick DNA base pairings. Consequently, AuNPs requires long DNA with enough flexibility to access the complementary on other nanoparticle surfaces. To clarify this hypothesis, I synthesized AuNPs with high surface curvature. The citric-capped

AuNPs shows a LSPR peak around 517 nm (**Figure 4. 14a**). The size of the citric-capped AuNPs was determined from TEM image using ImageJ software. The average diameter of 200 particles is 9.8 ± 1.5 nm (**Figure 4. 14b,c**).

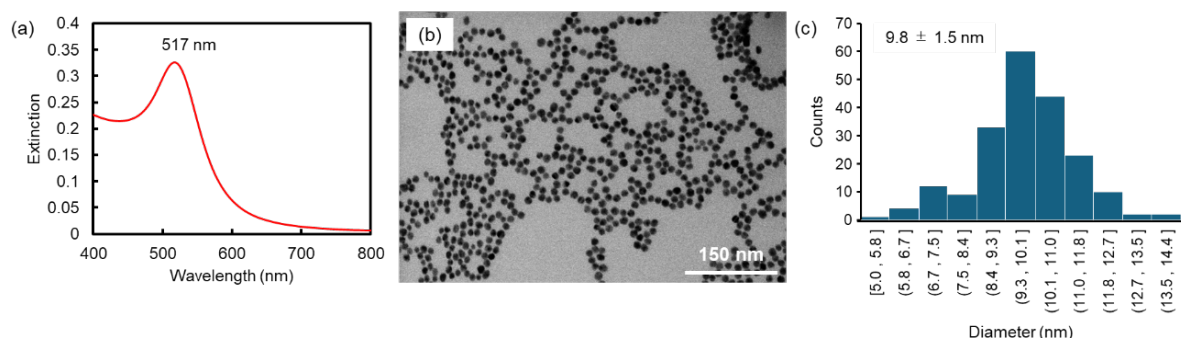


Figure 4. 14. (a) Extinction spectrum of citric-capped AuNPs dispersed in water. (b) TEM image of AuNPs dried at 25 °C. (c) Size distribution of AuNPs calculated 200 particles by ImageJ software.

Then, citric-capped AuNPs was modified with complementary 11-base DNA ligands ((+)-DNA and (-)-DNA) via butanol dehydration method. DNA-modified AuNPs has a plasmon band centered at 527 nm (**Figure 4. 15a,b**: blue), slightly red-shift than citric-capped AuNPs (**Figure 4. 15a**). To proceed DNA hybridization, salt concentration was subsequently increased (75 mM-1 M NaCl) by the sequential addition of 2 M NaCl to the mixed colloidal solutions (50:50 mixture of (+)-DNA-AuNP and (-)-DNA-AuNP). The formation of AuNPs assembly was monitored by measuring the surface plasmon band after 24 h incubation at 25 °C in each salt concentration. The surface plasmon band was maintained with the increase of salt concentration up to 0.8 M NaCl (**Figure 4. 15c**), indicating DNA hybridization did not occur. Further increased NaCl concentration up to 1 M slightly red-shifted the plasmon band to 537 nm (**Figure 4. 15d**, red), indicating the assembly formation of few AuNPs. Significantly, these results provide evidence that surface curvature greatly affects DNA interactions. Circular AuNPs with high curvature decreased the hybridization efficiency due to the limited access of short DNA on the particle surface to their complementary bases^{25,84}. Therefore, linker or longer DNA with spacer is required to increase the interparticle interaction^{98,99}. On the other hand, AuNDs with low curvature enables more DNA interactions, increasing the hybridization efficiency. Another factor that affects the hybridization efficiency is DNA surface coverage. Previous study reported that flat surface gold nanoparticle has lower grafting density than circular AuNPs, reducing the steric crowding of the bases and electrostatic repulsion force, enhancing the hybridization efficiency⁸⁴.

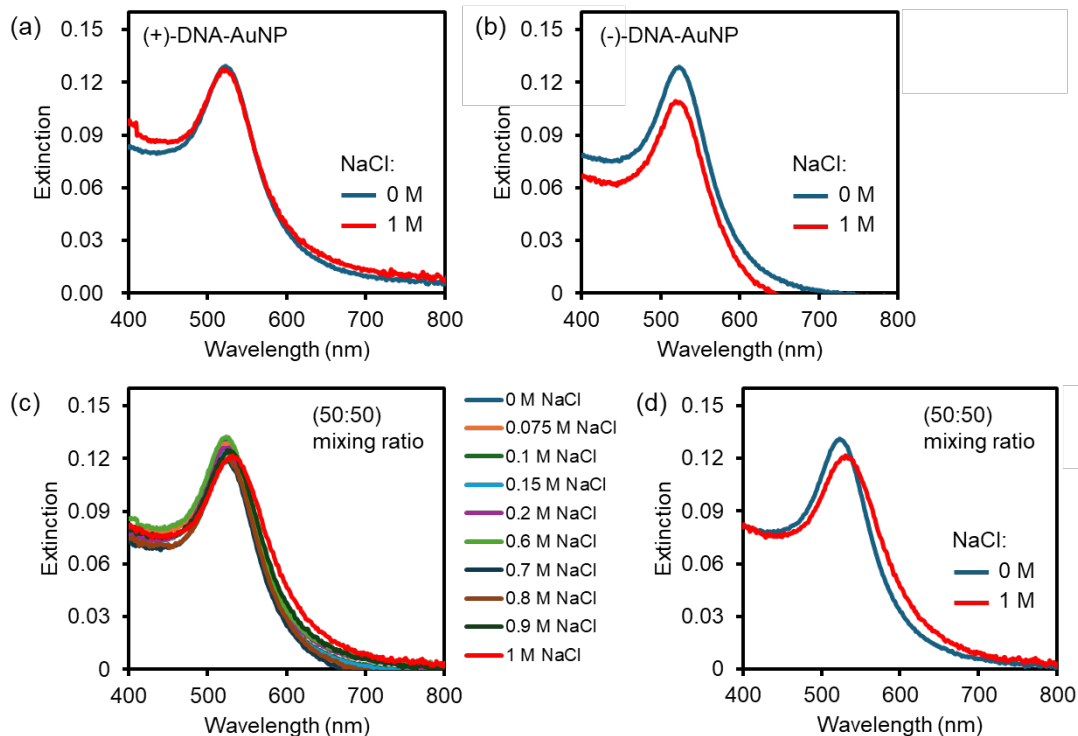


Figure 4. 15. Assembly of AuNPs modified with complementary 11-base DNA ligands ((+)-DNA and (-)-DNA). Extinction spectra of (a) (+)-DNA-AuNP and (b) (-)-DNA-AuNP in 0.5xTBE (blue) and 0.5xTBE with 1 M NaCl (red). (c) Extinction spectra of AuNP assemblies mixed at an equimolar ratio (50:50) in various NaCl concentrations after 24 h incubation at 25 °C. (d) Extinction spectra of dispersed (blue) and assembled (red) AuNP. A slightly red shift, decreased intensity, and broader spectrum indicating a limited assembly formation of AuNPs due to the strong steric hindrance of short DNA ligand on high surface curvature.

4.3.4. Controlled Multimerization Degree of Assembled AuNDs

Plasmonic coupling can be tuned by changing the sizes of assembled structures. Thus, the degree of AuND multimerization would be controlled by two methods: (i) changing the molar mixing ratio of AuNDs modified with (+)-DNA and (-)-DNA, and (ii) adjusting salt concentrations of the colloidal mixtures.

First, assembly size was regulated by adjusting the molar ratio of (+)-DNA-AuNDs and (-)-DNA-AuNDs, where excess of (+)-DNA-AuNDs terminate the multimerization reaction. This approach enables precise control of assembly size using hetero-DNA-modified AuNDs. The degree of multimerization (n) was estimated based on the chemical reaction theory from polymer chemistry (Eq. 4-1)⁹⁸.

$$n = \frac{1+r}{1+r-2pr} \quad \text{Eq. 4- 1}$$

where r is defined as the molar ratio of the monomers (Eq. 4-2), and p represents the extent of reaction, i.e., the fraction of monomers that have reacted at time t .

$$r = \frac{[(-)\text{DNA-AuND}]}{[(+)\text{DNA-AuND}]} \quad \text{Eq. 4- 2}$$

Assuming the reaction proceeds to completion ($p = 1$), meaning that AuNDs are fully consumed in the multimerization process via specific DNA hybridization, under the experimental conditions of sufficient salt concentration (150 mM) and incubation time (4 h), the expression for the degree of multimerization can be simplified as follows (**Eq. 4-3**):

$$n = \frac{1+r}{1-r} \quad \text{Eq. 4- 3}$$

As a benchmark for large multimers, 11-base (+)-DNA-AuND and 11-base (-)-DNA-AuND were assembled by mixing at the equimolar ratio (50:50, $r = 1$) in the same manner as described above. Evaluations using extinction spectra (**Figure 4. 16a**, red), DLS (**Figure 4. 16b**, red), and SEM (**Figure 4. 16c**, bottom) all indicated the formation of large AuND multimers, consistent with the results shown in **Figure 4. 2**. To investigate the degree of multimerization, each multimer in SEM images was analysed (total number counted: 1500 AuNDs as monomers, **Figure S 1-5**) and plotted the number of AuND monomers in each multimer as a histogram (**Figure 4. 16d**, red). The histogram exhibited a broad distribution of the multimers, analogous to the weight fraction distribution in condensation polymerization^{100,101}. The maximum degree of the multimerization was limited to a 39-mer with the length of assembly approximately 1000 nm (**Figure S 1**). The moderate degree of multimerization was possibly due to inaccurate assumptions regarding the factors influencing the multimerization: (i) the mixing ratio was not perfectly 50:50 ($r \approx 1$) even though a strictly equimolar ratio is require to complete the reaction for long multimerization (for instance, $n = 39$ when $r = 48.7/51.3 = 0.95$); or (ii) the multimerization reaction was not fully completed ($p < 1$) due to the steric hindrance between larger AuND assemblies that suppressed the interaction (for instance, $n = 38$ when $r = 1$ and $p = 0.974$). From another perspective, this limitation may reflect the physical constraints of the persistence length in the 1D cylindrical assembly that dispersed in solution under Brownian motions. Then, the multimerization degree was regulated by exceeding the molar ratio of (+)-DNA-AuND over (-)-DNA-AuND. Specifically, the mixing ratio of (+)-DNA-AuND to (-)-DNA-AuND was varied as 60:40 ($r = 2/3$), 67:33 ($r = 1/2$), 75:25 ($r = 1/3$), and 90:10 ($r = 1/9$). As the proportion of (+)-DNA-AuND increased, the plasmonic peak gradually red-shifted and intensified in the extinction spectra (**Figure 4. 16a**). Simultaneously, the hydrodynamic diameter exhibited a decrease in the peak corresponding to translational diffusion and the reappearance of the rotational diffusion peak at 18 nm (**Figure 4. 16b**). In both cases, the peak positions converged to those of individual DNA-AuNDs.

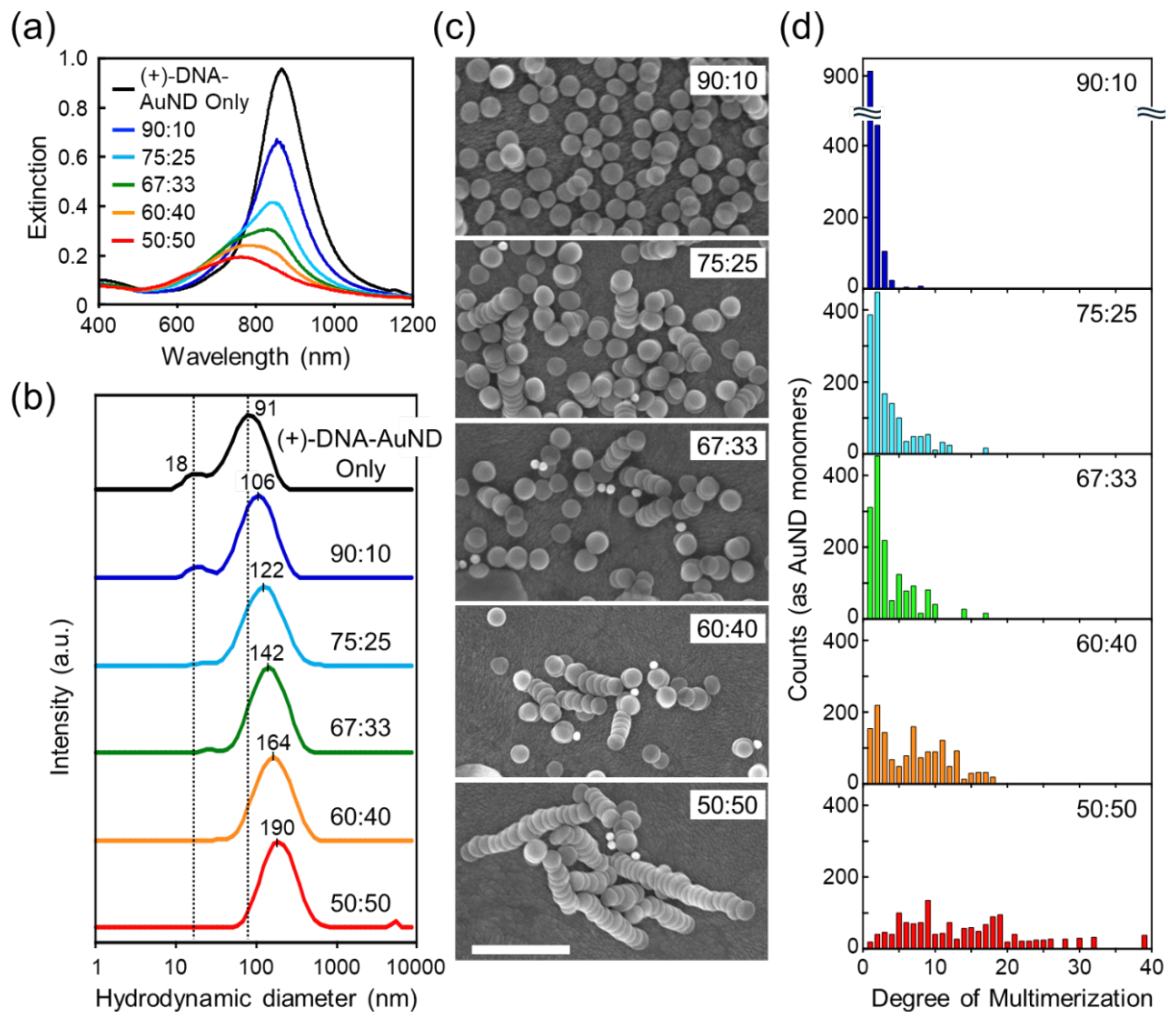


Figure 4. 16. Controlling the degree of AuND multimerization by varying the mixing molar ratios of 11-base (+)-DNA-AuNDs and 11-base (-)-DNA-AuNDs in the buffer solutions containing 150 mM NaCl. (a) Extinction spectra. (b) Hydrodynamic sizes. (c) SEM images. (d) Histograms of multimers obtained from image analysis of (c). The mixing molar ratios of (+)-DNA-AuNDs to (-)-DNA-AuNDs: blue, 90:10; aqua, 75:25; green, 67:33; orange, 60:40; red, 50:50. Black represents (+)-DNA-AuNDs only. Buffer composition: 0.01% SDS, 10 mM KPO₄, pH 7.0.

SEM imaging clearly confirmed that the degree of multimerization decreased as the ratio of (+)-DNA-AuND increased (i.e., as r decreased) (**Figure 4. 16c**). Histograms from SEM image analysis revealed that changes in the multimer distribution (**Figure 4. 16d**). In the case of DNA-AuNDs mixed at 60:40 ($r = 2/3$, $n = 5$), large multimers of more than 20-mers did not form while polydispersity was decreased. DNA-AuNDs mixed at 67:33 ($r = 1/2$, $n = 3$) and 75:25 ($r = 1/3$, $n = 2$) exhibited a significant reduction in longer multimers, with the majority consisting of short oligomers of fewer than 6-mers. DNA-AuNDs mixed at 90:10 ($r = 1/9$, $n = 1.3$) primarily consisted of monomers, with only a few oligomers observed. The abrupt decrease in larger multimers was consistent with the condensation polymerization theory,

although the estimated degree of polymerization was not precise with inconsistency of the values and broader distributions. These results indicated that adjusting the mixing molar ratio of (+)-DNA-AuNDs and (–)-DNA-AuNDs provides a simple means to control the assembly size, thereby enabling the tuning of plasmonic coupling.

Second, assembly sizes were regulated by adjusting salt concentrations. The ionic strength affects the efficiency of DNA hybridization because electrolytes shield the charge repulsion between the negatively charged phosphate groups⁶⁹. Consequently, the assembly of DNA-modified AuNDs driven by DNA hybridization can be modulated by adjusting the salt concentration (**Figure 4. 17a**). To investigate the salt effect, NaCl concentration was varied from 0 to 200 mM in a mixture of (+)-DNA-AuNDs and (–)-DNA-AuNDs at the equimolar ratio (50:50). Without NaCl, the AuNDs showed no plasmonic shift in the extinction spectrum (**Figure 4. 17b**, black) or any change in hydrodynamic size (**Figure 4. 17d**, black) compared to the individual DNA-AuNDs (**Figure 4. 2b,c**). Conversely, increasing the NaCl concentration resulted in a gradual blue shift in the plasmonic peaks (**Figure 4. 17b**) and an increase in the hydrodynamic diameter for translational diffusion, along with the disappearance of the rotational diffusion peak (**Figure 4. 17d**), indicating assembly control through salt concentration. It is noted that the measured temperature (25°C) was lower than each assembly temperature (detail of assembly temperature will be discussed in the next section).

SEM images confirmed that the degree of multimerization increased with increasing NaCl concentration (**Figure 4. 17d**). To evaluate multimerization, I quantified the abundance of multimers in SEM images (500 AuND monomers counted) and plotted histograms of the number of monomers in each multimer (**Figure 4. 17e**). Notably, the SEM distributions revealed larger multimers than those estimated by DLS. For example, assemblies formed at 100 mM NaCl exhibited a broad distribution of large multimers in the SEM images (**Figure 4. 17e**, middle), in contrast to the 67:33 mixed assemblies (**Figure 4. 16d,e**, green), which were predominantly composed of mono-, di-, and trimers. Nevertheless, both samples exhibited the same hydrodynamic diameter of 142 nm according to DLS (**Figure 4. 16c** and **Figure 4. 17d**). This discrepancy is probably due to the remaining accessible stacking surfaces that allowed further multimerization via hybridization in the assemblies formed at 100 mM NaCl, which were prepared by mixing (+)- and (–)-DNA-AuNDs at a 50:50 ratio. These uncapped surfaces may have promoted additional multimerization of the NaCl-induced assemblies during the drying process for SEM sample preparation. In contrast, the 67:33 mixture was capped by excess (+)-DNA-AuNDs, which prevented further association even under salt condensation.

This limitation complicates the accurate evaluation of the degree of multimerization, which I aim to address in future studies. Nonetheless, the salt-induced assembly method does not require sacrificial AuNDs (i.e., excess molar ratios of (+)-DNA-AuNDs) and is particularly advantageous for preparing lower-order AuND oligomers.

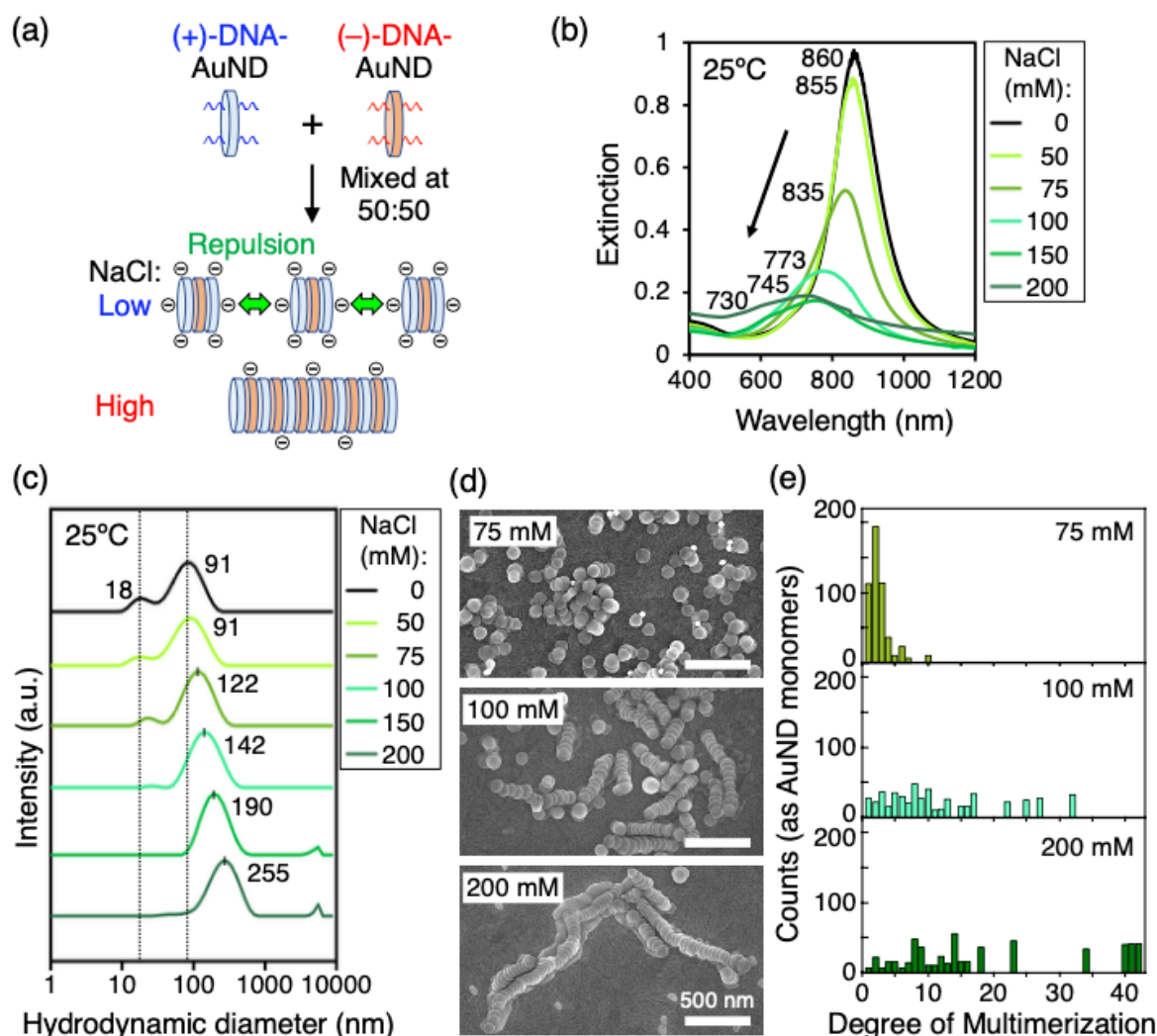


Figure 4.17. Salt concentration-dependent assembly of 11-base (+)-DNA-AuND and 11-base (-)-DNA-AuND mixed at 50:50. (a) Schematic illustration. (b–d) Extinction spectra (b), SEM images (c), and hydrodynamic size measurements (d) of the equimolar mixture in the buffer solutions containing 0–200 mM NaCl at 25°C. Buffer composition: 0.01% SDS, 10 mM KPO₄, pH 7.0.

Since sample preparation potentially affected the assembly formation of SEM images (**Figure 4.16c** and **Figure 4.17c**) due to the increase of NaCl concentrations upon drying and the interaction between nanoparticles and the substrate, the configuration of 67:33 mixing ratio was observed by Cryo-TEM (**Figure S 16**) and compared the histogram with the histogram of SEM images (**Figure 4.18**). Cryo-TEM offers high structural fidelity with minimal sample deformation and allows for more accurate classification of multimeric species due to its three-

dimensional imaging capability. Although the number of assemblies counted in Cryo-TEM was smaller than in SEM due to the experimental constraints—such as the field-of-view limitations and the upper size threshold of detectable assemblies—I analyzed a representative set of images of the 11-bp AuND assemblies mixed at 67:33 ratio. Importantly, the histogram obtained from the Cryo-TEM analysis exhibited a similar trend to that derived from SEM image analysis (**Figure 4. 18**, left), indicating consistency in the observed distribution patterns across methods. While SEM analysis may inherently possess lower precision due to the surface artifacts and projection limitations, the agreement between the two methods supports the validity of the statistical distributions obtained from SEM. Therefore, SEM-based statistical analysis is sufficiently robust to reflect the underlying assembly differences and can be considered valid for discussing comparative trends in the multimer distributions despite the limitations of sample preparation.

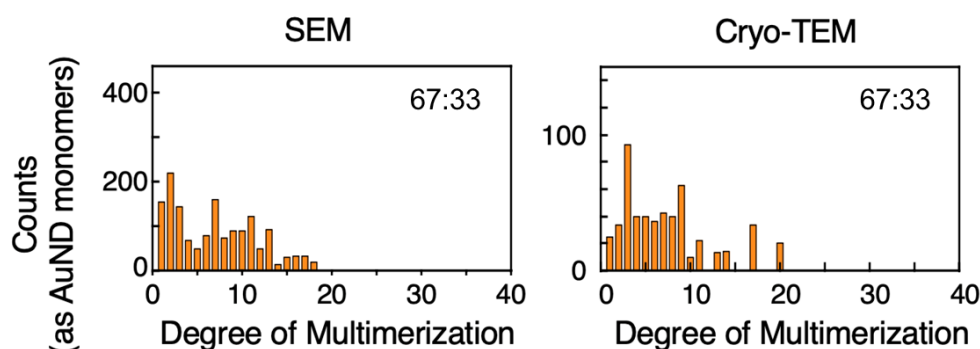


Figure 4. 18. Comparison of the multimer size distributions of 11-bp AuND assemblies mixed at a 67:33 ratio, obtained from SEM and cryo-TEM measurements. Left: histogram derived from SEM images (**Figure 4. 16d**, green). Right: histogram derived from cryo-TEM images (**Figure S 16**).

Then, various NaCl concentrations were applied for 20-base (+)-DNA-AuND and 20-base (–)-DNA-AuND at the equimolar ratio (50:50, $r = 1$). As expected, the assembly sizes increased by increasing NaCl concentrations (**Figure 4. 19b**). Remarkably, higher NaCl concentration was required to produce a similar assembly size of 20-base-DNA-AuND (**Figure 4. 19b**, 400 mM NaCl) than the assembly size of 11-base-DNA-AuND (**Figure 4. 17d**, 150 mM NaCl) due to the stronger electrostatic repulsion forces of longer DNA ligand. In addition, the larger plasmonic shift of 11-base-DNA-AuND (**Figure 4. 17b**, 150 mM NaCl) than 20-base-DNA-AuND (**Figure 4. 19a**, 400 mM NaCl) with comparable hydrodynamic size (190 nm) suggested that shorter interparticle gap distance more effectively enhanced the plasmonic coupling. Further effect of DNA lengths on the assembly properties would be discussed in the following section.

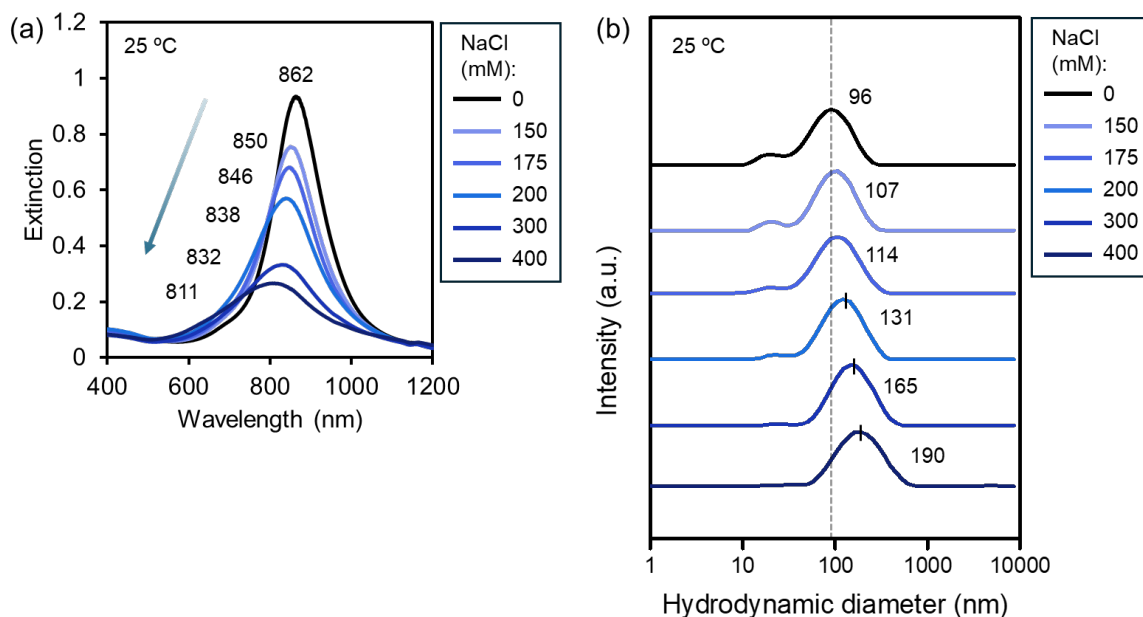


Figure 4. 19. Salt concentration-dependent assembly of 20-base (+)-DNA-AuND and 20-base (-)-DNA-AuND mixed at 50:50. (a) Extinction spectra and (b) hydrodynamic size measurements of the equimolar mixture in the buffer solutions containing 0–400 mM NaCl at 25 °C. Buffer composition: 0.01% SDS, 10 mM KPO₄, pH 7.0.

These findings revealed that salt concentration provides a simple means to control the assembly size, thereby enabling the tuning of plasmonic coupling modulating the strength of the charge repulsion between AuNDs.

