**TITLE:**

Assembly of gold nanorods into chiral plasmonic metamolecules using DNA origami templates

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DNA nanotechnology, gold nanorods, DNA origami, self-assembly, chiral plasmonics, circular dichroism.

**SUMMARY:**

We describe the detailed protocol for DNA origami-based assembly of gold nanorods into chiral plasmonic metamolecules with strong chiroptical responses. The protocol is not limited to chiral configurations and can be easily adapted for fabrication of various plasmonic architectures.

**ABSTRACT:**

Inherentaddressability of the DNA origami structures makes them ideal templates forarrangement of metal nanoparticles into complex plasmonic nanostructures. High spatial precision of