4.3.5. Tuning Interparticle Gap Distance of AuND Assembly by Different DNA Lengths

The plasmonic coupling between AuNDs can be tuned by modulating the interparticle gap distances via changing the lengths of DNA ligands. Specifically, three pairs of (+)-DNA and (-)-DNA ligands (8, 11, and 20 bases; all sequences are provided in **Table 4. 1**) were respectively modified onto AuNDs. The AuNDs modified with the 20-base DNA ligands exhibited good dispersion stability even at higher NaCl concentration (400 mM) (**Figure 4. 13a,b**), due to the strong charge repulsion from the larger number of phosphate anions in the longer DNA chains. Conversely, AuNDs modified with the 8-base DNA ligands were only dispersed at lower NaCl concentrations (< 100 mM) due to weaker charge repulsion (**Figure 4. 10a,b**), suggesting a limitation in colloidal stability with shorter DNA ligands. The assemblies of the three pairs of (+)-DNA-AuNDs and (-)-DNA-AuNDs were then conducted by mixing them at the equimolar ratio (50:50) to investigate their plasmonic properties. The assembled sizes of AuNDs were adjusted to a consistent hydrodynamic diameter of 164 nm, as measured by DLS (**Figure 4. 20a**), by varying the NaCl concentration at 25 °C, since size also influences the plasmonic coupling^{5,98}. Notably, the assembly of AuNDs with longer DNA (20 bp) required a higher salt concentration (400 mM) to effectively shield the electrostatic barrier. When the

interparticle gap distance was short with 8 bp-DNA ligands (theoretically <5.1 nm, **Table 3. 2**), the assembly of the AuNDs caused a larger plasmonic shift ($\Delta\lambda$) in the extinction spectra, reaching 98 nm (**Figure 4. 20b,c**, red), compared to 84 nm for AuNDs with 11 bp-DNA ligands (**Figure 4. 20b,c**, green). The significant plasmonic shift correlated to the strong plasmonic coupling of assembly with short interparticle gap distance. Conversely, in the case of 20 bp-DNA ligands (theoretically <9.2 nm, **Table 3. 2**) the plasmonic shift decreased to 49 nm due to the weaker plasmonic coupling resulting from the larger interparticle gap distance (**Figure 4. 20b,c**, orange), while the interparticle gap distance was still smaller than that in previous studies^{13,17,24}. These results revealed that the strong plasmonic coupling of AuNDs with short DNA ligands (<10 nm) sensitively responded to small distance change within a few nanometers, while decreasing of the length of DNA is limited by the colloidal stability of AuNDs. Importantly, this is the first report which successfully promoted Au nanoparticle assemblies by using shortest DNA ligands without linkers and unpaired spacer region between AuND and the base pairings.

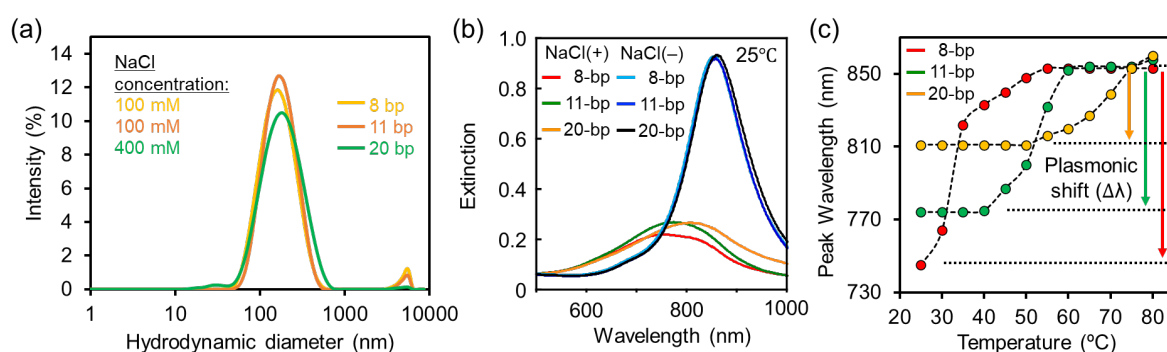


Figure 4. 20. Effects of modulating the interparticle gap distances in AuND assemblies using DNA ligands of three different lengths (8, 11, and 20 bases) on plasmonic coupling. (a) Hydrodynamic sizes of assembled AuNDs driven by hybridization of various DNA lengths and sequences: 8-bp in 100 mM NaCl (yellow), 11-bp in 100 mM NaCl (orange), and 20-bp in 400 mM NaCl (green). Different NaCl concentrations were required to form comparable assembly sizes due to the different electrostatic repulsion forces of different DNA lengths and sequences. (b) Extinction spectra of mixtures of (+)-DNA-AuNDs and (-)-DNA-AuNDs (50:50). Conditions with NaCl: 100 mM for 8-bp (red) and 11-bp (green), and 400 mM for 20-bp (orange). Conditions without NaCl: 8-bp (aqua), 11-bp (blue), and 20-bp (black). (c) Extinction peak wavelengths of the 50:50 mixtures as a function of temperature during the cooling process. DNA ligands: 8-bp (red), 11-bp (green), and 20-bp (orange).

To confirm that plasmonic shift was correlated with the plasmonic coupling, the interparticle gap distances of various DNA lengths were evaluated by Cryo-TEM images (**Figure 4. 21b**). The interparticle gap distance of 20-bp DNA-modified AuNDs was obtained by analyzing 72 interparticle distances across nine different regions under various tilting conditions (**Figure S 17**). The average interparticle gap distance was (8.8 ± 1.7) nm, which is

slightly shorter than the theoretical distance of a 20-bp DNA strand with two spacers (~9.2 nm). The interparticle gap distances observed for both 11-bp and 20-bp DNA are consistent with their respective theoretical values. The larger standard deviation for the 20-bp DNA (1.7 nm) compared to the 11-bp DNA (1.0 nm) is likely due to an increased probability of mismatched base pairing in the longer DNA strands, which contributes to greater variation in interparticle distances⁷⁸. Unfortunately, the interparticle distance for 8-bp DNA was difficult to identify due to the aggregation induced by weak repulsion forces of short DNA ligand (**Figure S 18**). The interparticle gap distance of 8-bp DNA-modified AuNDs was obtained by analyzing only 9 interparticle distances which did not meet the statistical requirements. These results suggest that the strong plasmonic coupling of AuNDs with short DNA ligands (<10 nm) are sensitive to the small changes of interparticle distance, within a few nanometers, but the decrease of DNA length is limited by the colloidal stability of AuNDs.

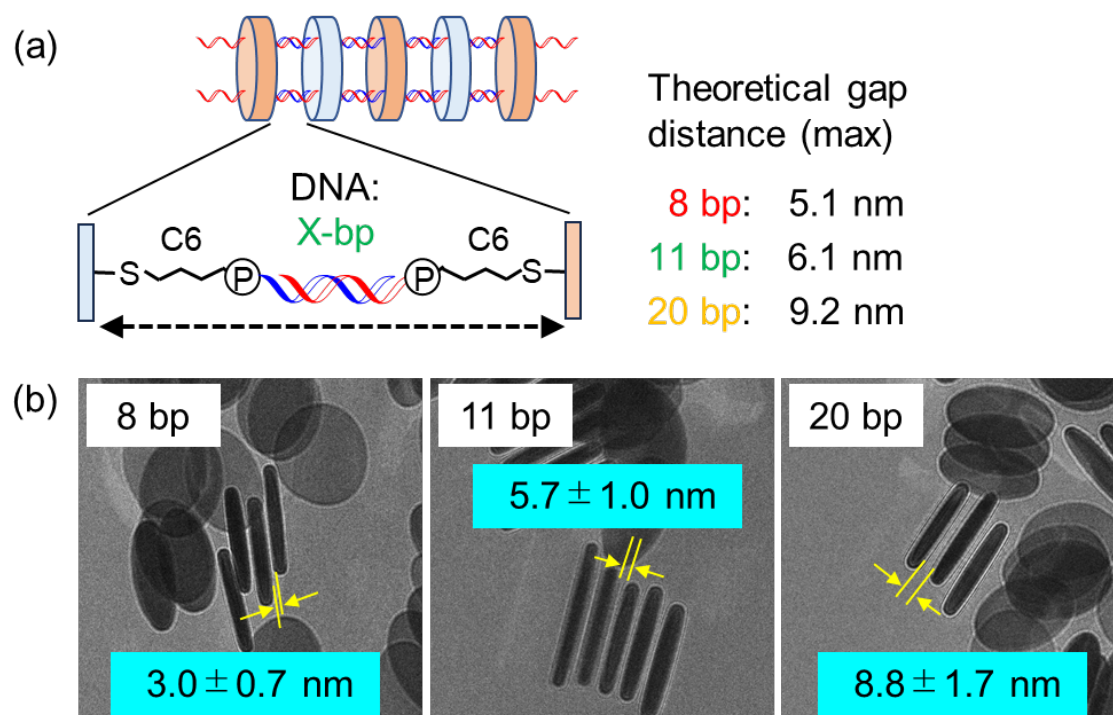


Figure 4. 21. (a) Schematic illustration of AuND assembly with various interparticle gap distances. (b) Cryo-TEM images of assembled AuND with different DNA lengths: 8, 11, and 20 bp. The assembly was obtained for 67:33 mixing ratio in different NaCl concentrations: 8 bp (100 mM NaCl), 11 bp (150 mM NaCl), and 20 bp (400 mM NaCl).

4.3.6. Kinetic and Thermodynamic Effects on AuNDs Assemblies

Since assembly is affected by kinetics and thermodynamics, a sufficient incubation time is required to exclude the kinetics effect. Therefore, the effect of incubation time on AuNDs dissociation was investigated by measuring the extinction intensity at the specific temperature every 15 min up to 60 min. Generally, the plot exhibited a significant increase in the extinction

intensity within 15 min, indicating that 15 min of waiting time upon cooling and heating was enough to equilibrate at each temperature and eliminate the effect of kinetic trapping. However, small increase of the extinction intensity appeared in the longer waiting time of 30 min for 11 bp-DNA-AuND in 75 mM NaCl (**Figure 4. 22b**) and 20 bp-DNA-AuND (**Figure 4. 22d**), suggesting that samples with higher electrostatic repulsion forces required longer incubation time to reach equilibrium state. These results are understandable since ionic strength affects the melting temperature, hybridization potential, and kinetics of assembly ¹⁰².

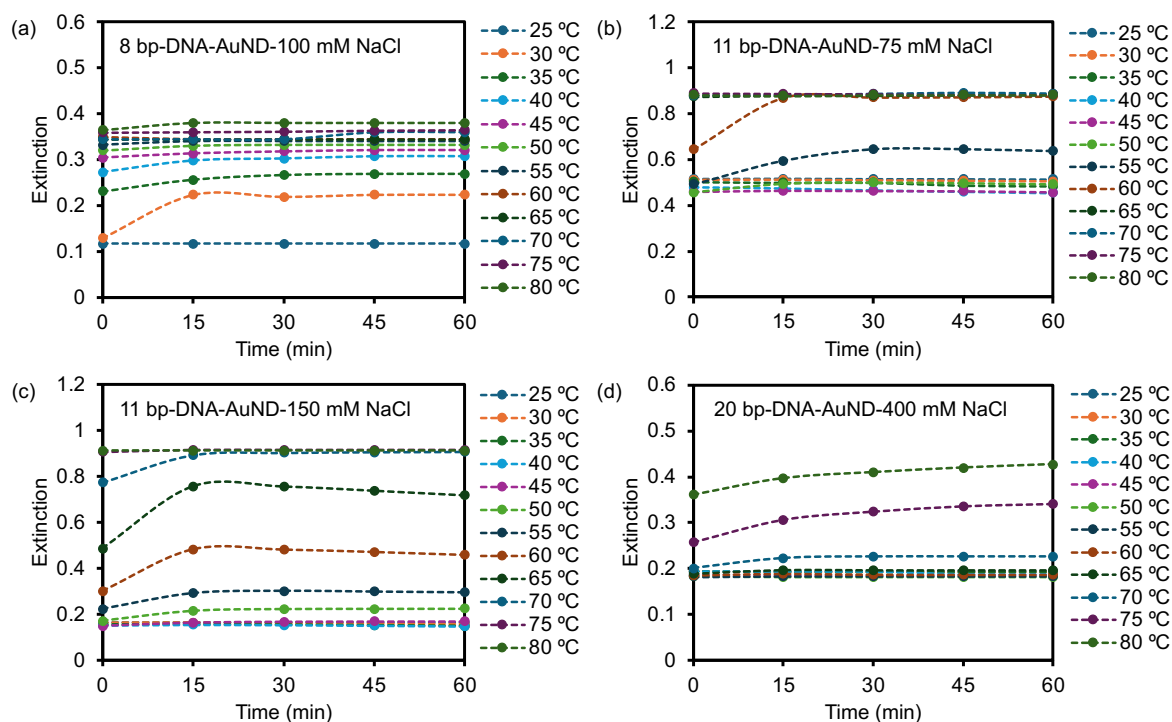


Figure 4. 22. The plot of changes in extinction during the time-dependent measurement of (a) 8-bp-DNA-AuND in 100 mM NaCl, (b) 11-bp-DNA-AuND in 75 mM NaCl, (c) 11-bp-DNA-AuND in 150 mM NaCl, and (d) 20-bp-DNA-AuND in 400 mM NaCl.

To reduce the kinetic effect, assembly of 11-base-(+)-DNA-AuND and 11-base-(−)-DNA-AuND with various mixing ratios (50:50, 60:40, and 67:33) was conducted by reducing the cooling rate from 0.25 °C/min to 0.08 °C/min. Compared to the assembly with 0.25 °C/min of cooling rate (**Figure 4. 16**), samples with 0.08 °C/min of cooling rate exhibited lower extinction intensities (**Figure 4. 23a** vs. **Figure 4. 16a**) and larger assembly sizes (**Figure 4. 23b** vs. **Figure 4. 16b**). Most assemblies with 0.08 °C/min of cooling rate exhibited similar multimer distributions as 0.25 °C/min of cooling rate (**Figure 4. 23c,d** vs. **Figure 4. 16c,d**), indicating that kinetic trapping is largely avoided even at the faster rate. In contrast, a minor fraction of assemblies showed higher multimerization degrees under the slower cooling rate (**Figure 4. 23d**). These multimers likely originated from localized clusters that were kinetically

hindered from further growth by electrostatic repulsion under faster cooling rate due to the electrostatic repulsion. By decreasing the cooling rate, these assemblies had more time to overcome the kinetic barriers, leading to the formation of larger multimers. These results suggested that the standard annealing condition (0.25 °C/min) was sufficient to reflect near-equilibrium multimer distributions for most of the sample, while also revealing that a small subpopulation may still be subject to kinetic limitations. These findings provide deeper insight into the interplay between kinetic and thermodynamic factors in the multimerization process.

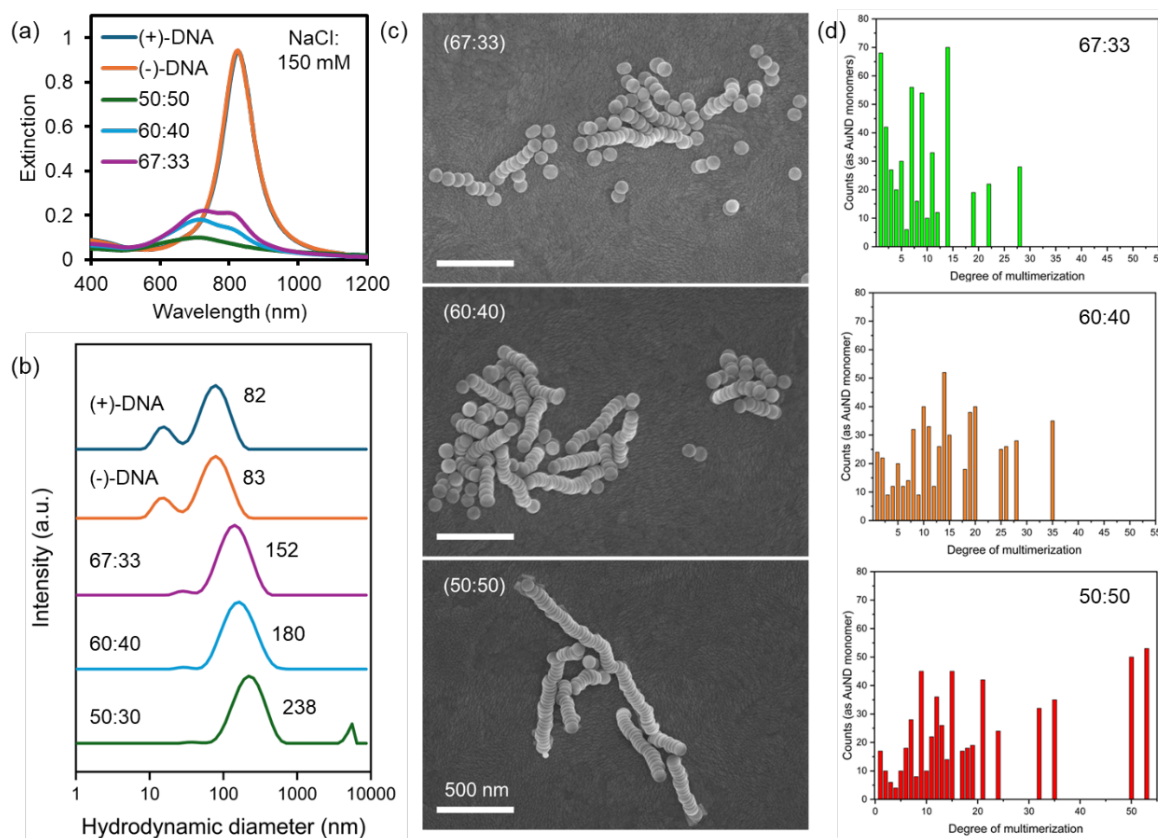


Figure 4. 23. The degree of AuND multimerization by varying the mixing molar ratios of 11-base (+)-DNA-AuNDs and 11-base (–)-DNA-AuNDs in the buffer solutions containing 150 mM NaCl with slower cooling rate (0.1 °C/min). (a) Extinction spectra. (b) Hydrodynamic size measurement. (c) SEM images. (d) Histograms of multimers obtained from image analysis by calculating 500 particles. The mixing molar ratio of 11-base-(+)-DNA-AuND to 11-base(–)-DNA-AuND: purple (67:33), blue (60:40), and green (50:50). Dark blue and orange represent (+)-DNA-AuNDs and (–)-DNA-AuNDs only, respectively.

4.3.7. Tuning Assembly and Disassembly Temperatures of Assembled AuNDs

Assembly (T_a) and disassembly (T_d) temperatures of DNA-AuNDs can be tuned by two methods: (i) adjusting salt concentration of colloidal solution and (ii) changing the length of DNA ligands. This tunability is potentially useful for the precise thermo-responsive control of biomedical and therapeutic materials¹⁰³. To determine the T_a and T_d , the extinction intensity of

plasmonic peaks of DNA-AuNDs was measured as a function of temperature during the heating and cooling processes, according to a previously reported method²⁴ (detailed calculation method was presented in **Figure S 19**). First, T_a and T_d of AuNDs modified with 11-bp DNA ligands were modulated by adjusting salt concentrations. To investigate the change of T_d , the assembled DNA-AuNDs were heated from 25 to 80°C in 5°C increments in the buffer solutions with varying NaCl concentrations (75-200 mM). It is noted that the extinction spectra were measured after allowing sufficient time (15 min) for thermal equilibrium at each temperature (**Figure 4. 22**). As a result, the plasmonic peaks increased in intensity under all conditions and eventually returned to the initial position, corresponding to the monomer state (**Figure 4. 24a**, red curves), indicating disassembly. The observed T_d gradually increased with the increasing of NaCl concentration (**Figure 4. 24a,b**: 75 mM, 55°C; 100 mM, 59°C; 150 mM, 62°C; 200 mM, 65°C), consistent with the general effect of salt concentration on the melting temperatures (T_m) of free DNA. These results reveal that the disassembly temperature of DNA-AuNDs can be finely tuned by adjusting the salt concentration. Subsequently, the T_a was determined by gradually lowering the temperature to 25 °C in 5°C increments, allowing equilibrium at each temperature before measurements (**Figure 4. 24a**, blue curves). Interestingly, under the lowest NaCl concentration (75 mM), a T_a of 48°C was observed (**Figure 4. 24a,b**), which was lower than the corresponding T_d of 55°C, indicating thermal hysteresis ($\Delta T = T_d - T_a = 7^\circ\text{C}$). In contrast, at the highest NaCl concentration (200 mM), no hysteresis was observed ($T_a = 65^\circ\text{C}$, $\Delta T = 0^\circ\text{C}$; **Figure 4. 24a,b**). Intermediate NaCl concentrations (100 and 150 mM) exhibited slight thermal hysteresis of 3°C and 1°C, respectively, showing gradual decrease as the NaCl concentration increased. The observed hysteresis is likely attributed to reduce the electrostatic shielding at low ionic strength, which increases repulsion between the negatively charged phosphate groups, thereby hindering DNA hybridization and DNA-AuND assembly.

To examine whether this thermal hysteresis—presumably caused by insufficient electrostatic shielding—represents a kinetically trapped state, the incubation times of 11-bp DNA at low salt concentration (75 mM NaCl) were extended (15, 30, and 60 min) and correspondingly reduced the heating and cooling rates (0.25, 0.15, and 0.08 °C/min). This condition was selected because it showed gradual changes in the extinction over time, suggesting the kinetically hindered assembly/disassembly. The hysteresis between disassembly and assembly curves was significantly reduced with extended incubation ($\Delta T = 2^\circ\text{C}$ at 0.08 °C/min; **Figure 4. 24c,d**), indicating that the kinetic traps were largely eliminated over

time. This supports the idea that electrostatic repulsion is the primary contributor to the kinetic delay under this condition.

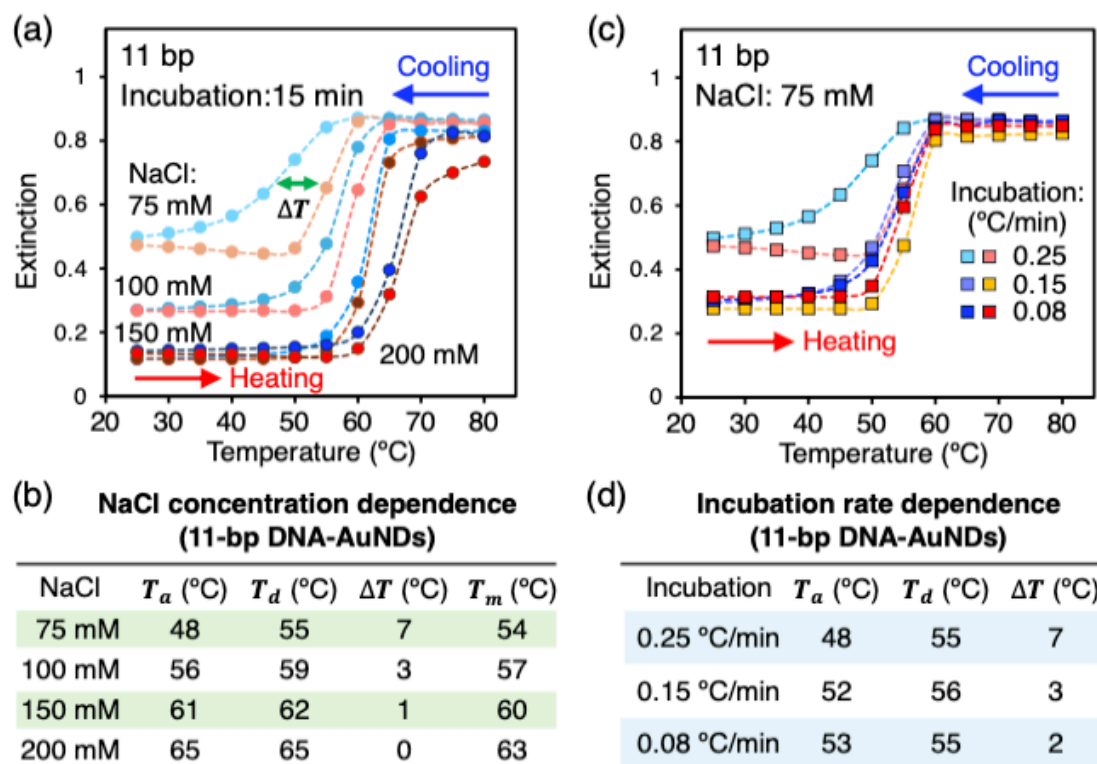


Figure 4. 24. (a, c) Extinction spectra obtained under varying conditions: (a) NaCl concentrations of 75, 100, 150, and 200 mM at a heating/cooling rate of 0.25 °C/min; and (c) heating/cooling rates of 0.25, 0.15, and 0.08 °C/min at 75 mM NaCl. Red curves: heating process; blue curves: cooling process. (b, d) Summary tables of the assembly (T_a) and disassembly (T_d) temperatures of the 11-bp DNA-AuND mixtures, along with the corresponding thermal hysteresis (ΔT): (b) obtained from (a) and (d) obtained from (c).

Second, T_a and T_d of DNA-AuNDs were modulated by adjusting the length of DNA ligands. Specifically, T_a and T_d of AuNDs modified with the DNA ligands of three different lengths (8, 11, and 20 bases) were determined using the same method described above, with consistent assembled sizes confirmed by DLS measurements (**Figure 4. 20a**). The obtained T_d value (**Figure 4. 25a-c**, blue) increased with increasing DNA ligand length (**Figure 4. 25a**: 8 bp, 38°C; **Figure 4. 25b**: 11 bp, 59°C; **Figure 4. 25c**: 20 bp, 80°C), consistent with the melting temperature of free DNA ligands (T_m) (**Figure 4. 27**). These results demonstrate that the disassembly temperature of DNA-AuNDs can be effectively tuned by modifying the DNA ligand length. In contrast, the measured T_a exhibited a significant thermal hysteresis ($\Delta T = 13^\circ\text{C}$) for 20-bp DNA-AuNDs, with a lower T_a of 67°C compared to its T_d of 80°C (**Figure 4. 25c**), even at a high NaCl concentration (400 mM). In comparison, 11-bp DNA-AuNDs exhibited a similar T_a of 56°C (**Figure 4. 25b**) to its T_d , with only slight thermal hysteresis (ΔT

= 3°C), while the 8-bp DNA-AuNDs showed no hysteresis ($T_a = 38^\circ\text{C}$, $\Delta T = 0^\circ\text{C}$, **Figure 4. 25a**). To further investigate the effects of DNA sequence on the hysteresis, T_a and T_d were measured using AuNDs modified with 11-bp DNA ligands composed of repetitive A and T sequences (**Figure 4. 25d**). The result showed a large hysteresis ($\Delta T = 15^\circ\text{C}$) even with the shorter ligands than 20-bp. Based on these results, the hysteresis is attributed to two factors. The first is strong electrostatic repulsions between the phosphates of DNA ligands on AuNDs, which is particularly significant for long DNA ligands. The second is increased probabilities of base-pairing during non-fully matched hybridization, which expand combination patterns between DNA strands on AuNDs, leading to greater entropic expansion. These factors likely create various metastable states and energy barriers, preventing the system from transitioning to its thermodynamically stable state—a fully overlapped configuration of AuNDs with perfectly matched DNA hybridization—thereby inducing critical undercooling in the colloidal crystals.²⁴

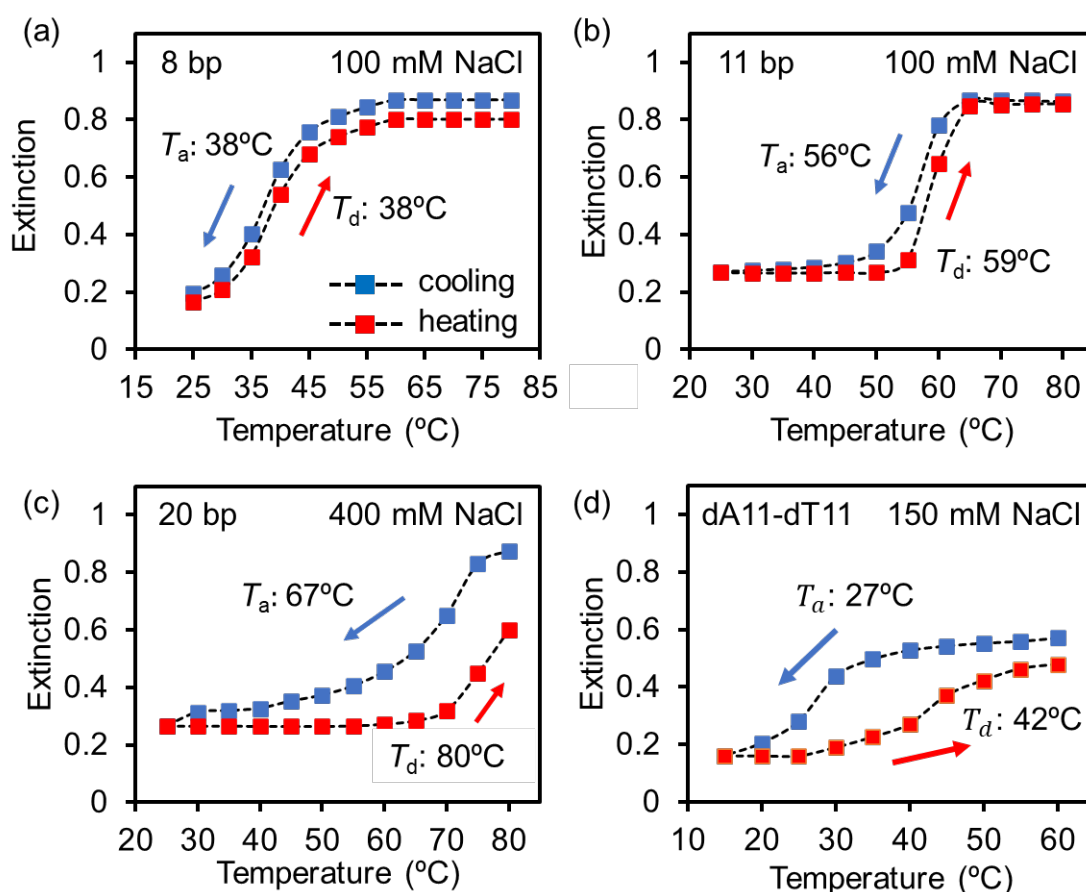


Figure 4. 25. Extinction spectra of 50:50 mixtures of AuNDs modified with DNA ligands of different lengths, plotted as a function of temperature. (a–d) Extinction spectra obtained at a heating/cooling rate of 0.25 °C/min for: (a) 8-bp DNA; (b) 11-bp DNA; (c) 20-bp DNA; and (d) 11-bp DNA composed of repetitive A and T bases. NaCl concentrations: (a, b) 100 mM; (c) 400 mM; and (d) 150 mM. Buffer: 10 mM phosphate (pH 7.0) containing 0.01% SDS.

To investigate whether the thermal hysteresis induced by elongating DNA ligands represents a kinetically trapped state, the heating and cooling rates of 20-bp DNA were reduced (0.25, 0.15, and 0.08 °C/min). The hysteresis persisted even after prolonged incubation ($\Delta T = 10$ °C at 0.08 °C/min; **Figure 4. 26a,b**). This finding suggests that the kinetic barrier is dominated by the time required for geometrical reconfiguration of AuND-bound DNA strands to form stable multivalent duplexes—a process influenced more by thermodynamic optimization than by electrostatic repulsion. Based on these results, there are two key factors which contribute to the observed kinetic barrier: (1) strong electrostatic repulsion between the negatively charged phosphate backbones of the DNA ligands on the AuND surfaces, and (2) a need for geometrical realignment to maximize the number of DNA base pairs formed between particle surfaces, particularly in cases of partially mismatched hybridization.

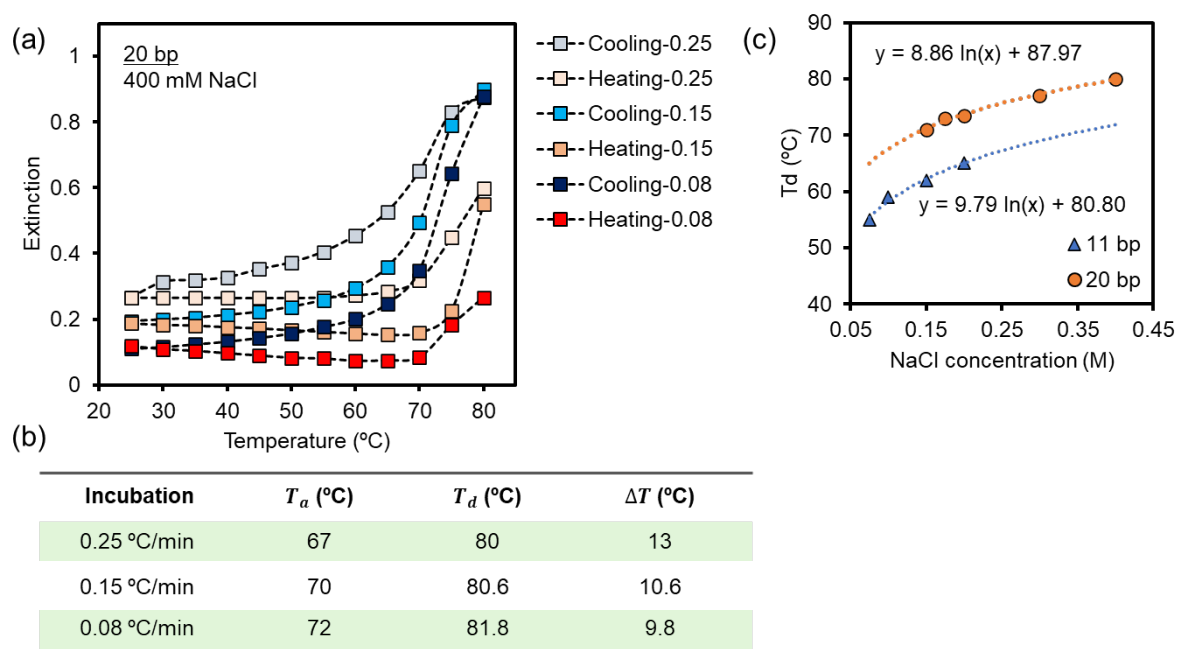


Figure 4. 26. (a) Extinction spectra of 50:50 mixtures of AuNDs modified with 20-bp DNA ligand at different heating/cooling rates: 0.25, 0.15, and 0.08 °C/min. (b) Summary table of the assembly (T_a) and disassembly (T_d) temperatures, as well as the corresponding thermal hysteresis (ΔT), obtained from (a). (c) Plot of T_d as a function of salt concentration for 11-bp DNA-AuNDs (blue) and 20-bp DNA-AuNDs at 0.25 °C/min of heating rate.

T_d represented the hybridization of free energy in DNA-mediated assembly gold nanoparticles. As an example, higher T_d of AuNTs was associated with the higher thermodynamic parameters, including the DNA hybridization free energy than AuNPs which presented lower T_d ²⁵. Therefore, the increase of DNA hybridization free energy could be estimated by enhancing the salt concentrations without the quantitative results. The plot of T_d as a function of salt concentration presents insignificantly different values of $dT_d/d \ln[Na^+]$

for 11-bp and 20-bp (**Figure 4. 26c**, 9.79 vs. 8.86), same as previous study which reported that $dT_d/d \ln[Na^+]$ was independent of DNA length for the duplexes with similar GC content¹⁰⁴. For DNA with different base pairs (11- and 20-bp) but the same fraction of GC bases (55%), T_d increases because of the larger number of hydrogen bonding as discussed in previous study¹⁰⁵. These observations qualitatively support the notion that the higher T_d values observed at elevated salt concentrations correspond to the increased DNA hybridization free energy, which would be expected to promote the formation of larger or more stable assemblies. In principle, increasing salt concentration screens electrostatic repulsion between negatively charged DNA backbones, thereby enhancing duplex stability, which results in higher the T_m (and T_d) values.

In this research, T_d (**Figure 4. 25**) is similar as T_m (**Figure 4. 27**), in contrary to the previous reports^{9,93} which exhibited much higher T_d than T_m . This result can be explained by two reasons. First, lower DNA grafting density (0.013 strands/nm²), which is four times lower than previous report with a comparable size of Au nanoplate⁹, inducing a similar behaviour of immobilized DNA as free DNA in the solution. Low grafting density also enhanced the effectiveness of hybridization due to the low steric hindrance, enabling the hybridization of short DNA ligand on the flat surface without spacer or additional linker. Second, short DNA ligands (<10 nm) with the absence of spacer reduced the flexibility of DNA ligands, significantly reducing the slipping effect of non-Watson-Crick base pairings⁷⁸. Compared to previous reports, assemblies of Au nanoplates that utilize a pair of DNA anchors for direct hybridization, showed a larger discrepancy from the corresponding T_m values.

- (i) Spacer: 20 bases (complementary region: 3 bases); T_d : 81.5 °C, T_m : < 22 °C⁷⁸
- (ii) Spacer: 10 bases (complementary region: 15 bases); T_d : 60.1 °C, T_m : ca. 45 °C⁹
- (iii) Spacer: 0 bases (complementary region: 11 bases); T_d : 62 °C, T_m : 61 °C (this research)

It shows that the spacer length affects the balance between the suppression and promotion of hybridization among DNA ligands immobilized on Au nanoplates. Flat surface of Au nanoplate reduced the DNA ligand density which reduced the steric hindrance, allowing access the complementary DNA strands on neighbouring nanoparticles and increasing the hybridization efficiency⁸⁴. The geometric characteristic facilitates DNA-directed assembly even in the absence of spacer sequences. On the other hand, spacer increased DNA flexibility, enhancing the probability of encountering the complementary strand which contributed to the multivalent hybridization and the dynamic reconfiguration of DNA duplexes, increasing the synergistic effects that increased T_d . This hypothesis was supported by changing the random

sequences with repetitive sequences (dA-dT) of the same length and design. As a result, T_d (**Figure 4. 25d**) is much higher than T_m (**Figure 4. 27f**) because slipping effect is more significant in the repetitive sequence. These results suggest that grafting density and design of DNA ligands greatly affected the assembly and disassembly behaviours of nanoparticles.

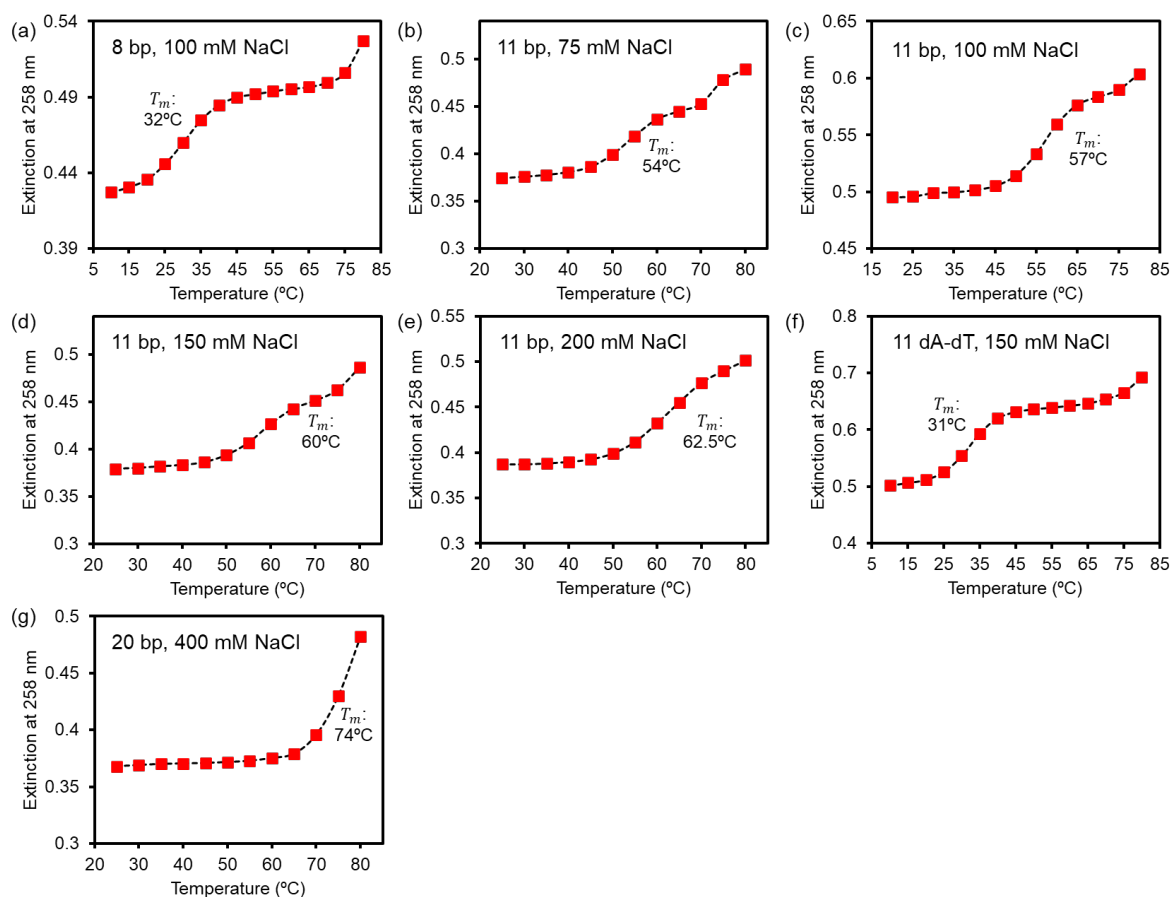


Figure 4. 27. Melting temperatures of free DNA. (a) 8 bp in 100 mM NaCl. (b-e) 11 bp in various NaCl concentrations: (b) 75 mM, (c) 100 mM, (d) 150 mM, and (e) 200 mM. (f) 11 bp of repetitive sequence in 150 mM NaCl. (g) 20 bp in 400 mM NaCl. Solution contained of 0.01% SDS, 10 mM KPO₄, pH 7.0.

Lastly, T_a and T_d of AuNDs modified with 20-bp DNA ligands were modulated by adjusting salt concentrations to examine the effect of strong repulsion force and geometrical realignment to maximize the number of DNA base pairs formed between particle surfaces. As the behaviours of 11-bp DNA-AuND explained above (**Figure 4. 24a,b**), T_d increased by increasing NaCl concentrations due to the stabilization of hydrogen bonding (**Figure 4. 28**). By reducing NaCl concentrations, T_a was gradually lowered than T_d , increasing the thermal hysteresis due to the enhancement of electrostatic repulsion forces. Thermal hysteresis is persistent in high NaCl concentration (400 mM) because 20-bp DNA requires a longer time for geometrical realignment to maximize the number of DNA base pairs formed between

particle surfaces. However, thermal hysteresis only slightly reduced by slower the heating and cooling rates (**Figure 4. 26a,b**), suggesting that the effect of electrostatic barrier is more significant than the geometrical realignment.

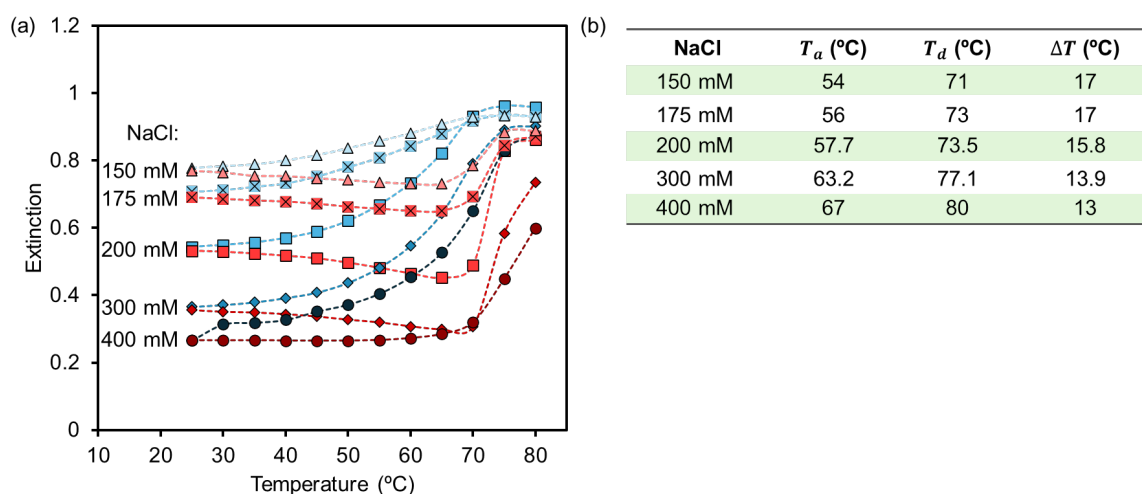


Figure 4. 28. (a) Extinction spectra of AuNDs assembly with 20-bp DNA ligand under various NaCl concentrations (150, 175, 200, 300, and 400 mM) at 0.25 °C/min of heating/cooling rate. Red curves: heating process; blue curves: cooling process. (b) Summary tables of the assembly (T_a) and disassembly (T_d) temperatures of the 20-bp DNA-AuND mixtures, along with the corresponding thermal hysteresis (ΔT) obtained from (a).

4.3.8. Separation of Assembled AuNDs by Gel-electrophoresis

Electrophoretic separation has been used to separate the hetero-structured AuNPs assemblies based on the cluster sizes¹⁰⁶. Here, agarose gel electrophoresis was performed to validate the multimeric assembly of gold nanodiscs (AuNDs). Three AuND samples (11-base (+)-DNA-AuNDs, 11-base (–)-DNA-AuNDs, and assembled AuNDs in 200 mM NaCl) were loaded onto 1 wt% agarose gel and run in 0.5× TAE buffer for 1 hour. All three AuND types migrated toward the positive electrode under the applied electric field. However, undistinguishable migration was observed between the assembled AuNDs and the monomeric controls (**Figure 4. 29a**). This result suggests that the assembled AuNDs dissociated into monomers during electrophoresis, likely due to the low ionic strength of the 0.5× TAE buffer, which weakens DNA hybridization. Assembly could not occur between the (+)- and (–)-DNA-AuNDs in the running buffer without NaCl (**Figure 4. 29b**), supporting the dissociation of assembled AuNDs in agarose gel. To prevent the dissociation of AuNDs assembly, electrophoretic separation was conducted in the buffer with 150 mM NaCl to maintain DNA hybridization. However, electrophoresis could not be stably conducted under high-salt concentration due to the limitations of the gel system and instrument setup.

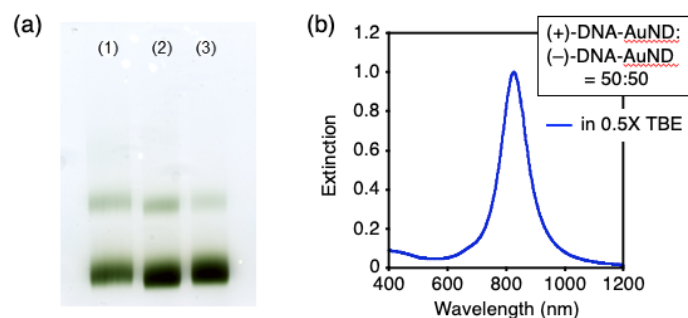


Figure 4. 29. (a) Photograph of a 1 wt% agarose gel after electrophoresis in 0.5× TAE buffer for 1 h, showing the following samples: (1) assembled 11-bp AuNDs prepared at a 1:1 mixing ratio in 200 mM NaCl; (2) 11-base (–)-DNA-AuNDs; and (3) 11-base (+)-DNA-AuNDs. The assembled AuNDs dissociated in low salt concentration. (b) Extinction spectrum of 1:1 mixing ratio in 0.5xTBE only.

4.3.9. Size-based Fractionation of AuND Multimer via Centrifugal Separation

Since the electrophoretic separation remains a technical challenge, another method is required to separate the sizes and broad population of oligomers which were inevitable due to the limited kinetics control during the assembly formation. Thus, multiple fractionations within assembled AuNDs would be separated into different clusters by applying different centrifugal forces. During centrifugation, larger colloidal particles sediment as a precipitate under higher centrifugal force, while smaller particles remain in the upper supernatant, enabling size-based separation. To demonstrate this, the 67:33 mixture ratio was selected which contained of a broad range of AuND multimers (**Figure 4. 16d**). Firstly, the mixture was centrifuged at the highest centrifugal force of 1000g, separating the supernatant, which contained lower-degree oligomers, from the precipitate. The precipitate was then redispersed in the same solution and subjected to centrifugation at 500g to further separate the supernatant and precipitate. This process was repeated with progressively decreasing the centrifugal forces, ultimately yielding seven fractions (supernatants from 1000g, 500g, 400g, 300g, 200g, and 100g, as well as the precipitate from 100g). Each fraction was analyzed using extinction spectroscopy, DLS, and SEM. The supernatant from 1000g exhibited a sharper extinction spectrum with a peak at a longer wavelength (850 nm) compared to the sample before separation (**Figure 4. 30a**). In the DLS spectra, the clear rotational diffusion peak at 18 nm was observed, along with a lower translational diffusion peak at 106 nm (**Figure 4. 30b**). Additionally, a histogram from SEM image analysis (**Figure S 9**) confirmed that the AuNDs primarily existed as monomers, with only a few dimers present (**Figure 4. 30c**). These results indicated that AuND monomers could be isolated in this fraction.

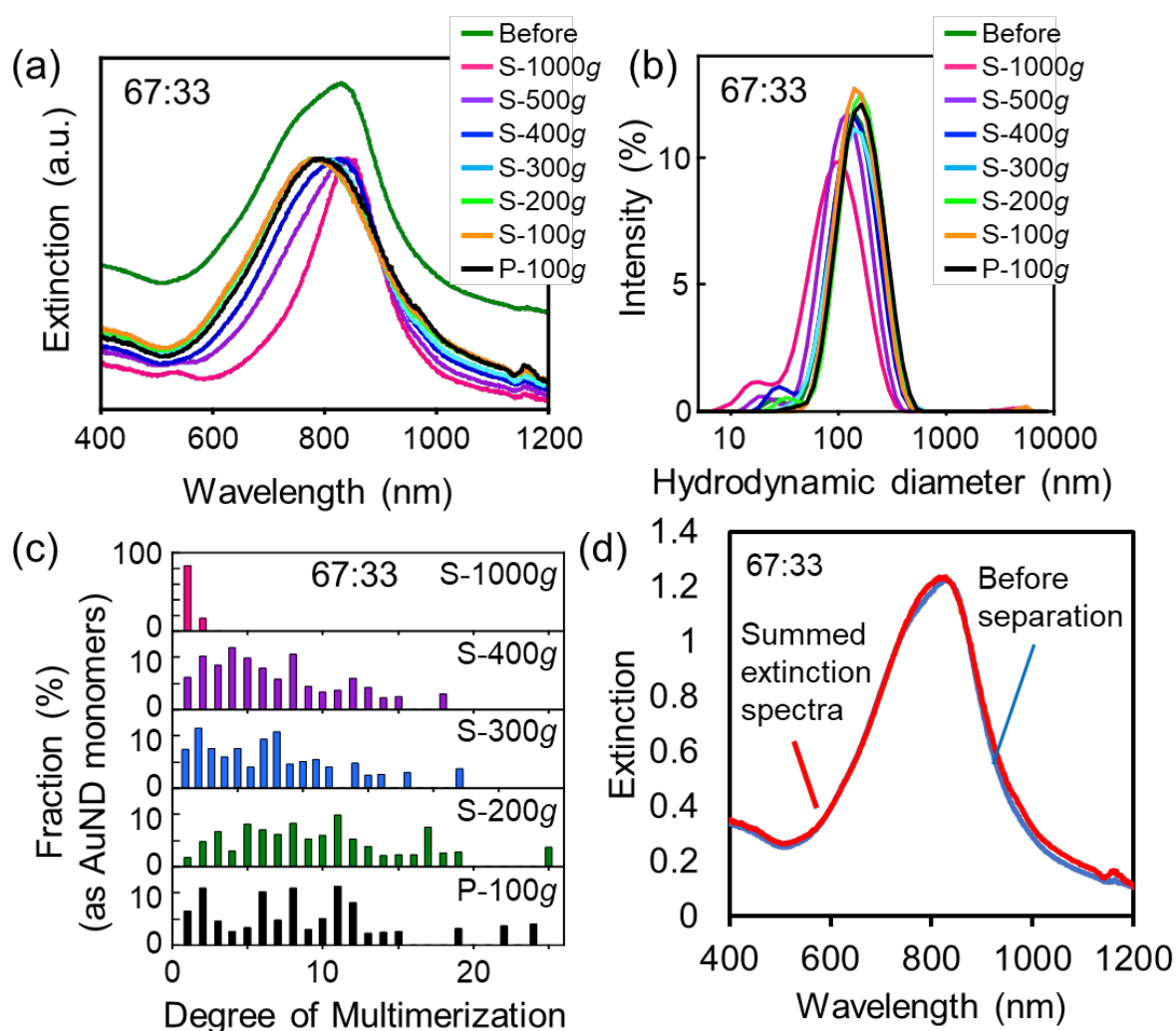


Figure 4. 30. Size-based fractionation of AuND multimers from assembled 11-bp DNA-AuNDs via centrifugal separation. (a–c) Characterization of fractions from the 67:33 mixture using (a) extinction spectroscopy, (b) DLS measurement, and (c) histograms of multimers obtained from SEM image analysis. Note: Representative histograms are shown in (c); for full histograms, see **Figure S9-15**. (d) Extinction spectrum of multimerization with 67:33 molar mixing ratio before centrifugal separation (blue) and the summed extinction spectrum of all seven fractions (red) in 150 mM NaCl at 25 °C. Note: S and P denote the supernatant and precipitate, respectively. The gravity conditions for sample preparation are indicated in the figures.

Supernatants obtained at decreasing centrifugal forces (500g, 400g, and 300g) exhibited a progressive broadening of the extinction peaks, accompanied by a blue shift (**Figure 4. 30a**). The hydrodynamic diameters for translational diffusion gradually increased, while the rotational diffusion peaks diminished in the DLS spectra (**Figure 4. 30b**). In histograms from SEM image analysis (**Figure S 9-15**), the supernatant at 400g showed a broad peak centered at 4-mers, whereas in the case of 300g, the distribution shifted toward larger oligomers, with an emerging peak corresponding to 8-mers (**Figure 4. 30c**). Monomers were eliminated in both cases. Meanwhile, the supernatants from 200g and 100g, as well as the precipitate from 100g,

all of which indicated larger oligomers contents, exhibited no significant differences in extinction peaks or hydrodynamic diameters (**Figure 4. 30a,b**). Histograms from SEM image analysis showed increased polydispersity and a shift in distribution toward larger multimers (**Figure 4. 30c**). Each separated multimer maintained its size without reorganizing the size distribution, indicating that the AuND assemblies were thermodynamically stable and not in dynamic equilibrium. Notably, the summed extinction spectrum of all seven fractions overlapped completely with the plasmonic spectrum before separation (**Figure 4. 30d**), demonstrating that this method enables size-based separation of AuND assemblies without loss. These results demonstrated that AuND assemblies were fractionated in order of increasing multimerization degree simply by varying centrifugal forces, although individual multimers were not completely distinct from one another.

Then, the size-based fractionation was applied to the mixture of 60:40, which also contained a broad range of AuND multimers (**Figure 4. 16d**), and compared the amounts of AuND multimers in individual fractions with those from the 67:33 mixture. Seven fractions from the 60:40 mixture were prepared in the same manner and evaluated them using DLS (**Figure 4. 31a**) and extinction spectroscopy (**Figure 4. 31b,c**). The hydrodynamic diameters of the fractions from the 60:40 mixture were comparable to those from the 67:33 mixture (**Figure 4. 31d-j**). Additionally, the wavelengths of the extinction peaks, which correspond to the degree of multimerization, were consistent with each other (**Figure 4. 31c**, x-axis), indicating that multimers of similar sizes were fractionated. In contrast, the intensities of the extinction peaks, which correspond to the amounts of AuND multimers, showed significant differences between the 60:40 and 67:33 mixtures (**Figure 4. 31c**, y-axis). Specifically, the fractions from the 67:33 mixture exhibited relatively consistent extinction intensities (approximately 0.2), except for the fraction from P-100g, which contained the largest multimers and showed a lower intensity (0.13). Conversely, the fractions from the 60:40 mixture exhibited variations in intensity, with a particularly higher intensity in the fraction from P-100g (0.30) corresponding the largest multimers and a lower intensity in the fraction from S-1000g (0.09) containing primarily AuND monomers. These trends were correlated with overall histogram of the distribution (**Figure 4. 16d**), suggesting that the distribution of AuND multimers can be estimated by combining centrifugal separation and extinction spectroscopy.

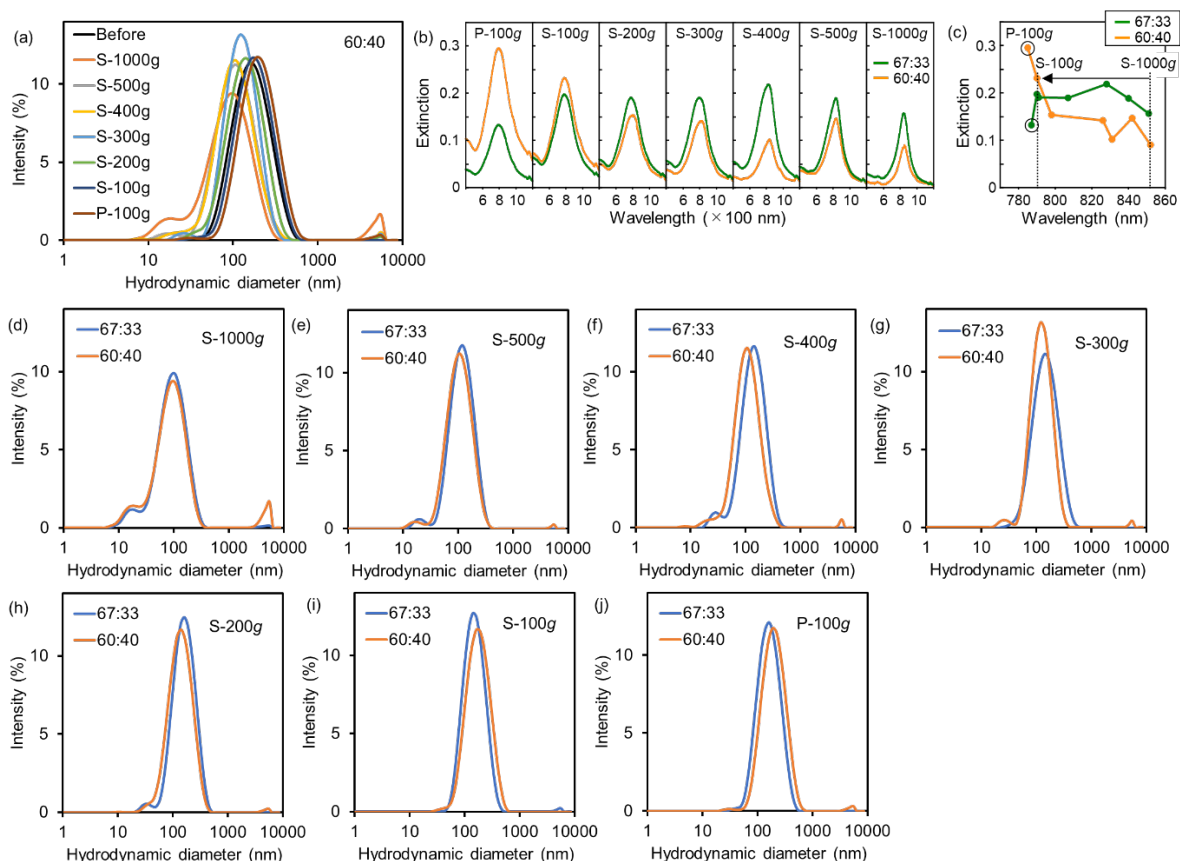


Figure 4.31. Size-based fractionation of AuND multimers from assembled 11-bp DNA-AuNDs via centrifugal separation. (a) Characterization of fractions from the 60:40 mixture using DLS measurement. (b-c) Comparison of fractions from the 67:33 and 60:40 mixtures using extinction spectroscopy. (b) Extinction spectra of the seven fractions. Green: 67:33. Orange: 60:40. (c) Plots of extinction peak intensities and wavelengths for the seven fractions in (b). (d-j) Comparison of hydrodynamic diameters between supernatants of 67:33 and 60:40 molar mixing ratios after separation by applying (d) 1000g, (e) 500g, (f) 400g, (g) 300g, (h) 200g, (i) 100g, including (j) precipitation of 100g. Note: S and P denote the supernatant and precipitate, respectively. The gravity conditions for sample preparation are indicated in the figures.

4.3.10. Crosslinking of Assembled AuNDs with Psoralen Molecule or HS-PEG-HS

In the previous sections, gel electrophoresis and centrifugal separation failed to isolate assemblies with defined oligomer numbers due to the challenges described above. It was correlated to the reversible assembly and disassembly driven by DNA hybridization which were highly sensitive to environmental factors such as temperature and salt concentration in the solution. Therefore, DNA-AuNDs were covalently cross-linked to obtain discrete oligomers of AuNDs. Specifically, the assembled AuNDs were cross-linked using (i) a psoralen-based crosslinker targeting DNA duplexes and (ii) dithiol-PEG (SH-PEG-SH) through Au-S bonds to stabilize the assembly structures.

First, the assembly structures were conjugated by cross-linking DNA duplexes with psoralen molecules. As a small-molecule crosslinker, 8-Methoxypsoralen (8-MOP) can

penetrate DNA duplexes and covalently bind to thymine bases. Psoralen undergoes [2 + 2] cycloaddition reactions to form mono- and bifunctional adducts with DNA. Under UV irradiation, psoralen molecules intercalate into double-stranded DNA. The pyrone ring forms a 3,4-monoadduct with thymine via a [2 + 2] cycloaddition reaction, while the furan ring forms a different 4',5'-monoadduct. These monoadducts can further absorb UV light and form interstrand cross-links between thymine bases¹⁰⁷. For example, 8-MOP has been used to cross-link DNA origami structures, successfully improving their thermal stability up to 85 °C.⁹⁶ Based on this approach, assembled AuNDs were mixed with 8-MOP (final concentration: 500 µM) irradiated under 100% UV intensity at various NaCl concentrations (0, 150, 200, and 250 mM). UV irradiation was applied every 5 min with 2 min intervals between exposures to prevent dehybridization of DNA duplexes caused by heating, for a total irradiation time of 40 min. After photoreaction, the solution was replaced with fresh buffer for further characterization. The extinction spectrum of AuNDs-150 was identical to that of the sample without NaCl, indicating that DNA duplexes were not effectively cross-linked under these conditions. In contrast, AuNDs-200 and AuNDs-250 exhibited slightly blue-shifted (~17 nm) and broader extinction spectra (**Figure 4. 32a**) compared to AuNDs-0, suggesting partial cross-linking of DNA duplexes that maintained the AuND assemblies even in the absence of NaCl. Slightly larger hydrodynamic diameters (**Figure 4. 32b**) further supported the successful cross-linking of AuNDs-200 and AuNDs-250. To confirm cross-linking more clearly, all samples were heated to 60 °C, followed by measurement of their hydrodynamic diameters (**Figure 4. 32c**). The reduced hydrodynamic diameters observed for AuNDs-200 and AuNDs-250 indicated that cross-linking was still incomplete under the initial irradiation conditions. Therefore, additional irradiation was performed for AuNDs-200 to optimize the cross-linking process.

Consequently, a slight increase in plasmon shift (~24 nm, **Figure 4. 32d**) suggested that prolonged irradiation cross-linked the DNA duplexes more effectively, consistent with the DLS measurements (**Figure 4. 32e**). However, the hydrodynamic diameters slightly decreased after heating at 60 °C (**Figure 4. 32f**), indicating partial dissociation of AuNDs upon heating. Accordingly, potential non-crosslinking interactions should be examined in future studies by irradiating AuNDs without DNA hybridization to confirm that the solution conditions alone did not induce AuND stacking. The cross-linked structures were then observed by SEM (**Figure 4. 32h-j**) after dispersing the AuNDs in water and rapidly drying the colloidal solutions at 60 °C. In the absence of crosslinking, AuNDs were expected to remain dispersed, as the lack of salt and heating above the T_m would promote dehybridization of DNA duplexes. AuNDs-

200 (**Figure 4. 32h**), which underwent intermittent heating, predominantly exhibited dimers and trimers (≤ 10 -mers). In contrast, AuNDs-200-40 (**Figure 4. 32i**) displayed longer oligomers (> 10 -mers), indicating that the extended irradiation improved crosslinking efficiency. Longer 1D assemblies were also observed for AuNDs-200-60 (**Figure 4. 32j**), although small oligomers (≤ 10 -mers) remained dominant. Overall, these results demonstrate that DNA duplex crosslinking was achieved, although further experiments are required to provide more definitive evidence. Moreover, the crosslinking efficiency should be improved to enable covalent linkage of larger numbers of AuNDs.

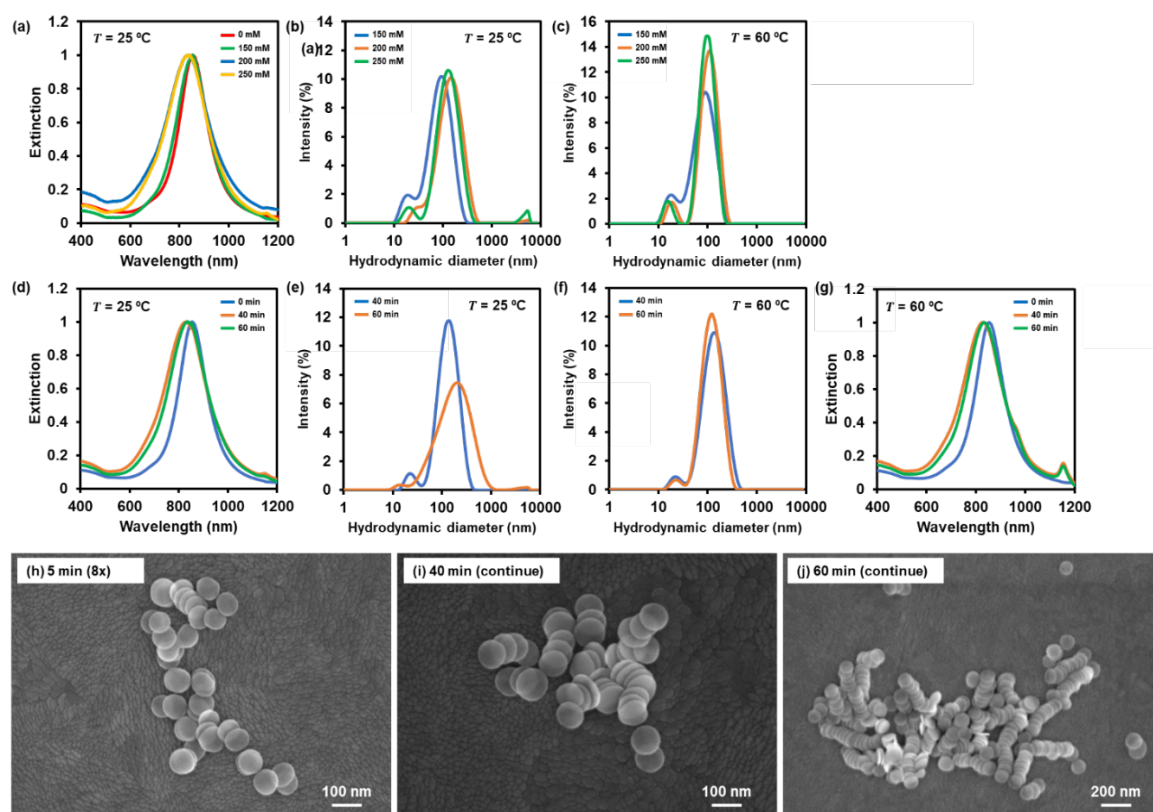


Figure 4. 32. (a-c) Crosslinking of DNA duplexes with 8-MOP (final concentration: 500 μ M) in various NaCl concentrations with 2-min interval irradiation. (a) Extinction spectra and (b) hydrodynamic diameters of AuNDs at 25 °C. (c) Hydrodynamic diameters of AuNDs after heating to 60 °C. (d-g) Crosslinking of DNA duplexes in 200 mM NaCl under continue irradiation for 40- and 60-min. (d) Extinction spectra and (e) hydrodynamic diameters of AuNDs at 25 °C. (f) Extinction spectra and (g) hydrodynamic diameters of AuNDs at 60 °C. AuNDs were dispersed in 0.01% SDS-10 mM KPO₄ (pH 7.0). (h-j) SEM images of conjugated AuNDs in H₂O after irradiation: (a) 40-min with interval, (b) 40-min continue, and (c) 60-min continue irradiation. Samples were dried at 60 °C (higher than T_m) to dehybridize all DNA duplexes.

Subsequently, SH-PEG-SH was employed to crosslink AuNDs via Au-S bonds. Previously, DNA–AuNP clusters were crosslinked with dithiothreitol (DTT) after the immobilization of oligonucleotides on AuNP surfaces⁷⁰. However, such systems could not form

aligned 1D assemblies without a template¹⁰⁸ and could not adjust the interparticle distance due to the short DTT linker (~1 nm). In contrast, DNA-modified AuNDs offer the advantages of tunable nanogaps—achieved by varying the DNA length—and the ability to assemble into 1D structures without the need for a template. To realize this, the amount of SH-PEG-SH used for crosslinking had to be optimized, as excessive concentrations of SH-PEG-SH induced AuNP aggregation¹⁰⁹. Therefore, various concentrations of SH-PEG-SH (0.1, 1, 10, 50, or 100 μM) were investigated for the modification of 20-nt(–)-DNA-AuND. The extinction spectra of all samples (**Figure 4. 33a**) indicated that 20-nt(–)-DNA-PEG(x)-AuNDs remained well-dispersed in phosphate buffer (pH 7.0), similar to 20-nt(–)-DNA-DNA-AuNDs prior to PEG modification. DLS measurements also revealed comparable hydrodynamic diameters between 20-nt(–)-DNA-DNA-PEG(x)-AuNDs and 20-nt(–)-DNA-DNA-AuNDs (**Figure 4. 33b**), suggesting that PEG modification at all tested concentrations did not promote aggregation.

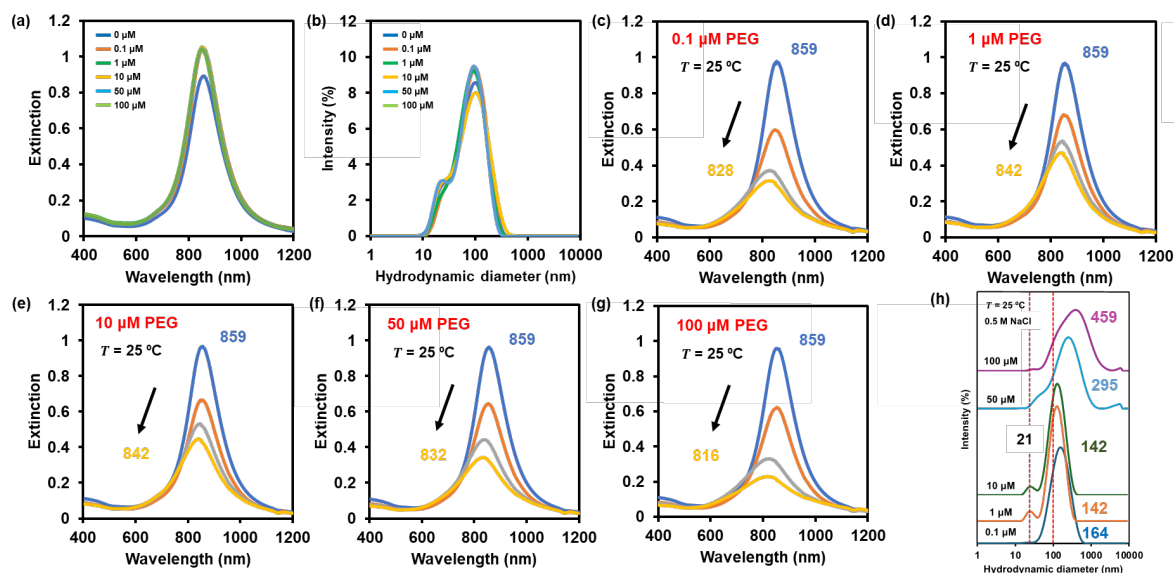


Figure 4. 33. Crosslinking of assembled AuNDs with various concentrations of SH-PEG-SH (0.1, 1, 10, 50, or 100 μM). (a) Extinction spectra and (b) hydrodynamic diameters of 20-nt(–)-DNA-PEG_x-AuND. (c-g) Assembly of 20-nt(–)-DNA-PEG_x-AuND and 20-nt(+)-DNA-AuND (1:1 ratio) in 0.5 M NaCl for 5 h incubation: (c) 0.1 μM PEG, (d) 1 μM PEG, (e) 10 μM PEG, (f) 50 μM PEG, and (g) 100 μM PEG. (h) Hydrodynamic diameters of assembled 20-bp-DNA-PEG_x-AuND. All solutions contained of 0.01% SDS-10 mM KPO₄ (pH 7.0). All measurements were conducted at 25 °C.

When 20-nt(–)-DNA-PEG(x)-AuND and 20-nt(+)-DNA-AuND (1:1 molar mixing ratio) were mixed at a 1:1 molar ratio, different degrees of plasmonic shifts were observed (**Figure 4. 33c-g**), indicating the formation of AuND assemblies with varying oligomeric states. The hydrodynamic diameter data further supported that SH-PEG-SH concentration affected the assembly behavior (**Figure 4. 33h**). Lower PEG concentration (20-bp-DNA-PEG(0.1)-

AuND, **Figure 4. 33c**) promoted larger plasmon shifting than 20-bp-DNA-PEG(1)-AuND (**Figure 4. 33d**) and 20-bp-DNA-PEG(10)-AuNDs (**Figure 4. 33e**). Possibly, 1 and 10 μM PEG passivated AuNDs surfaces, enhancing their stability in 0.5 M NaCl, consistent with previous findings¹¹⁰. This interpretation agrees with the DLS results (**Figure 4. 33h**), which showed bimodal hydrodynamic diameter distributions for 20-bp-DNA-PEG(1)-AuND and 20-bp-DNA-PEG(10)-AuND, whereas the smaller peak for 20-bp-DNA-PEG(0.1)-AuND was absent. In contrast, higher SH-PEG-SH concentrations (50 and 100 μM) likely caused aggregation due to excess thiol groups¹⁰⁹.

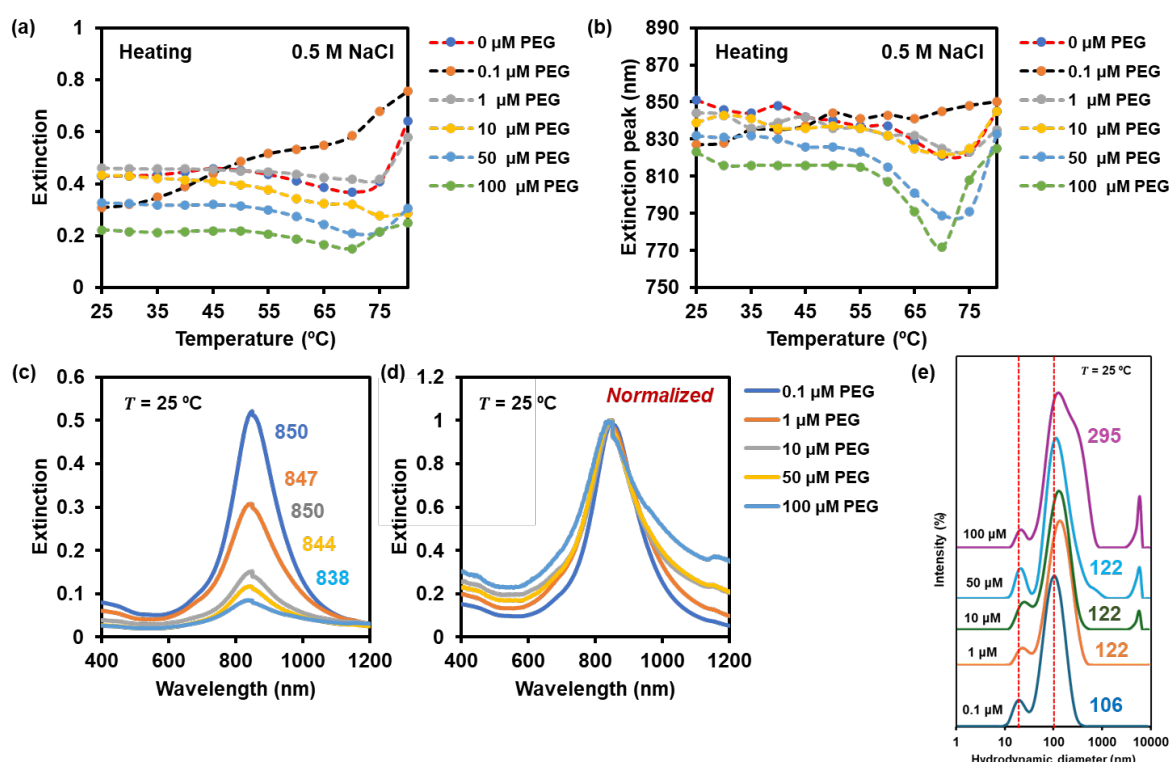


Figure 4. 34. Crosslinking of assembled AuNDs with various concentrations of SH-PEG-SH (0.1, 1, 10, 50, or 100 μM). (a) Extinction intensity and (b) extinction peak of R20-DNA-PEG_x-AuNDs upon heating from 25 to 80 °C. (c) Extinction spectra, (d) normalized extinction spectra, and (e) hydrodynamic diameters of R20-DNA-PEG_x-AuNDs after changing solution to the fresh buffer. All solutions contained of 0.01% SDS-10 mM KPO₄ (pH 7.0).

Then, all samples of 20-bp-DNA-PEG(x)-AuND were gradually heated to 80 °C. In the absence of PEG, the assembled AuNDs dissociated between 75 and 80 °C, as indicated by an increase in extinction intensity (**Figure 4. 34a**, red) and small plasmonic shift (**Figure 4. 34b**, red). The spectra of 20-bp-DNA-PEG(0.1)-AuND (**Figure 4. 34a**, black) and 20-bp-DNA-PEG(1)-AuND (**Figure 4. 34a**, grey) were also fully recovered after heating, suggesting insufficient crosslinking. At higher PEG concentrations (50 and 100 μM), the extinction intensities were maintained upon heating (**Figure 4. 34a**, blue and green), corresponding to

significant aggregation. In comparison, 10 μM SH-PEG-SH appeared sufficient to crosslink AuNDs without inducing aggregation (**Figure 4. 34a-b**, yellow). Subsequently, the solutions were exchanged with fresh buffer, followed by measurements of their extinction spectra and hydrodynamic diameters. The broader plasmonic spectra (**Figure 4. 34c,d**) and the increased hydrodynamic diameters observed with higher SH-PEG-SH concentrations suggested that the AuNDs were crosslinked through dithiol bonding. Overall, these results demonstrate that moderate concentrations of SH-PEG-SH, particularly around 10 μM , effectively crosslink DNA-modified AuNDs via dithiol bonds while maintaining colloidal stability and reversibility of assembly. However, additional experiments are required to clearly confirm and improve the crosslinking efficiency, as hydrophobic interactions during heating may have influenced the AuND assembly.

4.4. Conclusions

AuNDs modified with complementary short DNA ligands without spacers assembled into 1D cylindrical structures. Assemblies with shorter interparticle gap distances exhibited a significant blue shift in the extinction spectra, indicating a strong plasmonic coupling. The DNA-hybridization-driven assembly was confirmed by introducing blocking DNA strands, which suppressed hybridization, and by performing multiple heating and cooling cycles that demonstrated reversible assembly and disassembly under specific salt concentrations. The assembly sizes could be controlled by changing salts concentrations in the solution or adjusting the molar mixing ratios of (+)-DNA-AuND to (−)-DNA-AuND, resulting in different plasmonic shift by changing the plasmonic coupling. Although tuning the molar ratios successfully modulated the degree of multimerization, the inherent heterogeneity of the assembly reaction produced a broad multimer distribution due to the imbalance in AuND availability, resulting in deviations from the estimated values. Unlike the assemblies formed by varying the mixing ratios, salt-induced assembly proceeded further association of assembled structure by increasing salt concentration upon drying which limited the accurate evaluation of multimerization degree.

Then, plasmonic coupling could be altered by changing the lengths of DNA ligands of comparable assembly sizes. The plasmonic shift enhanced by reducing DNA length due to the stronger plasmonic coupling of shorter interparticle gap distance, whereas the decrease in the length of DNA ligands was limited by the colloidal stability of AuNDs. Besides, T_A and T_D could be tuned by changing salt concentrations and DNA lengths. T_D gradually increased with increasing salt concentration, consistent with general property of free DNA. Interestingly, T_A

was lower than T_D in the lower salt concentrations or longer DNA lengths, defined as hysteresis, which associated to the strong electrostatic repulsion between the phosphates of the DNA ligands on the AuNDs or the increased probability of the base pairing during partially mismatched hybridization.

Because a broad distribution of oligomers was inevitable due to the limited kinetic control when adjusting the mixing ratios or salt concentrations, size-based fractionation was conducted on mixtures of 67:33 and 60:40 ratios by applying different centrifugal forces. As a result, AuND assemblies were fractionated according to size, with lower centrifugal forces yielding larger assemblies. This centrifugation-based approach successfully separated AuND multimers by approximate size, although individual multimeric species were not completely distinct.

Finally, to stabilize the assemblies against environmental perturbations such as temperature and ionic strength, the DNA duplexes were crosslinked using psoralen molecules under UV irradiation or by connecting AuNDs with dithiol-PEG (SH-PEG-SH) via Au-S bonds. DNA duplex crosslinking was successfully achieved using 500 μM psoralen with more than 40 min of UV exposure, while moderate SH-PEG-SH concentrations (~ 10 μM) effectively crosslinked DNA-modified AuNDs through dithiol bonds, maintaining both colloidal stability and assembly reversibility. Nevertheless, further optimization will be required to improve the robustness of static assembly formation.

Chapter 5

Conclusions and Future Outlooks

Successful preparation of self-assembled structures has rendered remarkable improvements. However, regulating nanoparticle interactions to form simultaneous static and dynamic assembly structures is challenging. The use of thermo-responsive and click-reactive surface ligands to covalently conjugate AuNDs and concurrently enable reversible assembly and disassembly could not be achieved due to several factors. First, **Alk** and **N3** ligands increased the hydrophobicity, significantly enhancing the hydrogen bonding. Second, Cu^+ in the click solution reduced the electrostatic repulsion of deprotonated **COOH** ligand, inhibiting AuNDs dissociation. Third, large-flat surface AuNDs facilitated strong adhesion via van der Waals interaction and hydrogen bonding, maintaining the assembly structure. These factors inhibited to clearly confirm the formation of covalently cross-linking between **Alk**- and **N3**-modified AuNDs via click reaction through reversible extinction spectra and dynamic assembly structures. To overcome the strong face-to-face interactions, AuNDs were modified with DNA ligands to provide electrostatic repulsion and facilitate reversible assembly and disassembly via DNA hybridization.

Then, AuNDs were modified with DNA ligands using three methods: (i) click reaction between DBCO-DNA and **N3**-functionalized AuNDs, (ii) salt aging, and (iii) butanol dehydration. Among these methods, butanol dehydration was adopted as a simple and effective strategy for preparing DNA-modified AuNDs. Even AuNDs assembly have been studied previously by using organic and DNA ligands^{7,17,24,45}, the controllable sizes remained to be established. Besides, the use of spacer and linker DNA not only increased the interparticle gap distance, also enhanced DNA hybridization via cooperative and multivalent effects, significantly increased the disassembly temperature of nanoparticle compared to the melting temperature of free DNA. In addition, long DNA also reduced the directionality of assembly due to the large free volume of flexible DNA and increased the probability of slipping base-pairing interaction. In the present work, AuNDs assembly was controlled by regulating the assembly sizes and interparticle gap distances via direct hybridization of short DNA ligand without spacer or linker. The assembled AuNDs exhibited distinct thermodynamic and kinetic behaviours which offer valuable insights into nanoparticle assembly-optical property relationships. These findings successfully address a long-standing challenge in DNA-mediated AuNDs assembly, especially how to obtain oligomers with controllable size and well-defined interparticle distances to tune the plasmonic coupling.

The degree of AuND multimerization was controlled by adjusting (i) the molar mixing ratio of AuNDs modified with (+)-DNA and (–)-DNA and (ii) the salt concentration in the solution. The degree of multimerization significantly influences the plasmonic coupling

strength. Although tuning the molar ratios successfully modulated the degree of multimerization, the estimated degree of polymerization was not precise with inconsistency of the values and broader distributions. On the other hand, salt-induced assembly proceeded further association of assembled structure by increasing salt concentration upon drying which limited the accurate evaluation of multimerization degree. As a result, both methods did not achieve defined-number assemblies due to the limited kinetic control. Then, gel electrophoresis and centrifugal separation were applied to separate AuNDs assemblies according to the sizes. However, assembled AuNDs dissociated into monomers during electrophoresis because DNA hybridization was sensitive to the salt concentration and temperature. The centrifugation-based approach could not separate the individual multimers, preventing to obtain homogeneous assembly sizes. As introduced in this study, psoralen molecule and dithiol-PEG potentially crosslinked the assembled AuNDs. Nevertheless, further optimization is required to improve the static assembly formation. In the future, combining the crosslinking and gel electrophoresis will open the possibility to isolate discrete AuND oligomers, resulting in defined-number assemblies with well-defined plasmonic properties.

In addition, DNA-modified AuNDs exhibited a non-linear response to the applied stimulus, known as hysteresis. Hysteresis is classified in two categories, rate-dependent (kinetic) and rate-independent (thermodynamic) hysteresis. The knowledge of the type of hysteresis is central to predicting how the system responds to a stimulus. Thus, thermal hysteresis of assembled AuNDs was observed under conditions of lower salt concentrations, longer DNA ligands, or repetitive DNA sequences, likely due to the electrostatic repulsion and increased probability of base-pairing. Hysteresis is found in various applications such as memory devices¹¹¹ and piezoelectric actuators¹¹². Thermal hysteresis combined with asymmetry feedback loops was a crucial feature for developing nanoparticle-based oscillator¹¹³. Importantly, the observed T_d values matched the corresponding T_m values of free DNA owing to the spacer-free DNA ligand design, which optimally balances suppression factors (e.g., steric hindrance, limited hybridization zones) and promotion factors (e.g., flat surfaces, moderate DNA ligand density for improved accessibility). These results reveal that the disassembly temperature of DNA-AuNDs can be finely tuned by adjusting the salt concentration and remains consistent with the melting temperature of the corresponding free DNA strands, a feature advantageous for designing thermo-responsive plasmonic materials with targeted activation temperatures.

References

- (1) Roy, S.; Kashyap, R. K.; Pillai, P. P. Thermoplasmonics Enable the Coupling of Light into the Solvent-Mediated Self-Assembly of Gold Nanoparticles. *The Journal of Physical Chemistry C* **2023**, *127* (21), 10355–10365. <https://doi.org/10.1021/acs.jpcc.3c01316>.
- (2) Boles, M. A.; Engel, M.; Talapin, D. V. Self-Assembly of Colloidal Nanocrystals: From Intricate Structures to Functional Materials. *Chem Rev* **2016**, *116* (18), 11220–11289. <https://doi.org/10.1021/acs.chemrev.6b00196>.
- (3) Min, Y.; Akbulut, M.; Kristiansen, K.; Golan, Y.; Israelachvili, J. The Role of Interparticle and External Forces in Nanoparticle Assembly. *Nat Mater* **2008**, *7* (7), 527–538. <https://doi.org/10.1038/nmat2206>.
- (4) Bian, T.; Gardin, A.; Gemen, J.; Houben, L.; Perego, C.; Lee, B.; Elad, N.; Chu, Z.; Pavan, G. M.; Klajn, R. Electrostatic Co-Assembly of Nanoparticles with Oppositely Charged Small Molecules into Static and Dynamic Superstructures. *Nat Chem* **2021**, *13* (10), 940–949. <https://doi.org/10.1038/s41557-021-00752-9>.
- (5) Torii, Y.; Sugimura, N.; Mitomo, H.; Niikura, K.; Ijio, K. PH-Responsive Coassembly of Oligo(Ethylene Glycol)-Coated Gold Nanoparticles with External Anionic Polymers via Hydrogen Bonding. *Langmuir* **2017**, *33* (22), 5537–5544. <https://doi.org/10.1021/acs.langmuir.7b01084>.
- (6) Iida, R.; Mitomo, H.; Matsuo, Y.; Niikura, K.; Ijio, K. Thermoresponsive Assembly of Gold Nanoparticles Coated with Oligo(Ethylene Glycol) Ligands with an Alkyl Head. *Journal of Physical Chemistry C* **2016**, *120* (29), 15846–15854. <https://doi.org/10.1021/acs.jpcc.5b11687>.
- (7) Chidiebere Mba, J.; Mitomo, H.; Yonamine, Y.; Wang, G.; Matsuo, Y.; Ijio, K. Hysteresis in the Thermo-Responsive Assembly of Hexa(Ethylene Glycol) Derivative-Modified Gold Nanodiscs as an Effect of Shape. **2022**. <https://doi.org/10.3390/nano1209>.
- (8) Li, Z.; Zhu, Z.; Liu, W.; Zhou, Y.; Han, B.; Gao, Y.; Tang, Z. Reversible Plasmonic Circular Dichroism of Au Nanorod and DNA Assemblies. *J Am Chem Soc* **2012**, *134* (7), 3322–3325. <https://doi.org/10.1021/ja209981n>.
- (9) Millstone, J. E.; Georganopoulou, D. G.; Xu, X.; Wei, W.; Li, S.; Mirkin, C. A. DNA-Gold Triangular Nanoprism Conjugates. *Small* **2008**, *4* (12), 2176–2180. <https://doi.org/10.1002/sml.200800931>.
- (10) Xiong, K.; Mitomo, H.; Su, X.; Shi, Y.; Yonamine, Y.; Sato, S.; Ijio, K. Molecular Configuration-Mediated Thermo-Responsiveness in Oligo(Ethylene Glycol) Derivatives Attached on Gold Nanoparticles. *Nanoscale Adv* **2021**, *3* (13), 3762–3769. <https://doi.org/10.1039/D1NA00187F>.
- (11) Iida, R.; Kawamura, H.; Niikura, K.; Kimura, T.; Sekiguchi, S.; Joti, Y.; Bessho, Y.; Mitomo, H.; Nishino, Y.; Ijio, K. Synthesis of Janus-like Gold Nanoparticles with Hydrophilic/Hydrophobic Faces by Surface Ligand Exchange and Their Self-Assemblies in Water. *Langmuir* **2015**, *31* (14), 4054–4062. <https://doi.org/10.1021/la504647z>.

- (12) Iida, R.; Mitomo, H.; Niikura, K.; Matsuo, Y.; Ijiro, K. Two-Step Assembly of Thermoresponsive Gold Nanorods Coated with a Single Kind of Ligand. *Small* **2018**, *14* (14). <https://doi.org/10.1002/sml.201704230>.
- (13) Jones, M. R.; Macfarlane, R. J.; Lee, B.; Zhang, J.; Young, K. L.; Senesi, A. J.; Mirkin, C. A. DNA-Nanoparticle Superlattices Formed from Anisotropic Building Blocks. *Nat Mater* **2010**, *9* (11), 913–917. <https://doi.org/10.1038/nmat2870>.
- (14) Wang, G.; Yu, L.; Akiyama, Y.; Takarada, T.; Maeda, M. Reversible Shrinkage of DNA-Functionalized Gold Nanoparticle Assemblies Revealed by Surface Plasmon Resonance. *Biotechnol J* **2018**, *13* (12). <https://doi.org/10.1002/biot.201800090>.
- (15) Lermusiaux, L.; Sereda, A.; Portier, B.; Larquet, E.; Bidault, S. Reversible Switching of the Interparticle Distance in DNA-Templated Gold Nanoparticle Dimers. *ACS Nano* **2012**, *6* (12), 10992–10998. <https://doi.org/10.1021/nn304599d>.
- (16) Zhang, Q.; Tian, Y.; Liang, Z.; Wang, Z.; Xu, S.; Ma, Q. DNA-Mediated Au–Au Dimer-Based Surface Plasmon Coupling Electrochemiluminescence Sensor for BRCA1 Gene Detection. *Anal Chem* **2021**, *93* (6), 3308–3314. <https://doi.org/10.1021/acs.analchem.0c05440>.
- (17) Jones, M. R.; Kohlstedt, K. L.; O'Brien, M. N.; Wu, J.; Schatz, G. C.; Mirkin, C. A. Deterministic Symmetry Breaking of Plasmonic Nanostructures Enabled by DNA-Programmable Assembly. *Nano Lett* **2017**, *17* (9), 5830–5835. <https://doi.org/10.1021/acs.nanolett.7b03067>.
- (18) Chen, X.; Ding, L.; Wang, Y.; Gao, Z.; Li, J.; Liu, X.; Wang, L.; Zhu, Y.; Fan, C.; Jia, S.; Yao, G. Welded Gold Nanoparticle Assemblies Defined Plasmonic Coupling. *Nano Lett* **2024**, *24* (29), 8956–8963. <https://doi.org/10.1021/acs.nanolett.4c01887>.
- (19) Vial, S.; Nykypanchuk, D.; Deepak, F. L.; Prado, M.; Gang, O. Plasmonic Response of DNA-Assembled Gold Nanorods: Effect of DNA Linker Length, Temperature and Linker/Nanoparticles Ratio. *J Colloid Interface Sci* **2014**, *433*, 34–42. <https://doi.org/10.1016/j.jcis.2014.07.020>.
- (20) Shrestha, S.; Wang, B.; Dutta, P. Nanoparticle Processing: Understanding and Controlling Aggregation. *Adv Colloid Interface Sci* **2020**, *279*, 102162. <https://doi.org/10.1016/j.cis.2020.102162>.
- (21) Wang, Y.; Gao, Z.; Han, Z.; Liu, Y.; Yang, H.; Akkin, T.; Hogan, C. J.; Bischof, J. C. Aggregation Affects Optical Properties and Photothermal Heating of Gold Nanospheres. *Sci Rep* **2021**, *11* (1), 898. <https://doi.org/10.1038/s41598-020-79393-w>.
- (22) Lee, A.; Andrade, G. F. S.; Ahmed, A.; Souza, M. L.; Coombs, N.; Tumarkin, E.; Liu, K.; Gordon, R.; Brolo, A. G.; Kumacheva, E. Probing Dynamic Generation of Hot-Spots in Self-Assembled Chains of Gold Nanorods by Surface-Enhanced Raman Scattering. *J Am Chem Soc* **2011**, *133* (19), 7563–7570. <https://doi.org/10.1021/ja2015179>.
- (23) Cha, H.; Yoon, J. H.; Yoon, S. Probing Quantum Plasmon Coupling Using Gold Nanoparticle Dimers with Tunable Interparticle Distances Down to the Subnanometer Range. *ACS Nano* **2014**, *8* (8), 8554–8563. <https://doi.org/10.1021/nn5032438>.
- (24) O'Brien, M. N.; Brown, K. A.; Mirkin, C. A. Critical Undercooling in DNA-Mediated Nanoparticle Crystallization. *ACS Nano* **2016**, *10* (1), 1363–1368. <https://doi.org/10.1021/acsnano.5b06770>.

- (25) Jones, M. R.; Macfarlane, R. J.; Prigodich, A. E.; Patel, P. C.; Mirkin, C. A. Nanoparticle Shape Anisotropy Dictates the Collective Behavior of Surface-Bound Ligands. *J Am Chem Soc* **2011**, *133* (46), 18865–18869. <https://doi.org/10.1021/ja206777k>.
- (26) Nykypanchuk, D.; Maye, M. M.; van der Lelie, D.; Gang, O. DNA-Guided Crystallization of Colloidal Nanoparticles. *Nature* **2008**, *451* (7178), 549–552. <https://doi.org/10.1038/nature06560>.
- (27) Zhang, Z.; Wen, Y.; Zhao, D.; Zhang, X. Stable End-to-End Assembly of Gold Nanorods Directed by Cyclic Disulfide-Modified DNA. *Appl Phys Lett* **2012**, *101* (21), 213701. <https://doi.org/10.1063/1.4767392>.
- (28) Ma, W.; Kuang, H.; Xu, L.; Ding, L.; Xu, C.; Wang, L.; Kotov, N. A. Attomolar DNA Detection with Chiral Nanorod Assemblies. *Nat Commun* **2013**, *4* (1), 2689. <https://doi.org/10.1038/ncomms3689>.
- (29) Pan, B.; Ao, L.; Gao, F.; Tian, H.; He, R.; Cui, D. End-to-End Self-Assembly and Colorimetric Characterization of Gold Nanorods and Nanospheres via Oligonucleotide Hybridization. *Nanotechnology* **2005**, *16* (9), 1776–1780. <https://doi.org/10.1088/0957-4484/16/9/061>.
- (30) Calcaterra, H. A.; Chellam, N. S.; Lee, B.; Schatz, G. C.; Mirkin, C. A. High Temperature, Isothermal Growth Promotes Close Packing and Thermal Stability in DNA-Engineered Colloidal Crystals. *ACS Nano* **2024**, *18* (41), 28268–28278. <https://doi.org/10.1021/acsnano.4c09308>.
- (31) Generalova, A. N.; Oleinikov, V. A.; Khaydukov, E. V. One-Dimensional Necklace-like Assemblies of Inorganic Nanoparticles: Recent Advances in Design, Preparation and Applications. *Adv Colloid Interface Sci* **2021**, *297*, 102543. <https://doi.org/10.1016/j.cis.2021.102543>.
- (32) Gentili, D.; Ori, G. Reversible Assembly of Nanoparticles: Theory, Strategies and Computational Simulations. *Nanoscale* **2022**, *14* (39), 14385–14432. <https://doi.org/10.1039/D2NR02640F>.
- (33) Trinh, H. D.; Kim, S.; Park, J.; Yoon, S. Core-Satellite-Satellite Hierarchical Nanostructures: Assembly, Plasmon Coupling, and Gap-Selective Surface-Enhanced Raman Scattering. *Nanoscale* **2022**, *14* (45), 17003–17012. <https://doi.org/10.1039/d2nr04621k>.
- (34) Liu, Y.; Zeng, T.; Liu, C.; Fang, X.; Li, S.; Cao, X.; Lu, C.; Yang, H. DNA Origami-Based Letterpress Printing of Gold Nanostructures with Predesigned Morphologies. *Nano Lett* **2023**, *23* (24), 11569–11577. <https://doi.org/10.1021/acs.nanolett.3c03307>.
- (35) Buchkremer, A.; Linn, M. J.; Timper, J. U.; Eckert, T.; Mayer, J.; Richtering, W.; von Plessen, G.; Simon, U. Synthesis and Internal Structure of Finite-Size DNA–Gold Nanoparticle Assemblies. *The Journal of Physical Chemistry C* **2014**, *118* (13), 7174–7184. <https://doi.org/10.1021/jp412283q>.
- (36) O'Brien, M. N.; Girard, M.; Lin, H.-X.; Millan, J. A.; Olvera de la Cruz, M.; Lee, B.; Mirkin, C. A. Exploring the Zone of Anisotropy and Broken Symmetries in DNA-Mediated Nanoparticle Crystallization. *Proceedings of the National Academy of Sciences* **2016**, *113* (38), 10485–10490. <https://doi.org/10.1073/pnas.1611808113>.
- (37) Klinkova, A.; Thérien-Aubin, H.; Ahmed, A.; Nykypanchuk, D.; Choueiri, R. M.; Gagnon, B.; Muntyanu, A.; Gang, O.; Walker, G. C.; Kumacheva, E. Structural and

- Optical Properties of Self-Assembled Chains of Plasmonic Nanocubes. *Nano Lett* **2014**, *14* (11), 6314–6321. <https://doi.org/10.1021/nl502746h>.
- (38) Cao, G.; Wang, Y. *Nanostructures and Nanomaterials*; World Scientific Series in Nanoscience and Nanotechnology; WORLD SCIENTIFIC, 2011; Vol. 2. <https://doi.org/10.1142/7885>.
 - (39) Cui, X.; Qin, F.; Ruan, Q.; Zhuo, X.; Wang, J. Circular Gold Nanodisks with Synthetically Tunable Diameters and Thicknesses. *Adv Funct Mater* **2018**, *28* (11). <https://doi.org/10.1002/adfm.201705516>.
 - (40) Zorić, I.; Zäch, M.; Kasemo, B.; Langhammer, C. Gold, Platinum, and Aluminum Nanodisk Plasmons: Material Independence, Subradiance, and Damping Mechanisms. *ACS Nano* **2011**, *5* (4), 2535–2546. <https://doi.org/10.1021/nn102166t>.
 - (41) Ghosh, S. K.; Pal, T. Interparticle Coupling Effect on the Surface Plasmon Resonance of Gold Nanoparticles: From Theory to Applications. *Chem Rev* **2007**, *107* (11), 4797–4862. <https://doi.org/10.1021/cr0680282>.
 - (42) Mayer, K. M.; Hafner, J. H. Localized Surface Plasmon Resonance Sensors. *Chem Rev* **2011**, *111* (6), 3828–3857. <https://doi.org/10.1021/cr100313v>.
 - (43) Jana, J.; Ganguly, M.; Pal, T. Enlightening Surface Plasmon Resonance Effect of Metal Nanoparticles for Practical Spectroscopic Application. *RSC Adv* **2016**, *6* (89), 86174–86211. <https://doi.org/10.1039/C6RA14173K>.
 - (44) Wi, J.-S.; Park, J.; Kang, H.; Jung, D.; Lee, S.-W.; Lee, T. G. Stacked Gold Nanodisks for Bimodal Photoacoustic and Optical Coherence Imaging. *ACS Nano* **2017**, *11* (6), 6225–6232. <https://doi.org/10.1021/acsnano.7b02337>.
 - (45) Cui, X.; Qin, F.; Ruan, Q.; Zhuo, X.; Wang, J. Circular Gold Nanodisks with Synthetically Tunable Diameters and Thicknesses. *Adv Funct Mater* **2018**, *28* (11). <https://doi.org/10.1002/adfm.201705516>.
 - (46) O'Dwyer, C.; Gay, G.; Viaris de Lesegno, B.; Weiner, J. The Nature of Alkanethiol Self-Assembled Monolayer Adsorption on Sputtered Gold Substrates. *Langmuir* **2004**, *20* (19), 8172–8182. <https://doi.org/10.1021/la049103b>.
 - (47) Xiong, K.; Nagayama, M.; Ijio, K.; Mitomo, H. Fair Surface Modification with Mixed Alkanethiols on Gold Nanoparticles through Minimal Unfair Ligand Exchange. *Nanoscale Adv* **2024**, *6* (18), 4583–4590. <https://doi.org/10.1039/D4NA00270A>.
 - (48) Xiong, K.; Mitomo, H.; Su, X.; Shi, Y.; Yonamine, Y.; Sato, S.; Ijio, K. Molecular Configuration-Mediated Thermo-Responsiveness in Oligo(Ethylene Glycol) Derivatives Attached on Gold Nanoparticles. *Nanoscale Adv* **2021**, *3* (13), 3762–3769. <https://doi.org/10.1039/d1na00187f>.
 - (49) Van Der Meer, S. B.; Loza, K.; Wey, K.; Heggen, M.; Beuck, C.; Bayer, P.; Eppe, M. Click Chemistry on the Surface of Ultrasmall Gold Nanoparticles (2 Nm) for Covalent Ligand Attachment Followed by NMR Spectroscopy. *Langmuir* **2019**, *35* (22), 7191–7204. <https://doi.org/10.1021/acs.langmuir.9b00295>.
 - (50) Lebitania, J. A.; Inada, N.; Morimoto, M.; You, J.; Shahiduzzaman, M.; Taima, T.; Hirata, K.; Fukuma, T.; Ohta, A.; Asakawa, T.; Asakawa, H. Local Cross-Coupling Activity of Azide-Hexa(Ethylene Glycol)-Terminated Self-Assembled Monolayers Investigated by Atomic Force Microscopy. *Langmuir* **2021**, *37* (50), 14688–14696. <https://doi.org/10.1021/acs.langmuir.1c02451>.

- (51) Brunel, D.; Jangid, V.; Sanchez Adaime, E.; Duché, D.; Bharwal, A. K.; Abel, M.; Koudia, M.; Buffeteau, T.; Lebouin, C.; Simon, J. J.; Sauvage, R. M.; Berginc, G.; Escoubas, L.; Gigmes, D.; Dumur, F. Click Chemistry: An Efficient Tool to Control the Functionalization of Metallic Surfaces with Alkyl Chains Possessing Two Reactive End Groups. *Appl Surf Sci* **2021**, 566. <https://doi.org/10.1016/j.apsusc.2021.150731>.
- (52) Kaur, J.; Saxena, M.; Rishi, N. An Overview of Recent Advances in Biomedical Applications of Click Chemistry. *Bioconjugate Chemistry*. American Chemical Society August 18, 2021, pp 1455–1471. <https://doi.org/10.1021/acs.bioconjchem.1c00247>.
- (53) Scarabelli, L.; Liz-Marzán, L. M. An Extended Protocol for the Synthesis of Monodisperse Gold Nanotriangles. *ACS Nano* **2021**, 15 (12), 18600–18607. <https://doi.org/10.1021/acsnano.1c10538>.
- (54) Sun, Z.; Umar, A.; Zeng, J.; Luo, X.; Song, L.; Zhang, Z.; Chen, Z.; Li, J.; Su, F.; Huang, Y. Highly Pure Gold Nanotriangles with Almost 100% Yield for Surface-Enhanced Raman Scattering. *ACS Appl Nano Mater* **2022**, 5 (1), 1220–1231. <https://doi.org/10.1021/acsanm.1c03827>.
- (55) Sun, Z.; Umar, A.; Zeng, J.; Luo, X.; Song, L.; Zhang, Z.; Chen, Z.; Li, J.; Su, F.; Huang, Y. Highly Pure Gold Nanotriangles with Almost 100% Yield for Surface-Enhanced Raman Scattering. *ACS Appl Nano Mater* **2022**, 5 (1), 1220–1231. <https://doi.org/10.1021/acsanm.1c03827>.
- (56) O'Brien, M. N.; Jones, M. R.; Kohlstedt, K. L.; Schatz, G. C.; Mirkin, C. A. Uniform Circular Disks with Synthetically Tailorable Diameters: Two-Dimensional Nanoparticles for Plasmonics. *Nano Lett* **2015**, 15 (2), 1012–1017. <https://doi.org/10.1021/nl5038566>.
- (57) Huang, Y.; Ferhan, A. R.; Gao, Y.; Dandapat, A.; Kim, D. H. High-Yield Synthesis of Triangular Gold Nanoplates with Improved Shape Uniformity, Tunable Edge Length and Thickness. *Nanoscale* **2014**, 6 (12), 6496–6500. <https://doi.org/10.1039/c4nr00834k>.
- (58) Hassan, N. A.; Hashim, A. M. The Effect of Size of Au Nanodisc on the Localized Surface Plasmon Resonance Simulated Using Finite-Difference Time-Domain (FDTD) Method. *Mater Today Proc* **2019**, 7, 607–611. <https://doi.org/10.1016/j.matpr.2018.12.014>.
- (59) O'Brien, M. N.; Jones, M. R.; Kohlstedt, K. L.; Schatz, G. C.; Mirkin, C. A. Uniform Circular Disks with Synthetically Tailorable Diameters: Two-Dimensional Nanoparticles for Plasmonics. *Nano Lett* **2015**, 15 (2), 1012–1017. <https://doi.org/10.1021/nl5038566>.
- (60) Li, N.; Binder, W. H. Click-Chemistry for Nanoparticle-Modification. *J Mater Chem* **2011**, 21 (42), 16717. <https://doi.org/10.1039/c1jm11558h>.
- (61) Brennan, J. L.; Hatzakis, N. S.; Tshikhudo, T. R.; Nijole Dirvianskyte; Razumas, V.; Patkar, S.; Vind, J.; Svendsen, A.; Nolte, R. J. M.; Rowan, A. E.; Brust, M. Bionanoconjugation via Click Chemistry: The Creation of Functional Hybrids of Lipases and Gold Nanoparticles. *Bioconjug Chem* **2006**, 17 (6), 1373–1375. <https://doi.org/10.1021/bc0601018>.
- (62) van der Kooij, F. M.; van der Beek, D.; Lekkerkerker, H. N. W. Isotropic–Nematic Phase Separation in Suspensions of Polydisperse Colloidal Platelets. *J Phys Chem B* **2001**, 105 (9), 1696–1700. <https://doi.org/10.1021/jp0031597>.

- (63) Zhang; Horsch, M. A.; Lamm, M. H.; Glotzer, S. C. Tethered Nano Building Blocks: Toward a Conceptual Framework for Nanoparticle Self-Assembly. *Nano Lett* **2003**, 3 (10), 1341–1346. <https://doi.org/10.1021/nl034454g>.
- (64) Liu, B.; Liu, J. Methods for Preparing DNA-Functionalized Gold Nanoparticles, a Key Reagent of Bioanalytical Chemistry. *Analytical Methods* **2017**, 9 (18), 2633–2643. <https://doi.org/10.1039/C7AY00368D>.
- (65) Storhoff, J. J.; Elghanian, R.; Mirkin, C. A.; Letsinger, R. L. Sequence-Dependent Stability of DNA-Modified Gold Nanoparticles. *Langmuir* **2002**, 18 (17), 6666–6670. <https://doi.org/10.1021/la0202428>.
- (66) Ha, C. E.; Bhagavan, N. V. Structure and Properties of DNA. In *Essentials of Medical Biochemistry*; Elsevier, 2023; pp 453–476. <https://doi.org/10.1016/B978-0-323-88541-6.00026-0>.
- (67) Wang, M. D. Ruler of Life. *Nat Phys* **2021**, 17 (8), 976–976. <https://doi.org/10.1038/s41567-021-01300-5>.
- (68) Barrow, S. J.; Wei, X.; Baldauf, J. S.; Funston, A. M.; Mulvaney, P. The Surface Plasmon Modes of Self-Assembled Gold Nanocrystals. *Nat Commun* **2012**, 3 (1), 1275. <https://doi.org/10.1038/ncomms2289>.
- (69) Jin, R.; Wu, G.; Li, Z.; Mirkin, C. A.; Schatz, G. C. What Controls the Melting Properties of DNA-Linked Gold Nanoparticle Assemblies? *J Am Chem Soc* **2003**, 125 (6), 1643–1654. <https://doi.org/10.1021/ja021096v>.
- (70) Kim, J.-Y.; Lee, J.-S. Synthesis and Thermally Reversible Assembly of DNA–Gold Nanoparticle Cluster Conjugates. *Nano Lett* **2009**, 9 (12), 4564–4569. <https://doi.org/10.1021/nl9030709>.
- (71) Trantakis, I. A.; Bolisetty, S.; Mezzenga, R.; Sturla, S. J. Reversible Aggregation of DNA-Decorated Gold Nanoparticles Controlled by Molecular Recognition. *Langmuir* **2013**, 29 (34), 10824–10830. <https://doi.org/10.1021/la401211u>.
- (72) Hurst, S. J.; Hill, H. D.; Mirkin, C. A. “Three-Dimensional Hybridization” with Polyvalent DNA–Gold Nanoparticle Conjugates. *J Am Chem Soc* **2008**, 130 (36), 12192–12200. <https://doi.org/10.1021/ja804266j>.
- (73) Wang, L.; Shi, Y.; Wang, G.; Liang, X.; Takarada, T.; Maeda, M. Range-Tunable Plasmon Switching of Gold Nanorods by Terminal Breathing of Surface-Grafted DNA in Alcoholic Solvents. *J Mater Chem C Mater* **2021**, 9 (15), 5105–5112. <https://doi.org/10.1039/D1TC00588J>.
- (74) Dujardin, E.; Hsin, L. B.; Wang, C. R. C.; Mann, S. DNA-Driven Self-Assembly of Gold Nanorods. *Chemical Communications* **2001**, No. 14, 1264–1265. <https://doi.org/10.1039/b102319p>.
- (75) Vial, S.; Nykypanchuk, D.; Yager, K. G.; Tkachenko, A. V.; Gang, O. Linear Mesosstructures in DNA–Nanorod Self-Assembly. *ACS Nano* **2013**, 7 (6), 5437–5445. <https://doi.org/10.1021/nn401413b>.
- (76) O’Brien, M. N.; Jones, M. R.; Lee, B.; Mirkin, C. A. Anisotropic Nanoparticle Complementarity in DNA-Mediated Co-Crystallization. *Nat Mater* **2015**, 14 (8), 833–839. <https://doi.org/10.1038/nmat4293>.
- (77) Jones, M. R.; Mirkin, C. A. Bypassing the Limitations of Classical Chemical Purification with DNA-Programmable Nanoparticle Recrystallization. *Angewandte*

- Chemie International Edition* **2013**, 52 (10), 2886–2891. <https://doi.org/10.1002/anie.201209504>.
- (78) Hill, H. D.; Hurst, S. J.; Mirkin, C. A. Curvature-Induced Base Pair “Slipping” Effects in DNA-Nanoparticle Hybridization. *Nano Lett* **2009**, 9 (1), 317–321. <https://doi.org/10.1021/nl8030482>.
 - (79) Wasserman, S. R.; Tao, Y. T.; Whitesides, G. M. Structure and Reactivity of Alkylsiloxane Monolayers Formed by Reaction of Alkyltrichlorosilanes on Silicon Substrates. *Langmuir* **1989**, 5 (4), 1074–1087. <https://doi.org/10.1021/la00088a035>.
 - (80) Hao, Y.; Li, Y.; Song, L.; Deng, Z. Flash Synthesis of Spherical Nucleic Acids with Record DNA Density. *J Am Chem Soc* **2021**, 143 (8), 3065–3069. <https://doi.org/10.1021/jacs.1c00568>.
 - (81) Łaszewski, H. J.; Palpant, B.; Buckle, M.; Nogues, C. Influence of the Sequestration Effect of CTAB on the Biofunctionalization of Gold Nanorods. *ACS Appl Bio Mater* **2021**, 4 (6), 4753–4759. <https://doi.org/10.1021/acsabm.0c01522>.
 - (82) Monego, D.; Kister, T.; Kirkwood, N.; Mulvaney, P.; Widmer-Cooper, A.; Kraus, T. Colloidal Stability of Apolar Nanoparticles: Role of Ligand Length. *Langmuir* **2018**, 34 (43), 12982–12989. <https://doi.org/10.1021/acs.langmuir.8b02883>.
 - (83) Kanayama, N.; Sekine, T.; Ozasa, K.; Kishi, S.; Nyu, T.; Hayashi, T.; Maeda, M. Terminal-Specific Interaction between Double-Stranded DNA Layers: Colloidal Dispersion Behavior and Surface Force. *Langmuir* **2016**, 32 (49), 13296–13304. <https://doi.org/10.1021/acs.langmuir.6b03470>.
 - (84) Demers, L. M.; Mirkin, C. A.; Mucic, R. C.; Reynolds, R. A.; Letsinger, R. L.; Elghanian, R.; Viswanadham, G. A Fluorescence-Based Method for Determining the Surface Coverage and Hybridization Efficiency of Thiol-Capped Oligonucleotides Bound to Gold Thin Films and Nanoparticles. *Anal Chem* **2000**, 72 (22), 5535–5541. <https://doi.org/10.1021/ac0006627>.
 - (85) Hurst, S. J.; Lytton-Jean, A. K. R.; Mirkin, C. A. Maximizing DNA Loading on a Range of Gold Nanoparticle Sizes. *Anal Chem* **2006**, 78 (24), 8313–8318. <https://doi.org/10.1021/ac0613582>.
 - (86) Soares, D. M.; Gomes, W. E.; Tenan, M. A. Sodium Dodecyl Sulfate Adsorbed Monolayers on Gold Electrodes. *Langmuir* **2007**, 23 (8), 4383–4388. <https://doi.org/10.1021/la063508+>.
 - (87) Wang, G.; Zhang, Y.; Liang, X.; Takarada, T.; Maeda, M. Regioselective DNA Modification and Directed Self-Assembly of Triangular Gold Nanoplates. *Nanomaterials* **2019**, 9 (4), 581. <https://doi.org/10.3390/nano9040581>.
 - (88) Hurst, S. J.; Lytton-Jean, A. K. R.; Mirkin, C. A. Maximizing DNA Loading on a Range of Gold Nanoparticle Sizes. *Anal Chem* **2006**, 78 (24), 8313–8318. <https://doi.org/10.1021/ac0613582>.
 - (89) Demers, L. M.; Mirkin, C. A.; Mucic, R. C.; Reynolds, R. A.; Letsinger, R. L.; Elghanian, R.; Viswanadham, G. A Fluorescence-Based Method for Determining the Surface Coverage and Hybridization Efficiency of Thiol-Capped Oligonucleotides Bound to Gold Thin Films and Nanoparticles. *Anal Chem* **2000**, 72 (22), 5535–5541. <https://doi.org/10.1021/ac0006627>.

- (90) Cederquist, K. B.; Keating, C. D. Curvature Effects in DNA: Au Nanoparticle Conjugates. *ACS Nano* **2009**, *3* (2), 256–260. <https://doi.org/10.1021/nn9000726>.
- (91) Macfarlane, R. J.; Jones, M. R.; Senesi, A. J.; Young, K. L.; Lee, B.; Wu, J.; Mirkin, C. A. Establishing the Design Rules for DNA-Mediated Programmable Colloidal Crystallization. *Angewandte Chemie International Edition* **2010**, *49* (27), 4589–4592. <https://doi.org/10.1002/anie.201000633>.
- (92) Macfarlane, R. J.; Lee, B.; Hill, H. D.; Senesi, A. J.; Seifert, S.; Mirkin, C. A. Assembly and Organization Processes in DNA-Directed Colloidal Crystallization. *Proceedings of the National Academy of Sciences* **2009**, *106* (26), 10493–10498. <https://doi.org/10.1073/pnas.0900630106>.
- (93) Hurst, S. J.; Hill, H. D.; Mirkin, C. A. “Three-Dimensional Hybridization” with Polyvalent DNA–Gold Nanoparticle Conjugates. *J Am Chem Soc* **2008**, *130* (36), 12192–12200. <https://doi.org/10.1021/ja804266j>.
- (94) Johnson, P. B.; Christy, R. W. Optical Constants of the Noble Metals. *Phys Rev B* **1972**, *6* (12), 4370–4379. <https://doi.org/10.1103/PhysRevB.6.4370>.
- (95) Gen_Tsutsui_2001_Jpn._J._Appl._Phys._40_346.
- (96) Rajendran, A.; Endo, M.; Katsuda, Y.; Hidaka, K.; Sugiyama, H. Photo-Cross-Linking-Assisted Thermal Stability of DNA Origami Structures and Its Application for Higher-Temperature Self-Assembly. *J Am Chem Soc* **2011**, *133* (37), 14488–14491. <https://doi.org/10.1021/ja204546h>.
- (97) Spadavecchia, J.; Barras, A.; Lyskawa, J.; Woisel, P.; Laure, W.; Pradier, C.-M.; Boukherroub, R.; Szunerits, S. Correction to “Approach for Plasmonic Based DNA Sensing: Amplification of the Wavelength Shift and Simultaneous Detection of the Plasmon Modes of Gold Nanostructures.” *Anal Chem* **2024**, *96* (17), 6860–6861. <https://doi.org/10.1021/acs.analchem.4c01810>.
- (98) Storhoff, J. J.; Lazarides, A. A.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L.; Schatz, G. C. What Controls the Optical Properties of DNA-Linked Gold Nanoparticle Assemblies? *J Am Chem Soc* **2000**, *122* (19), 4640–4650. <https://doi.org/10.1021/ja993825l>.
- (99) Lytton-Jean, A. K. R.; Mirkin, C. A. A Thermodynamic Investigation into the Binding Properties of DNA Functionalized Gold Nanoparticle Probes and Molecular Fluorophore Probes. *J Am Chem Soc* **2005**, *127* (37), 12754–12755. <https://doi.org/10.1021/ja052255o>.
- (100) Flory, P. J. Fundamental Principles of Condensation Polymerization. *Chem Rev* **1946**, *39* (1), 137–197. <https://doi.org/10.1021/cr60122a003>.
- (101) Meaurio, E. Generalization of the Carothers Equation for Linear Step Growth Polymers. *Sci Rep* **2024**, *14* (1), 27281. <https://doi.org/10.1038/s41598-024-78476-2>.
- (102) Hueckel, T.; Woo, S.; Macfarlane, R. J. Controlling the Thermally-Driven Crystallization of DNA-Coated Nanoparticles with Formamide. *Soft Matter* **2024**, *20* (34), 6723–6729. <https://doi.org/10.1039/D4SM00854E>.
- (103) Yuan, Y.; Raheja, K.; Milbrandt, N. B.; Beilharz, S.; Tene, S.; Oshabaheebwa, S.; Gurkan, U. A.; Samia, A. C. S.; Karayilan, M. Thermoresponsive Polymers with LCST Transition: Synthesis, Characterization, and Their Impact on Biomedical Frontiers. *RSC Applied Polymers* **2023**, *1* (2), 158–189. <https://doi.org/10.1039/D3LP00114H>.

- (104) Owczarzy, R.; You, Y.; Moreira, B. G.; Manthey, J. A.; Huang, L.; Behlke, M. A.; Walder, J. A. Effects of Sodium Ions on DNA Duplex Oligomers: Improved Predictions of Melting Temperatures. *Biochemistry* **2004**, *43* (12), 3537–3554. <https://doi.org/10.1021/bi034621r>.
- (105) Markarian, M. Z.; Schlenoff, J. B. Effect of Molecular Crowding and Ionic Strength on the Isothermal Hybridization of Oligonucleotides. *J Phys Chem B* **2010**, *114* (32), 10620–10627. <https://doi.org/10.1021/jp103213w>.
- (106) Cao, A.; Tan, J.; Liu, D.; Chen, Z.; Dou, L.; Liu, Z.; Li, Y. Mass-Determining Role in the Electrophoretic Separation of Colloidal Plasmonic Nanoparticle Oligomers. *Nanoscale* **2022**, *14* (38), 14161–14168. <https://doi.org/10.1039/D2NR03585E>.
- (107) Wielenberg, K.; Wang, M.; Yang, M.; Ozer, A.; Lis, J. T.; Lin, H. An Improved 4'-Aminomethyltrioxsalen-Based Nucleic Acid Crosslinker for Biotinylation of Double-Stranded DNA or RNA. *RSC Adv* **2020**, *10* (65), 39870–39874. <https://doi.org/10.1039/D0RA07437C>.
- (108) Kim, P. Y.; Oh, J.-W.; Nam, J.-M. Controlled Co-Assembly of Nanoparticles and Polymer into Ultralong and Continuous One-Dimensional Nanochains. *J Am Chem Soc* **2015**, *137* (25), 8030–8033. <https://doi.org/10.1021/jacs.5b04714>.
- (109) Fu, Y.; Feng, Q.; Shen, Y.; Chen, M.; Xu, C.; Cheng, Y.; Zhou, X. A Feasible Strategy for Self-Assembly of Gold Nanoparticles via Dithiol-PEG for Photothermal Therapy of Cancers. *RSC Adv* **2018**, *8* (11), 6120–6124. <https://doi.org/10.1039/C7RA12735A>.
- (110) Deka, J.; Měch, R.; Ianeselli, L.; Amenitsch, H.; Cacho-Nerin, F.; Parisse, P.; Casalis, L. Surface Passivation Improves the Synthesis of Highly Stable and Specific DNA-Functionalized Gold Nanoparticles with Variable DNA Density. *ACS Appl Mater Interfaces* **2015**, *7* (12), 7033–7040. <https://doi.org/10.1021/acsami.5b01191>.
- (111) Kruse, J.; Merkens, S.; Chuvilin, A.; Grzelczak, M. Kinetic and Thermodynamic Hysteresis in Clustering of Gold Nanoparticles: Implications for Nanotransducers and Information Storage in Dynamic Systems. *ACS Appl Nano Mater* **2020**, *3* (9), 9520–9527. <https://doi.org/10.1021/acsanm.0c02249>.
- (112) Gan, J.; Mei, Z.; Chen, X.; Zhou, Y.; Ge, M.-F. A Modified Duhem Model for Rate-Dependent Hysteresis Behaviors. *Micromachines (Basel)* **2019**, *10* (10), 680. <https://doi.org/10.3390/mi10100680>.
- (113) Rao, A.; Iglesias, A. S.; Grzelczak, M. Choreographing Oscillatory Hydrodynamics with DNA-Coated Gold Nanoparticles. *J Am Chem Soc* **2024**, *146* (27), 18236–18240. <https://doi.org/10.1021/jacs.4c06868>.

Acknowledgements

I would like to express my deepest gratitude to my supervisors (Prof. Kuniharu Ijiro, Prof. Hideyuki Mitomo, and Prof. Yusuke Yonamine), for their unwavering support and insightful critiques throughout my research journey. Their meticulous attention, constructive feedback, and essential suggestions enhanced the quality of my work. I truly appreciate Prof. Xu Shi for conducting finite-difference time-domain (FDTD) simulations which support the experimental results. I am equally thankful to Takehiro Yachi and Advanced Imaging and Modeling Center for Soft Materials at Tohoku University (Prof. Koji Yonekura, Daisuke Unabara, and Tasuku Hamaguchi), for cryo-TEM and 3D construction images which strengthen the discussion of this research. I acknowledge the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for the financial support during my doctoral studies. My appreciation also goes to Ms. Takeuchi, Ms. Okuhara, and all members in Ijiro-Lab for their continues help in the laboratory. I must express my deepest appreciation for my family, who always stands by me. I am also thankful for my friends, who encouraged me when it seemed difficult to reach my academic goals. This dissertation reflects not only my work but also the collective support of everyone who has touched my life academically and personally. The journey has taught me the value of questioning and the importance of diverse perspectives in enriching my understanding to solve the problems. Most importantly, I must express my gratitude for all the blessings from Allah, who always gives me everything I need in life. I still vividly remember how I felt when I first arrived in Hokkaido University, mesmerized by the stunning views of the early spring. The beautiful landscapes reduced my stress, improved my mood, gave me more motivation, and helped me to adapt to the new environment. I collect all moments and valuable insights during my journey to improve myself in the future.

Supporting Information

I. SEM Images of AuNDs Multimers Prepared with Various Mixing Ratios

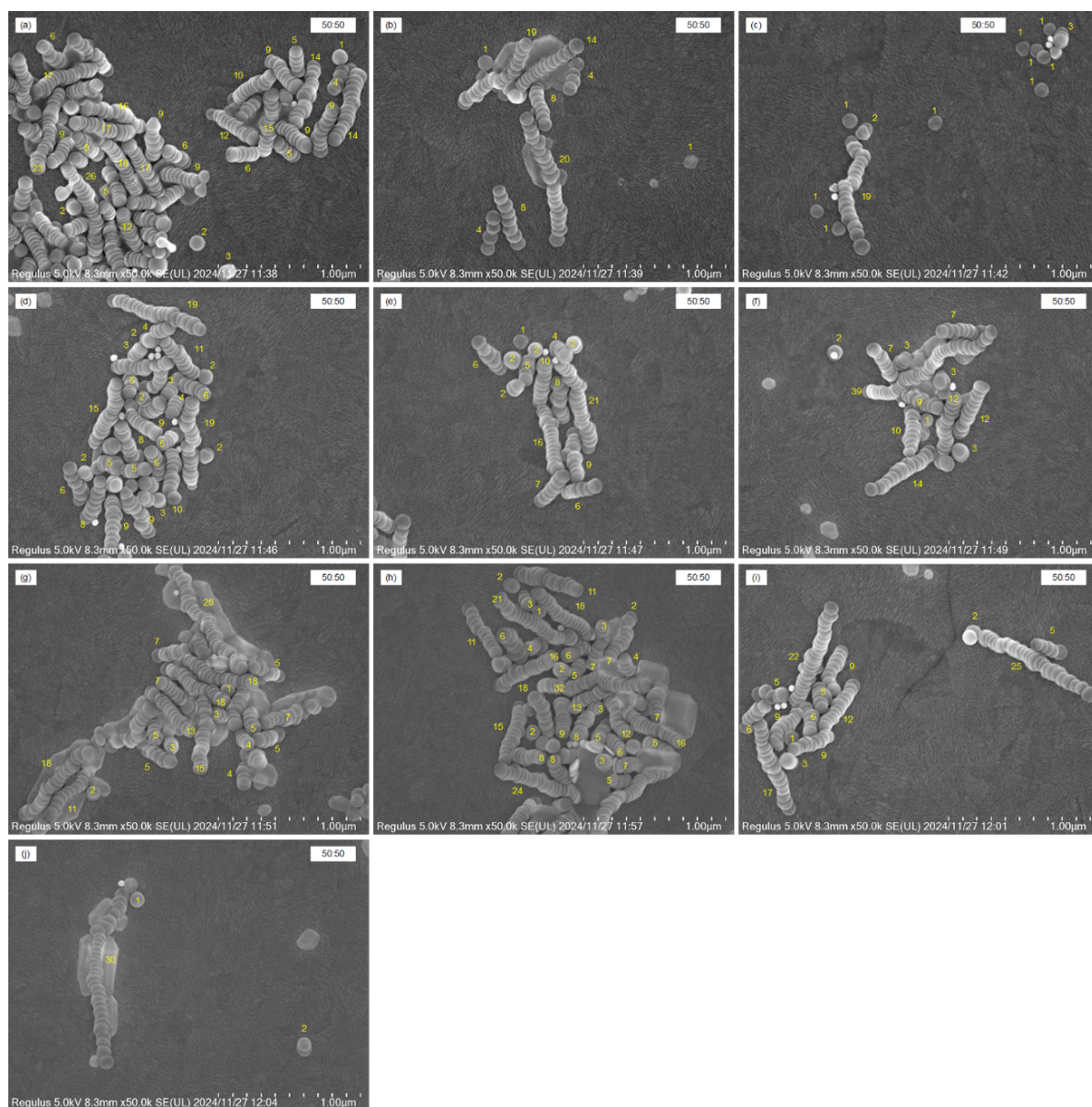


Figure S 1. SEM images of multimerization with same ratio of 11-base-(+)-DNA-AuND to 11-base-(−)-DNA-AuND (50:50, $r = 1$) in buffer with 150 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C. Number of oligomers was counted in each assembly cluster.

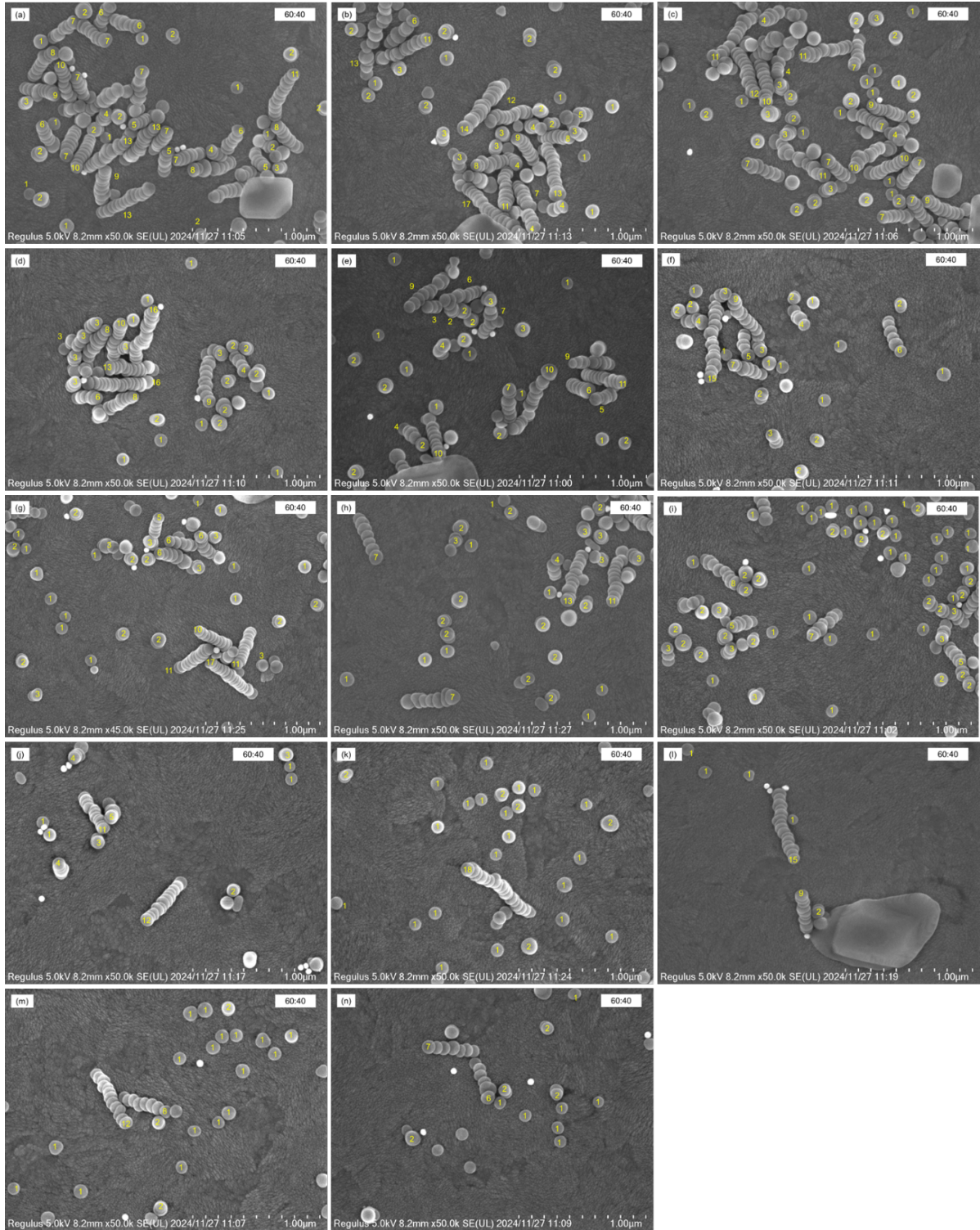


Figure S 2. SEM images of multimerization with ratio of 11-base-(+)-DNA-AuND : 11-base-(-)-DNA-AuND = 60:40 ($r = 2/3$) in buffer with 150 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C. Number of oligomers was counted in each assembly cluster.

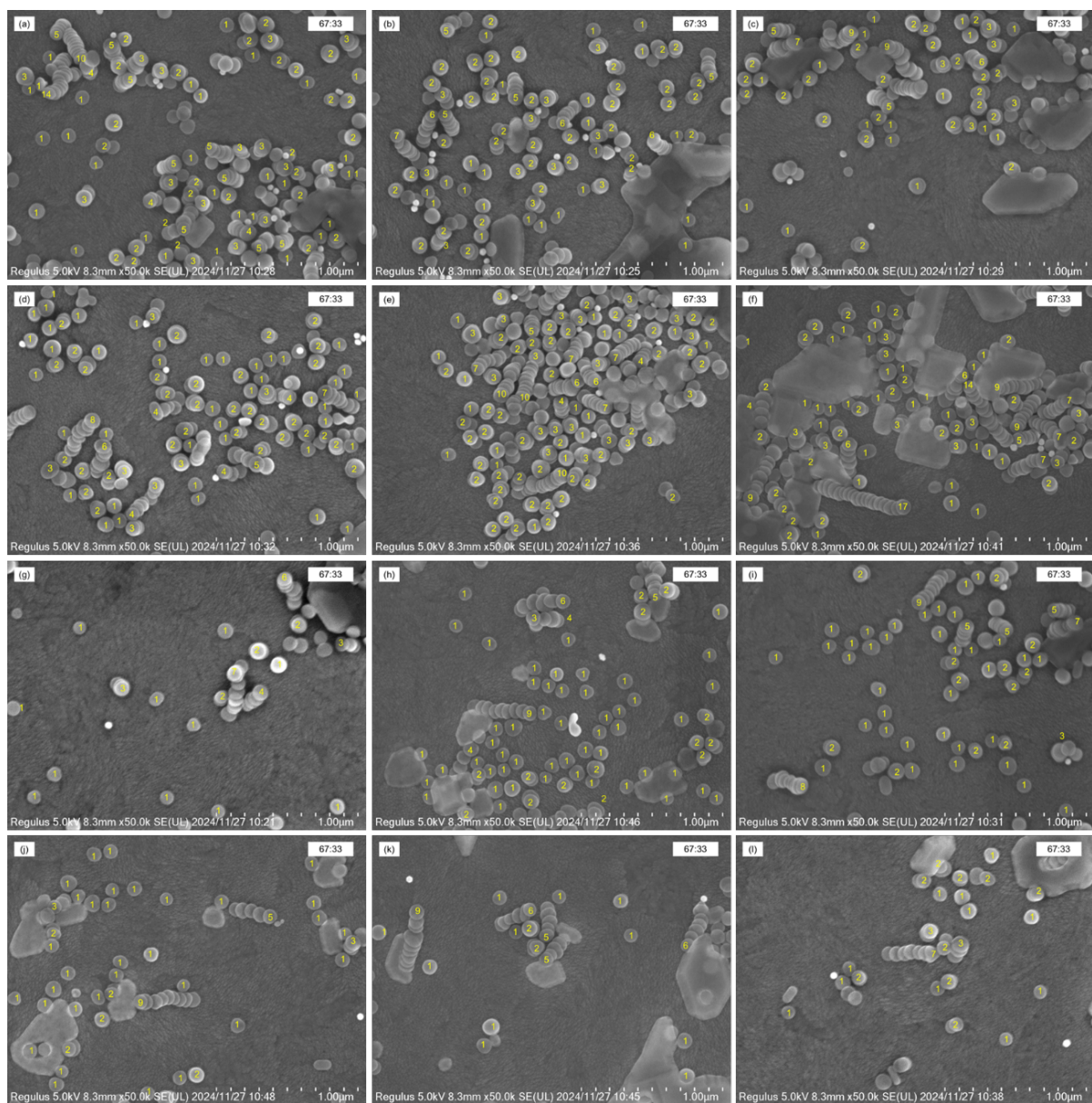


Figure S 3. SEM images of multimerization with ratio of 11-base-(+)-DNA-AuND : 11-base-(-)-DNA-AuND = 67:33 ($r = 1/2$) in buffer with 150 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C. Number of oligomers was counted in each assembly cluster.

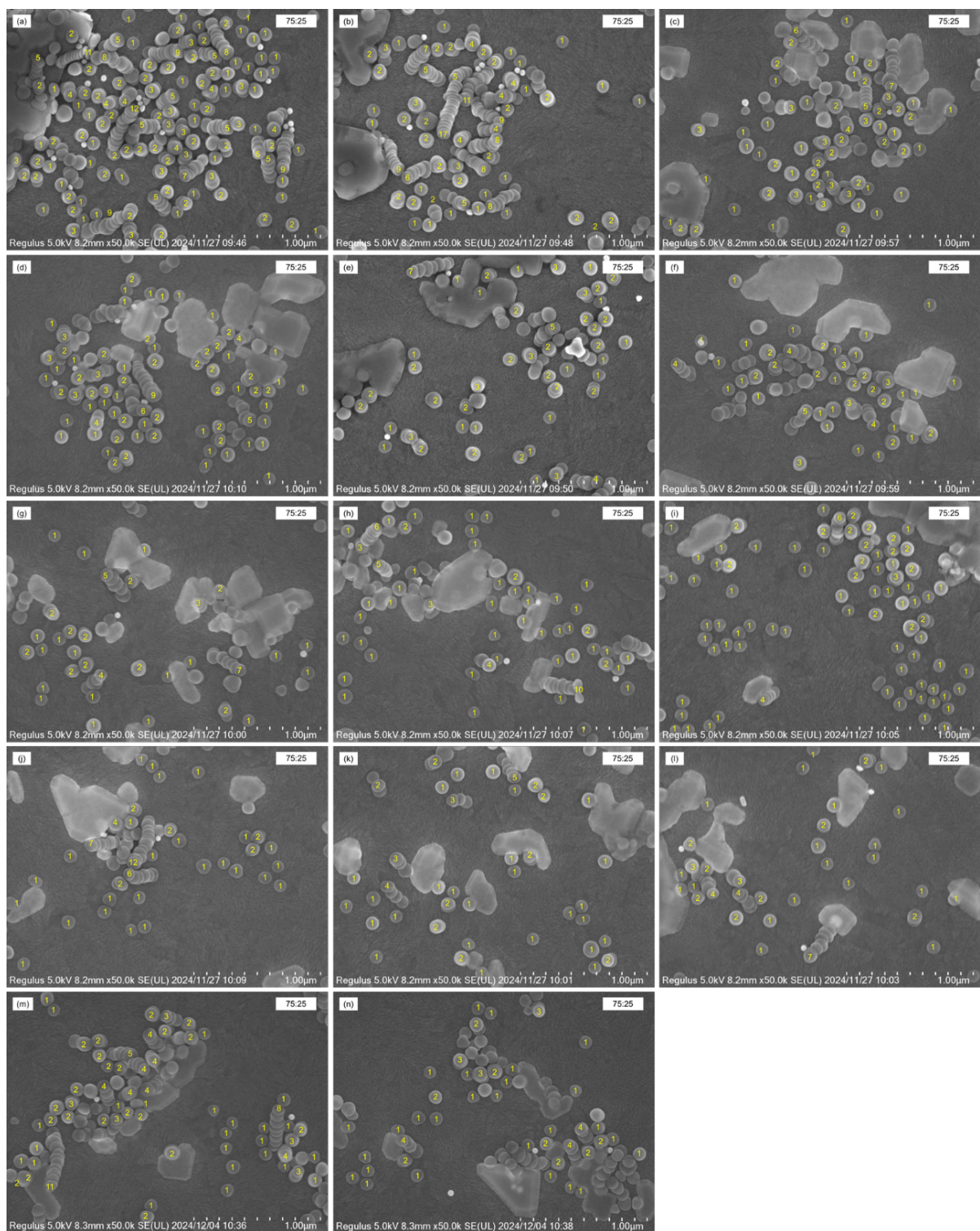


Figure S 4. SEM images of multimerization with ratio of 11-base-(+)-DNA-AuND : 11-base-(-)-DNA-AuND = 75:25 ($r = 1/3$) in buffer with 150 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C. Number of oligomers was counted in each assembly cluster.

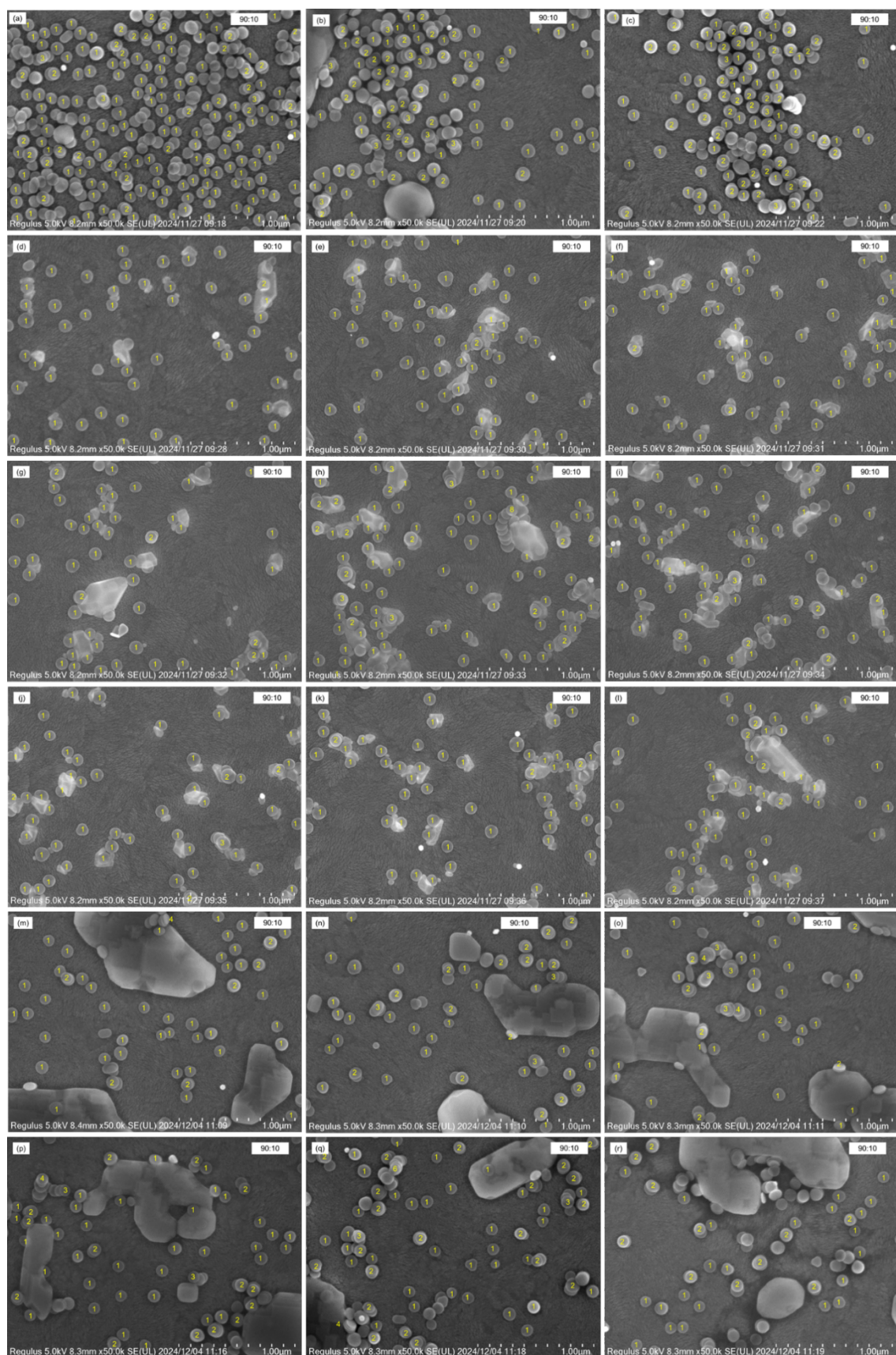


Figure S 5. SEM images of multimerization with ratio of 11-base-(+)-DNA-AuND : 11-base-(-)-DNA-AuND = 90:10 ($r = 1/9$) in buffer with 150 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C. Number of oligomers was counted in each assembly cluster.

II. SEM Images of AuNDs Multimers Prepared with Various NaCl Concentrations

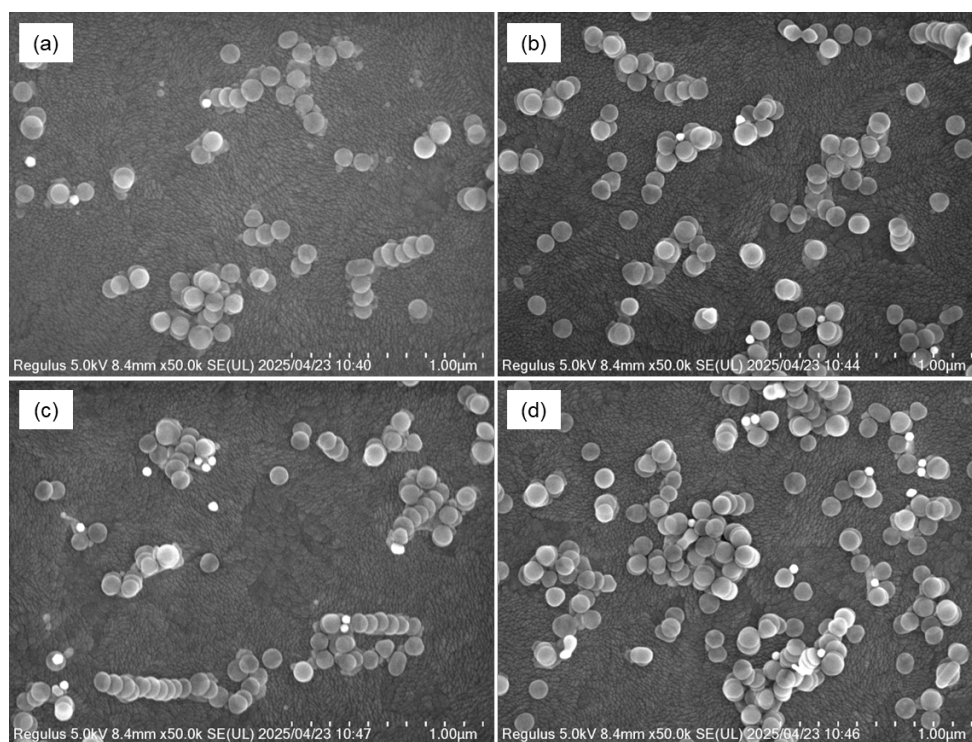


Figure S 6. SEM images of assembled AuNDs with equimolar ratio of 11-base-(+)-DNA-AuND : 11-base(-)-DNA-AuND (50:50) in buffer with 75 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C.

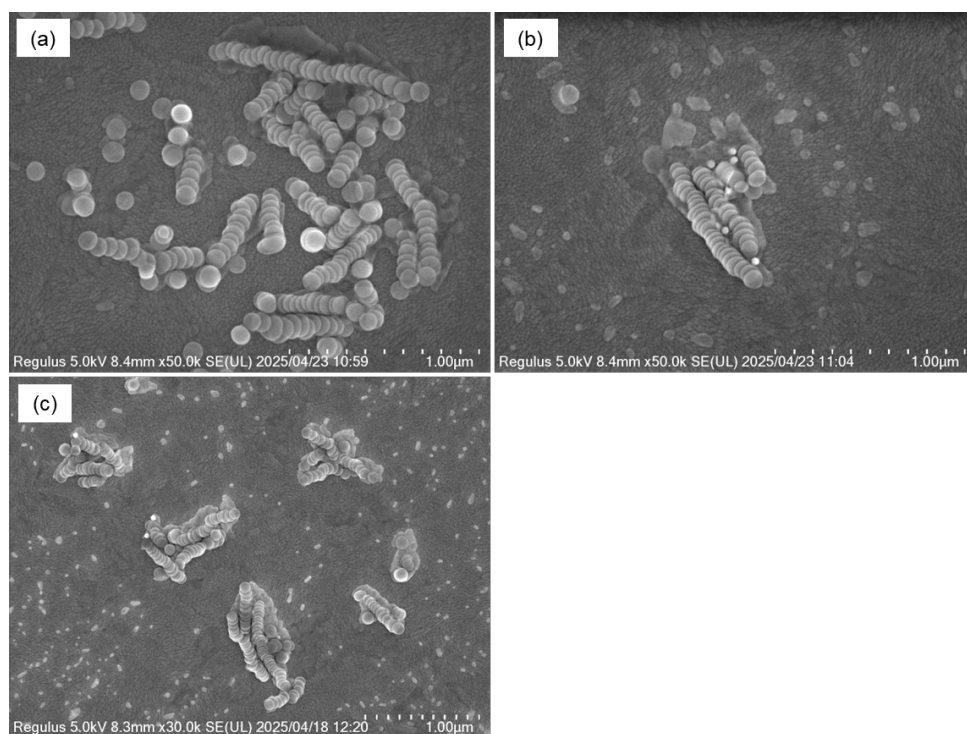


Figure S 7. SEM images of assembled AuNDs with equimolar ratio of 11-base-(+)-DNA-AuND : 11-base(-)-DNA-AuND (50:50) in buffer with 100 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C.

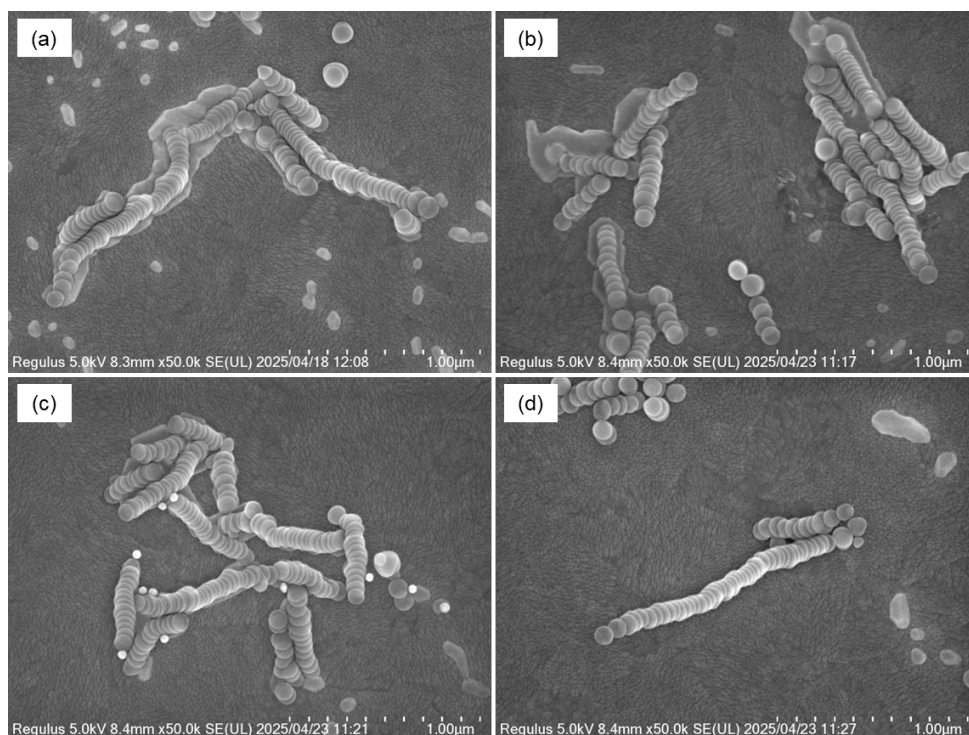


Figure S 8. SEM images of assembled AuNDs with equimolar ratio of 11-base-(+)-DNA-AuND : 11-base(-)-DNA-AuND (50:50) in buffer with 200 mM NaCl after hybridization for 4 h upon gradually cooling from 80 to 25 °C.

III. SEM Images of AuNDs Multimers Separated by Different Centrifugal Forces

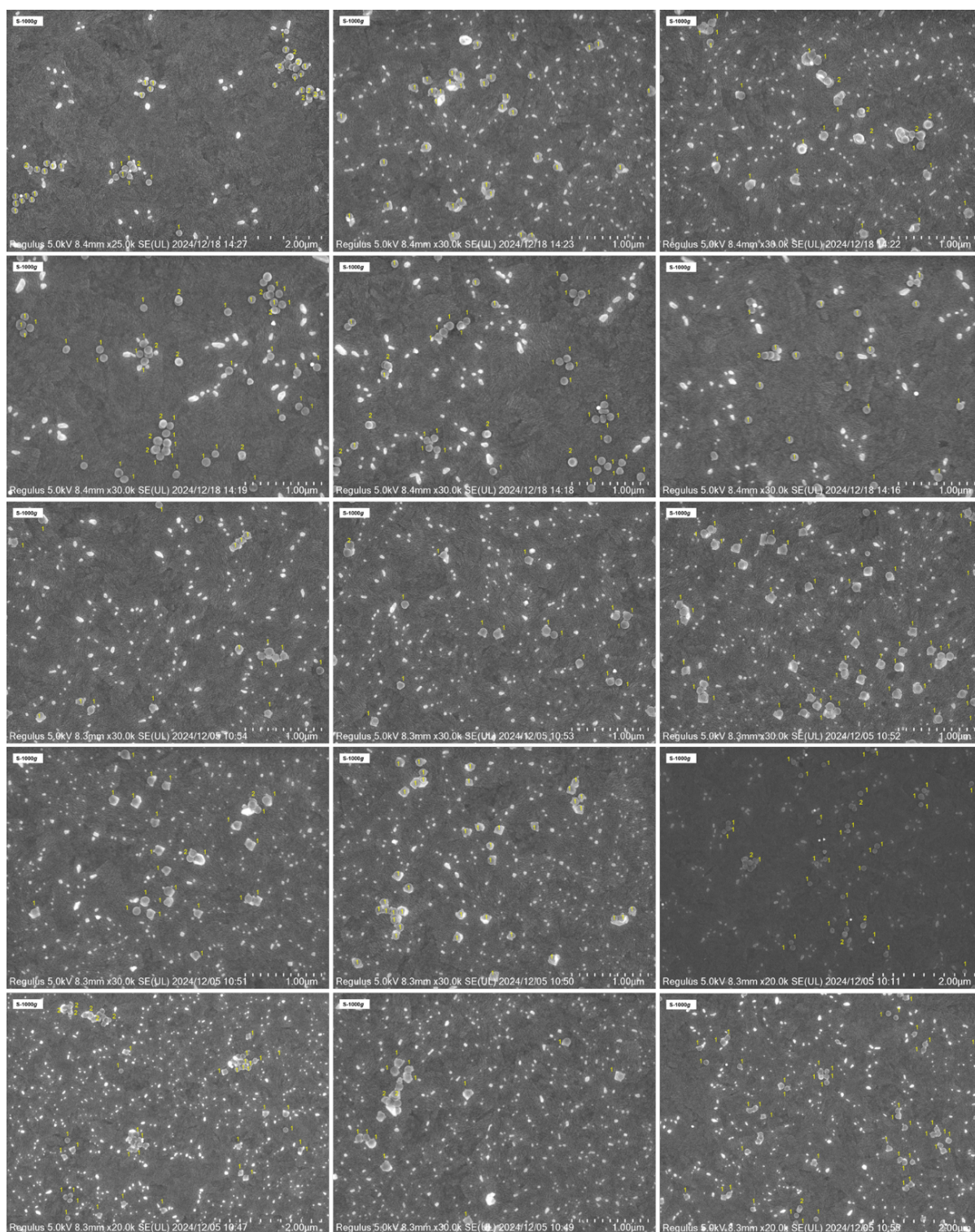


Figure S 9. SEM images of supernatant obtained from separation of 67:33 ratio by applying 1000g centrifugal force. Number of oligomers was counted in each assembly cluster. All particles are monomers and dimers AuNDs.

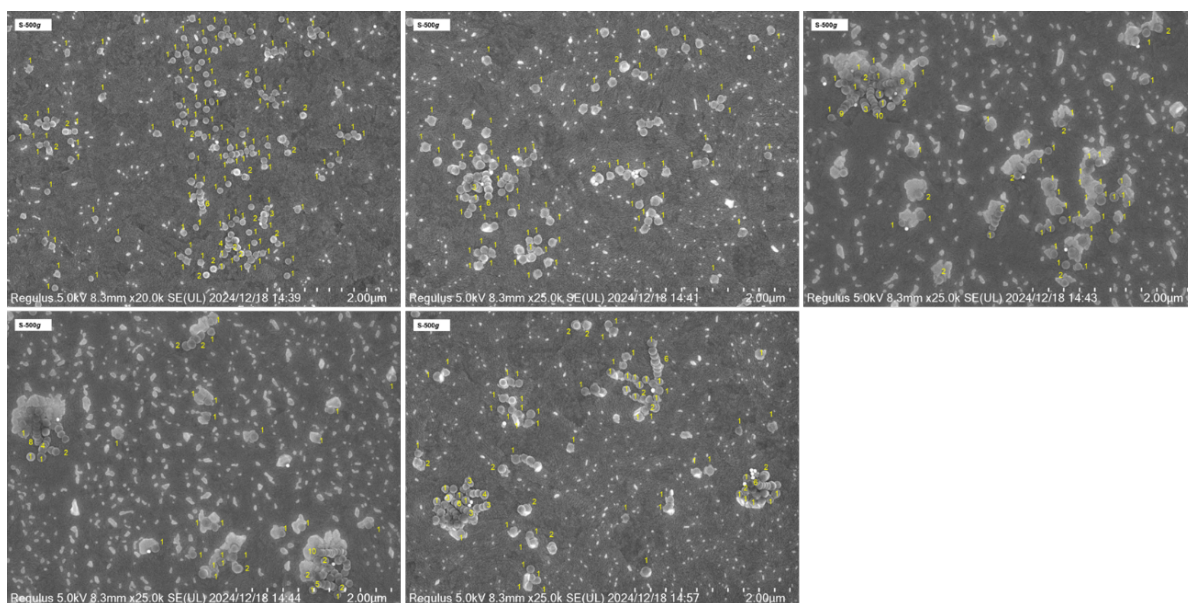


Figure S 10. SEM images of supernatant obtained from separation of 67:33 ratio by applying 500g centrifugal force. Number of oligomers was counted in each assembly cluster. Few larger oligomers (≤ 10 -mers) were observed which correlated to the broader extinction spectrum.

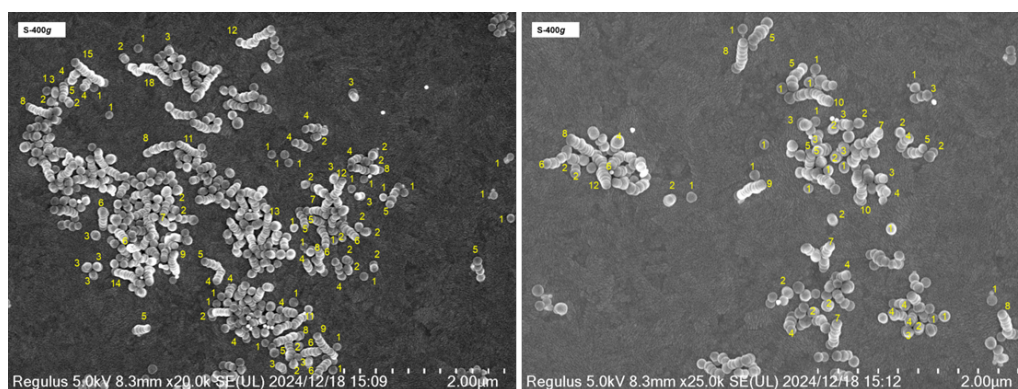


Figure S 11. SEM images of supernatant obtained from separation of 67:33 ratio by applying 400g centrifugal force. Number of oligomers was counted in each assembly cluster.

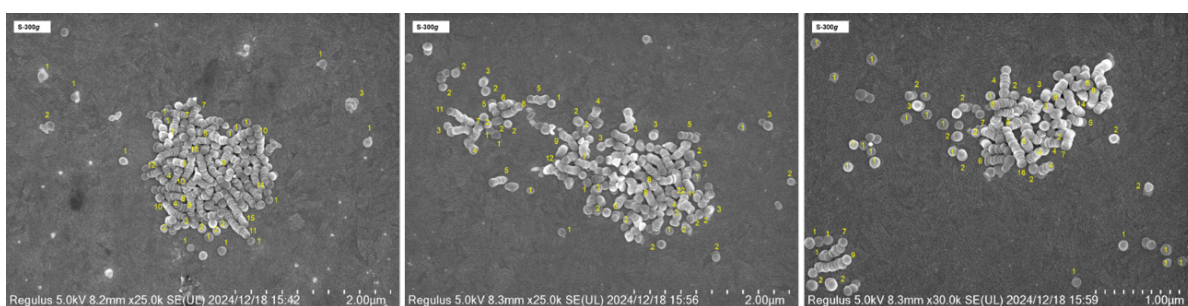


Figure S 12. SEM images of supernatant obtained from separation of 67:33 ratio by applying 300g centrifugal force. Number of oligomers was counted in each assembly cluster.

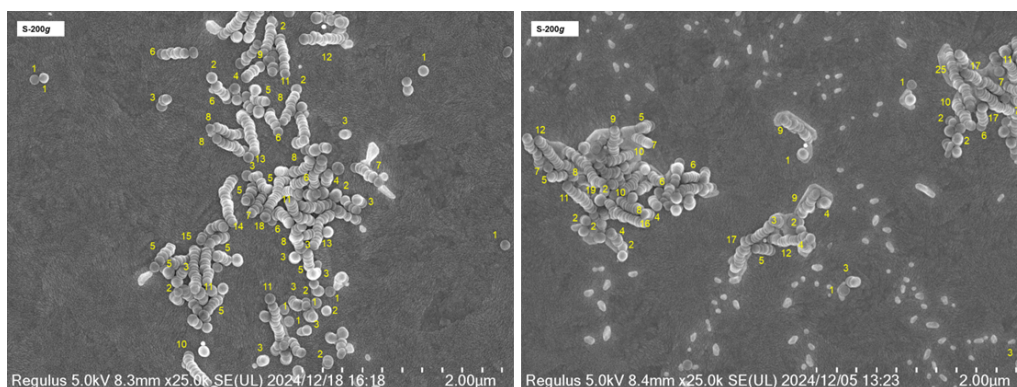


Figure S 13. SEM images of supernatant obtained from separation of 67:33 ratio by applying 200g centrifugal force. Number of oligomers was counted in each assembly cluster.

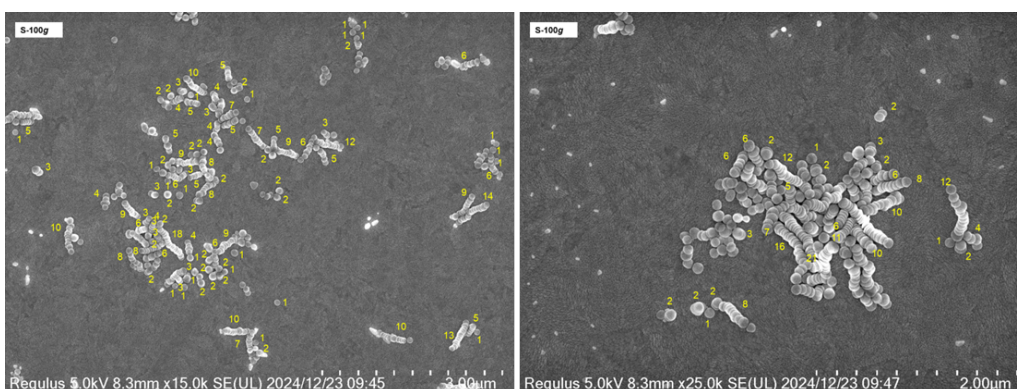


Figure S 14. SEM images of supernatant obtained from separation of 67:33 ratio by applying 100g centrifugal force. Number of oligomers was counted in each assembly cluster.

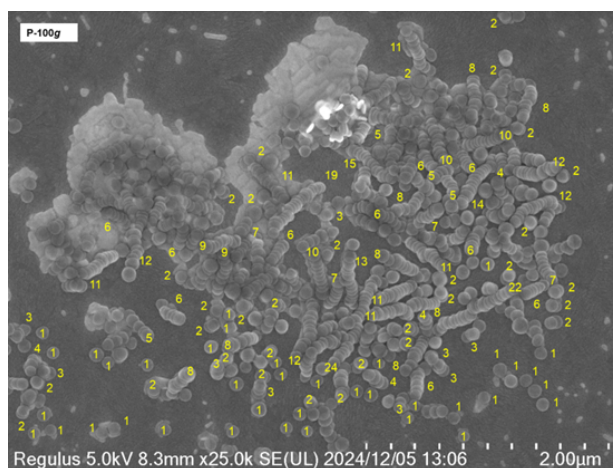
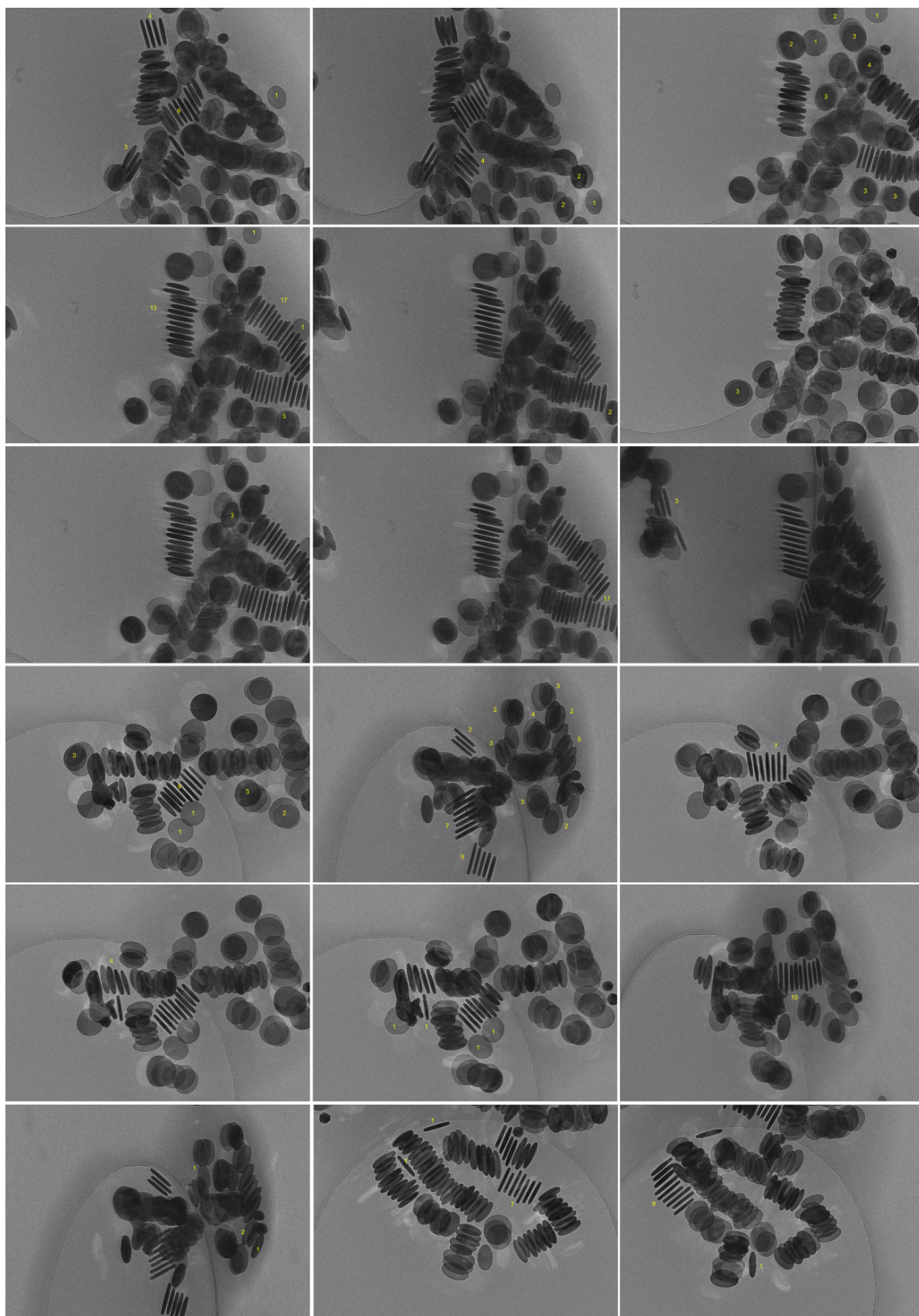
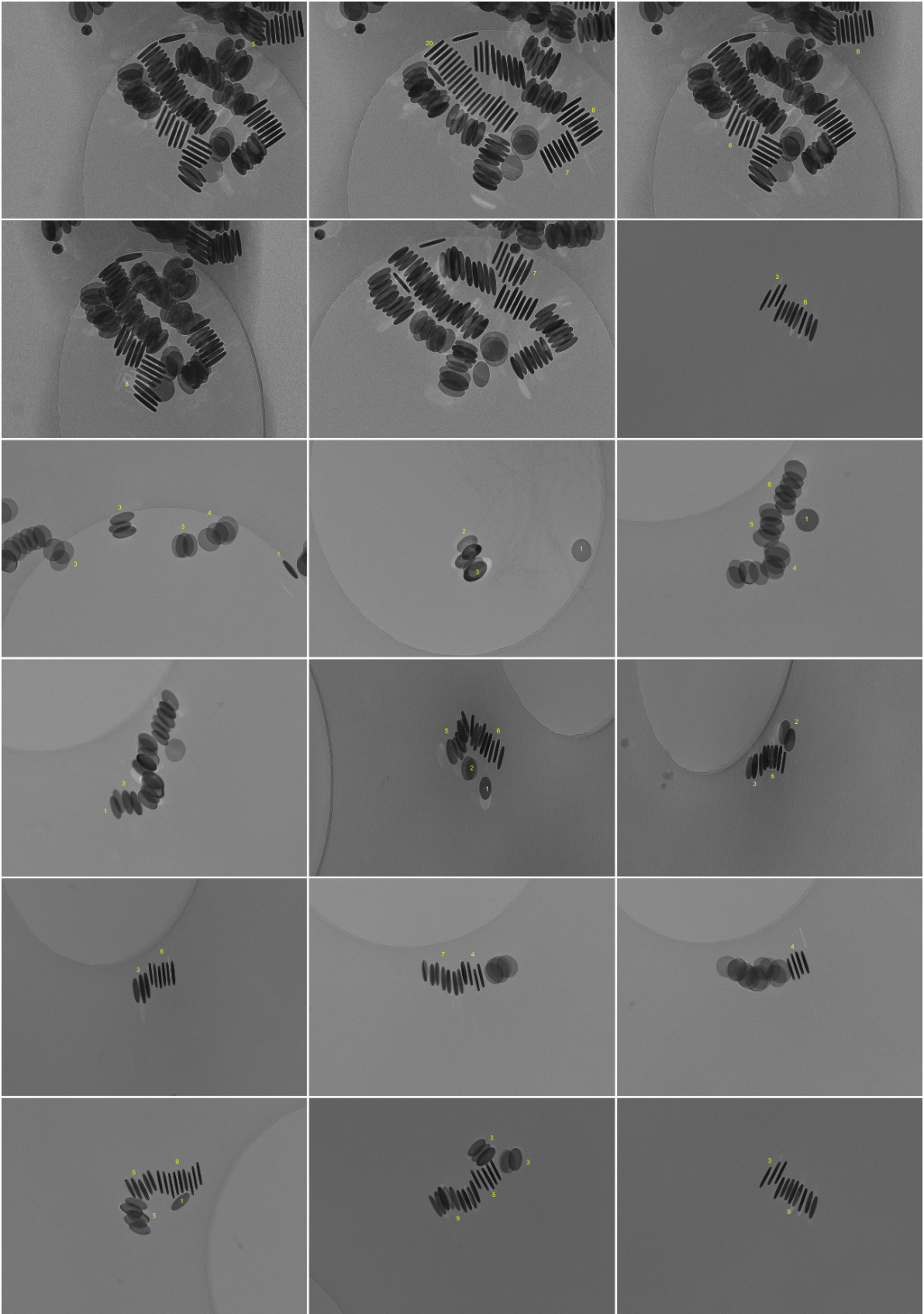


Figure S 15. SEM images of precipitation obtained from separation of 67:33 ratio by applying 100g centrifugal force. Number of oligomers was counted in each assembly cluster.

IV. Cryo-TEM Images of 11 bp-DNA-AuND Multimers with (67:33) Molar Ratio





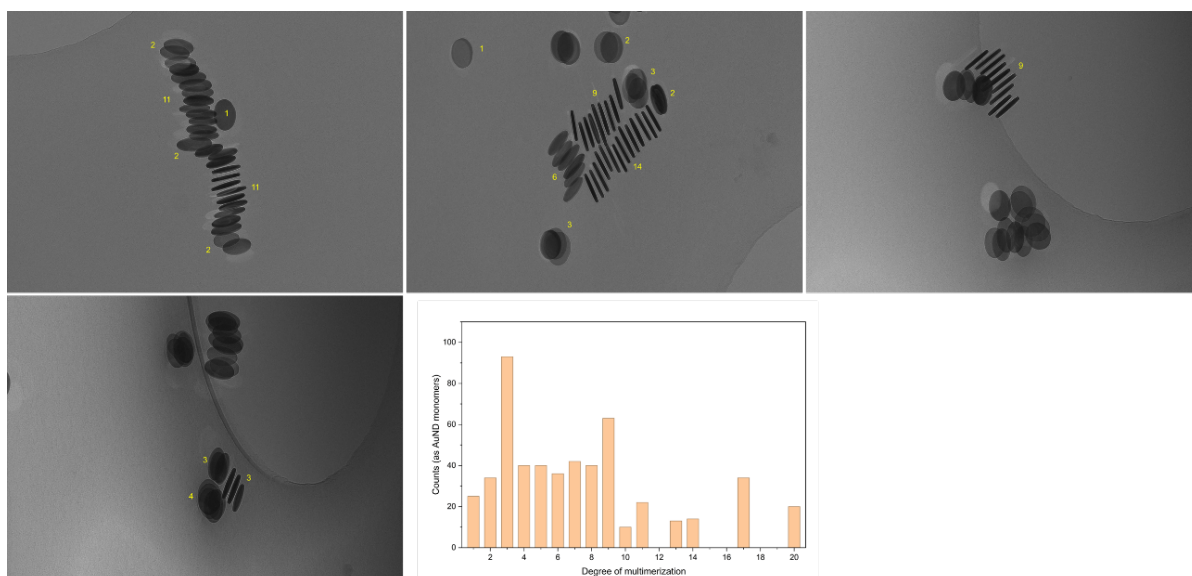
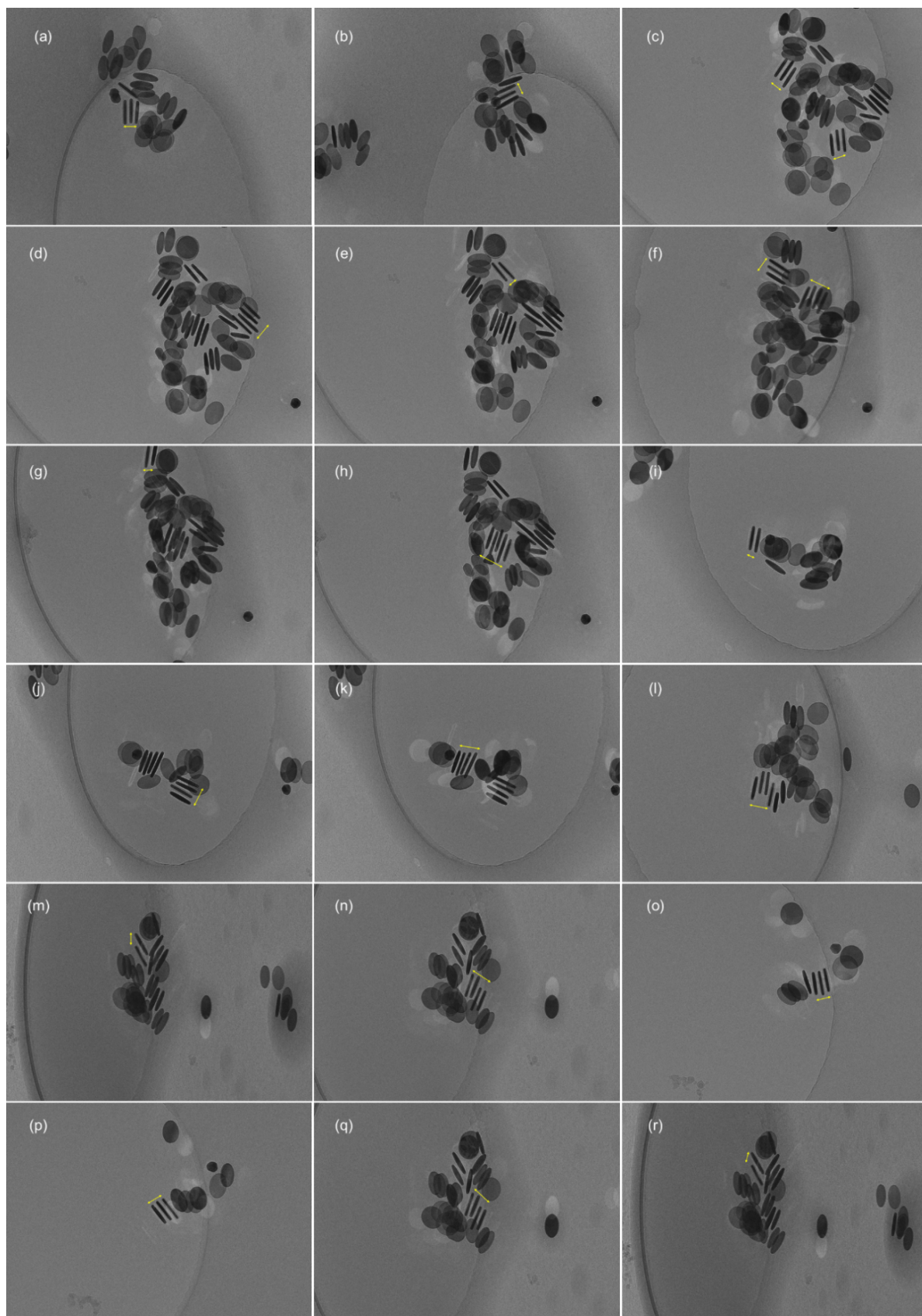


Figure S 16. Cryo-TEM images of AuND multimers prepared by mixing 11-base (+)-DNA-AuND and 11-base (–)-DNA-AuND at a 67:33 molar ratio. The numbers indicate the number of AuNDs counted in each assembly cluster.

V. Cryo-TEM Images of 20 bp-DNA-AuND Multimers with (67:33) Molar Ratio



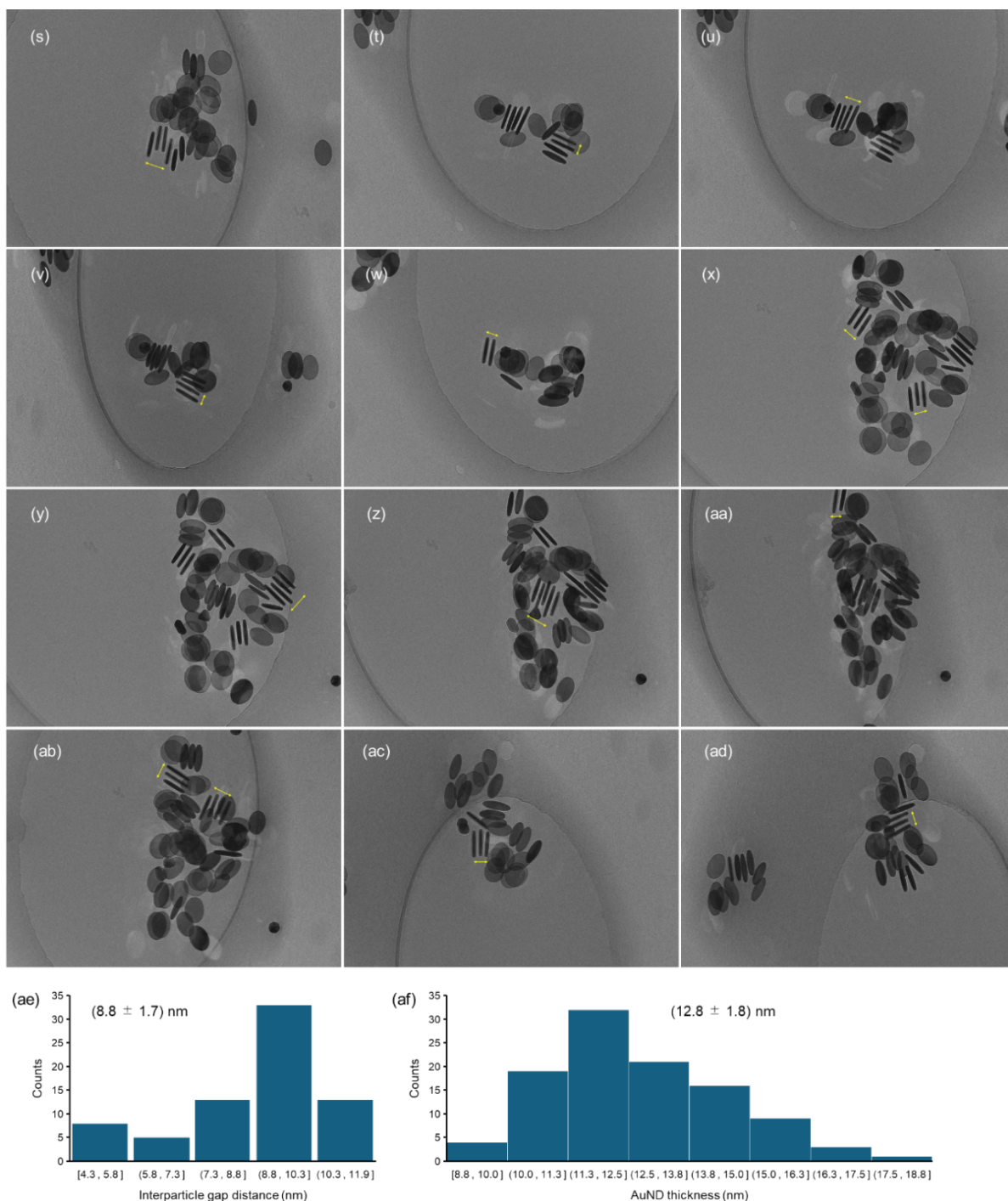


Figure S 17. Cryo-TEM images of assembled 20-bp DNA-AuNDs prepared at a 67:33 molar mixing ratio of (+)-DNA-AuND to (-)-DNA-AuND in 400 mM NaCl. Images were acquired from nine different positions: (a–b) first position, (c–h) second position, (i–k) third position, (l–n) fourth position, (o–p) fifth position, (q–s) sixth position, (t–w) seventh position, (x–ab) eighth position, and (ac–ad) ninth position at various tilt angles. Distances were measured in pixels using ImageJ. Histograms of (ae) interparticle gap distances and (af) AuND thicknesses were generated based on measurements from 105 particles.

VI. Cryo-TEM Images of 8 bp-DNA-AuND Multimers with (67:33) Molar Ratio

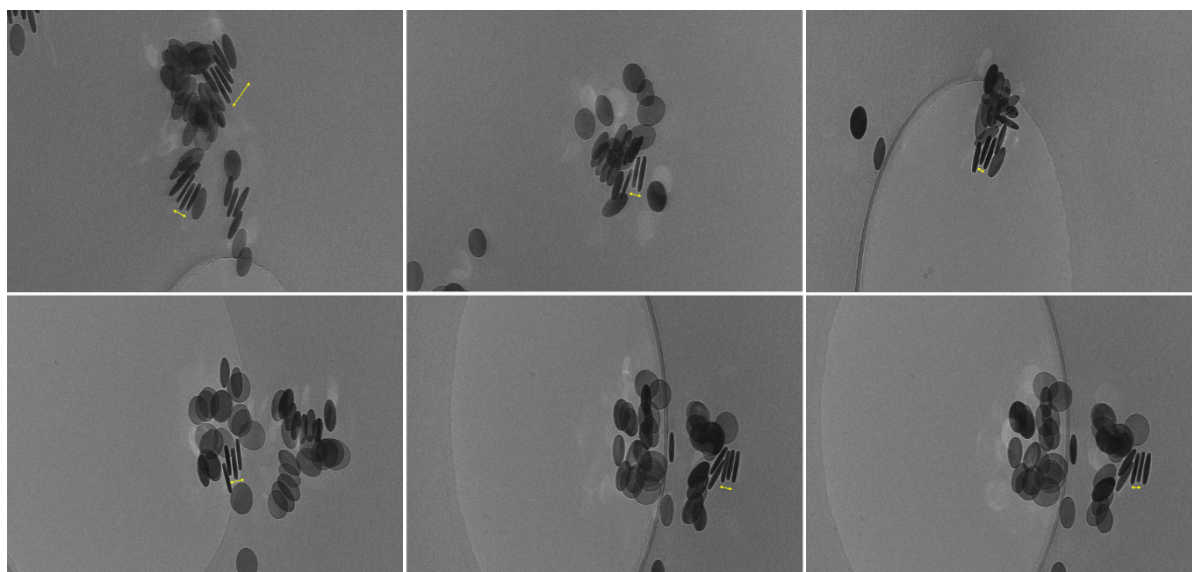
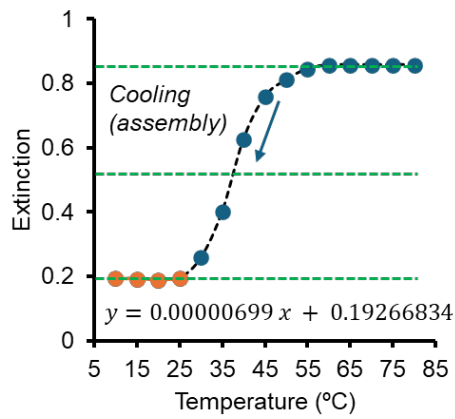


Figure S 18. Cryo-TEM images of assembled 8-bp DNA-AuNDs prepared at a 67:33 molar mixing ratio of (+)-DNA-AuND to (-)-DNA-AuND in 100 mM NaCl. Most particles are aggregated due to the instability of short DNA ligand.

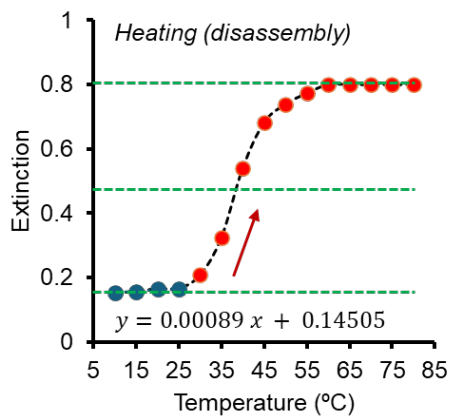
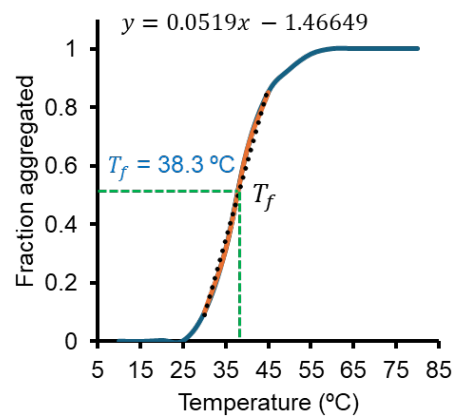
VII. Determination of Assembly (T_a) and Disassembly (T_d) Temperatures of AuNDs

Assembly (T_a) and disassembly (T_d) were defined as freezing (T_f) and melting (T_m) temperatures. To simplify calculate the baseline subtraction method, I assumed that hybridization of 8 bp-DNA was saturated at temperature lower than 25 °C. This approach did not affect T_f and T_m of oligonucleotides. Besides, dehybridization of 20 bp-DNA continued at temperature higher than 80 °C but the limitation of instrument inhibited to fully observe AuNDs dissociation. I believe that 20 base pairings DNA fully dehybridize at 100 °C, promoting all AuNDs dispersed in solution.

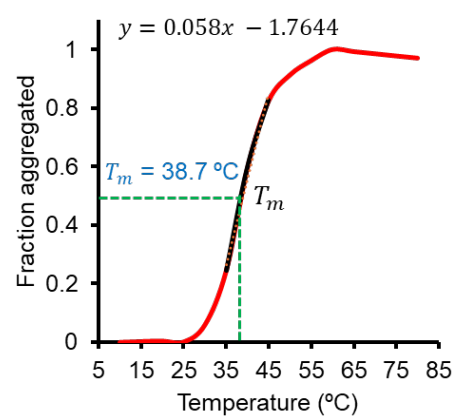
(a) 8 bp-DNA-AuND in 100 mM NaCl



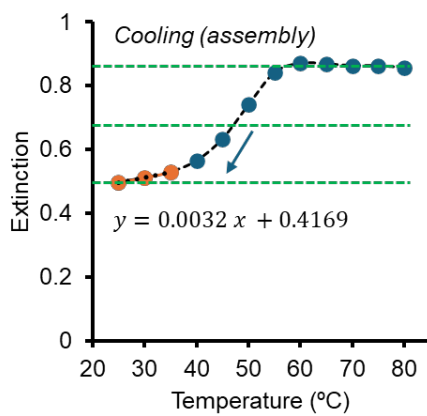
Baseline subtraction



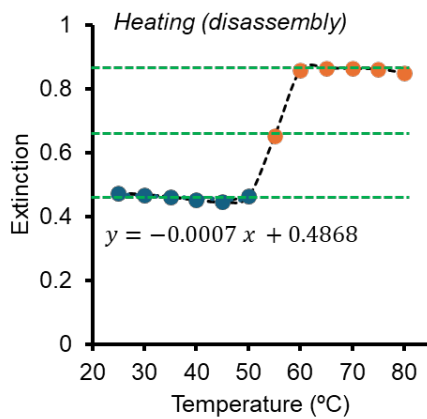
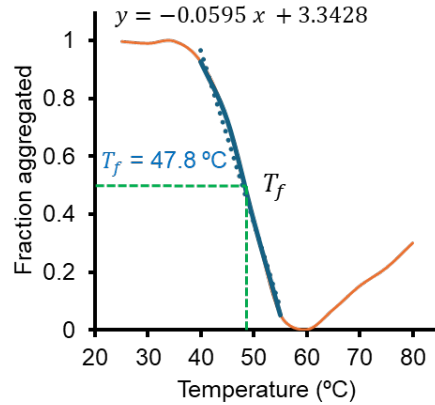
Baseline subtraction



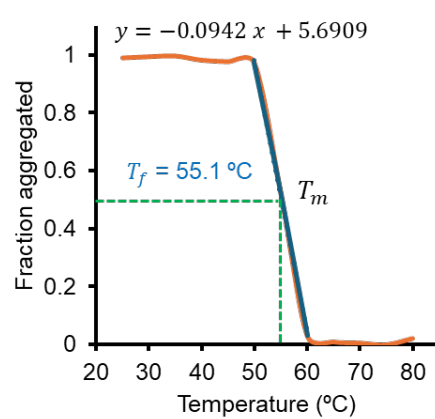
(b) 11 bp-DNA-AuND in 75 mM NaCl



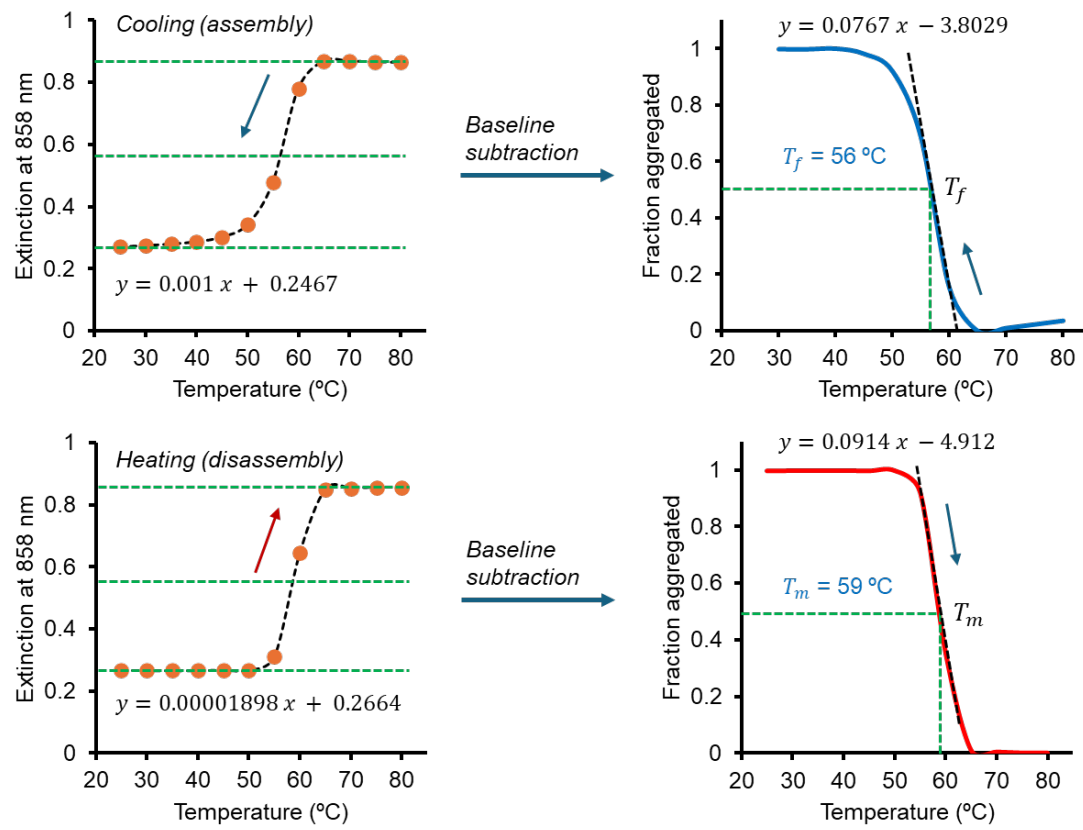
Baseline subtraction



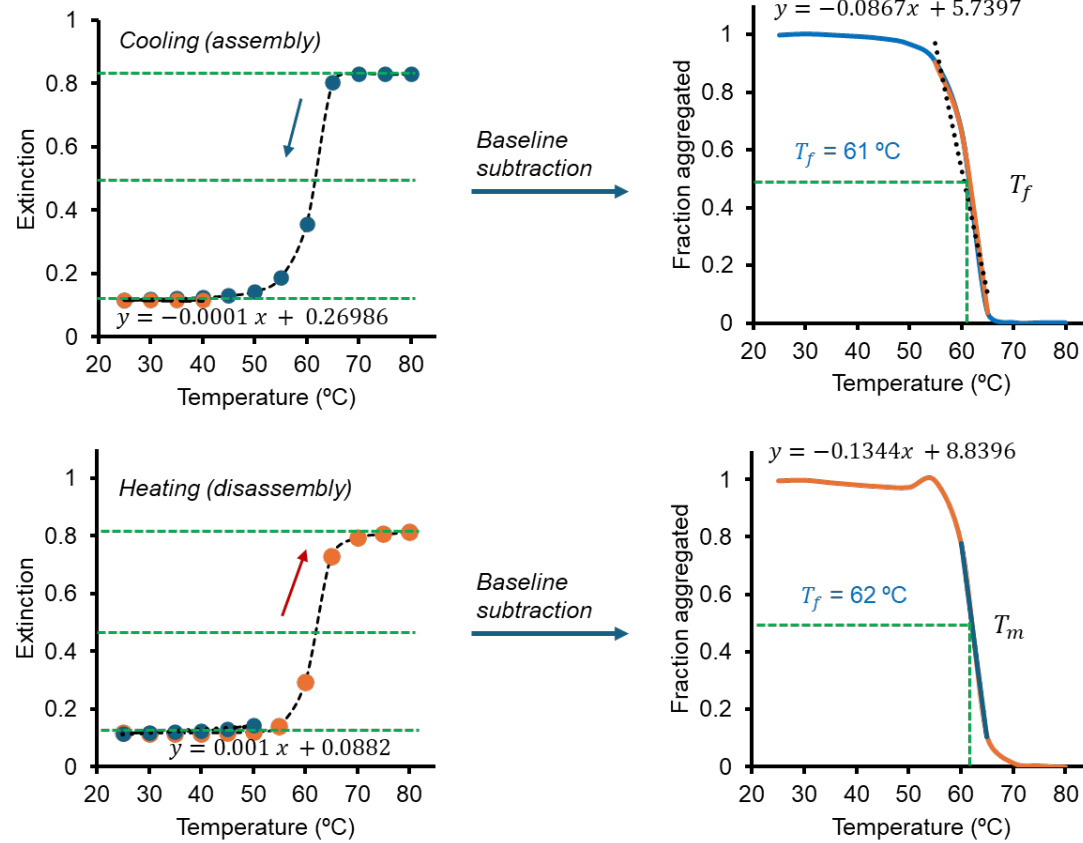
Baseline subtraction



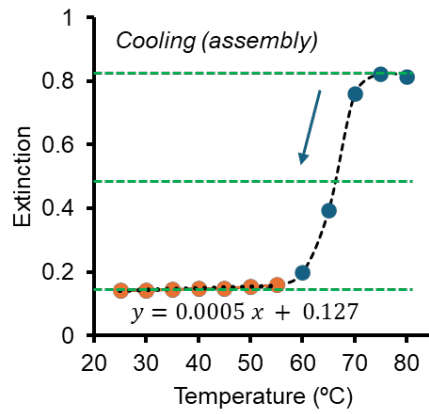
(c) 11 bp-DNA-AuND in 100 mM NaCl



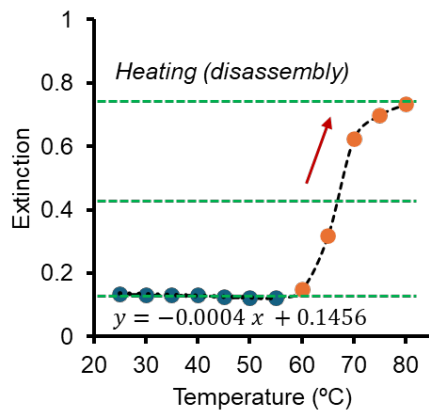
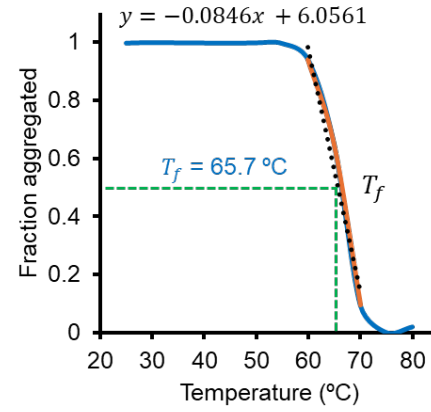
(d) 11 bp-DNA-AuND in 150 mM NaCl



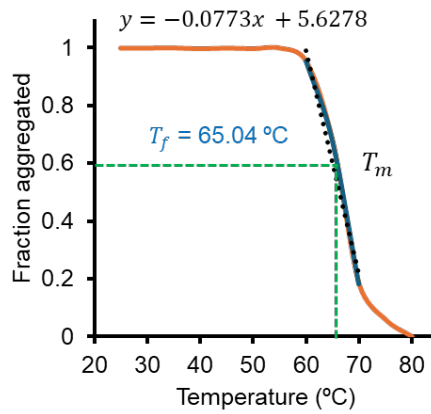
(e) 11 bp-DNA-AuND in 200 mM NaCl



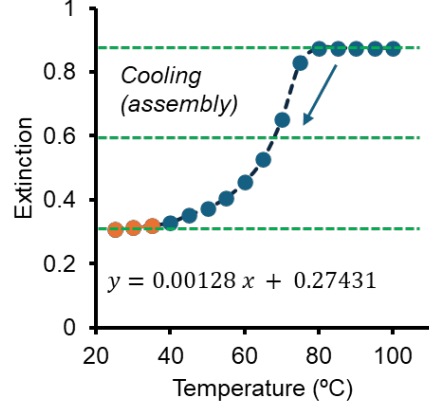
Baseline subtraction



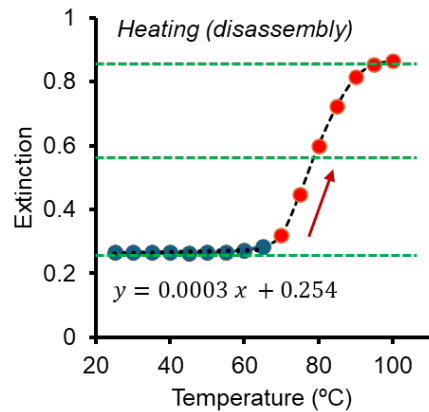
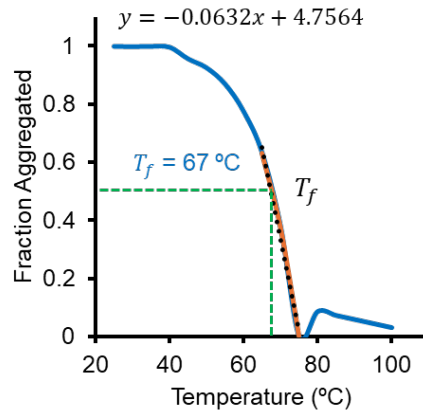
Baseline subtraction



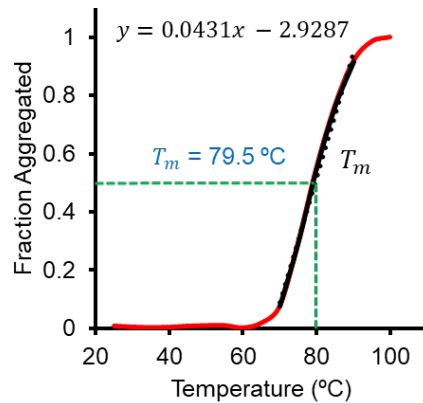
(f) 20 bp-DNA-AuND in 400 mM NaCl



Baseline subtraction



Baseline subtraction



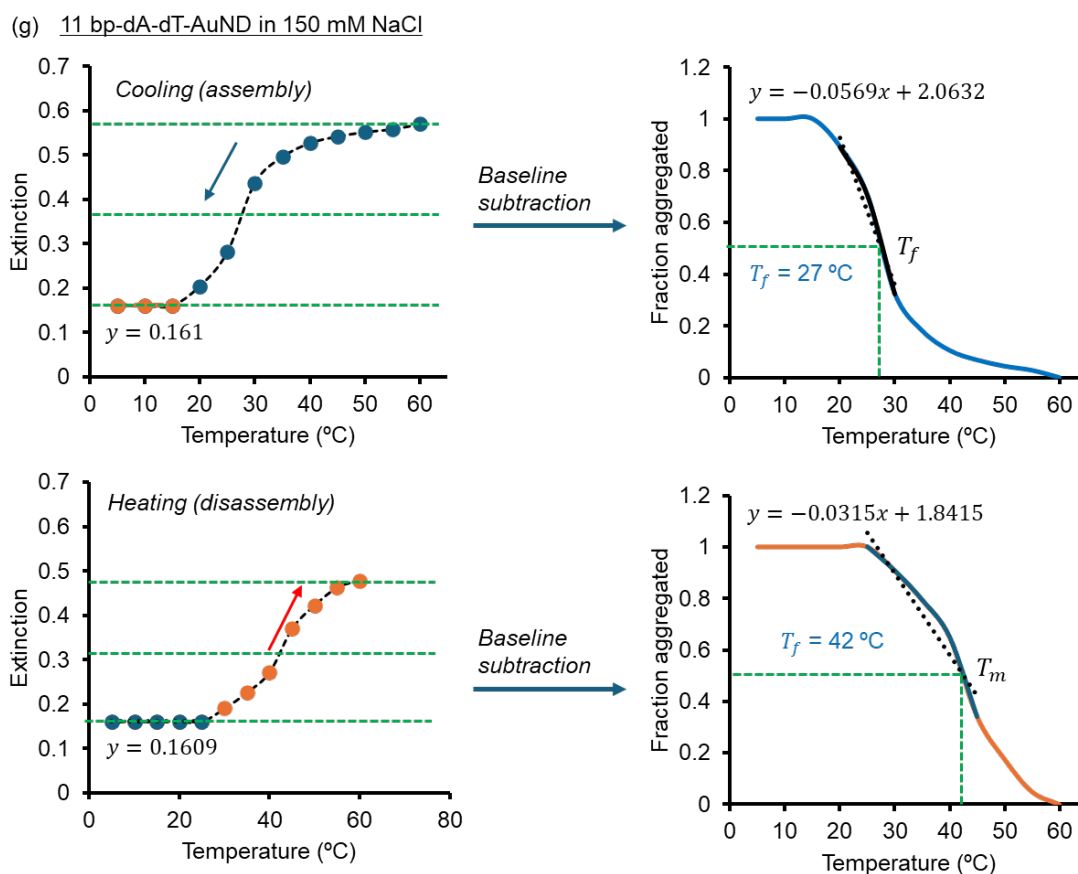


Figure S 19. Conversion from extinction to fraction assembled in temperature-dependent UV-Vis measurements of random and repetitive DNA sequences to define freezing (T_f) and melting (T_m) temperatures following the reported method.²⁴ (a) 8 bp-, (b-e) 11 bp-, and (f) 20 bp-DNA length of random sequences. (g) 11 bp-DNA length of repetitive sequences.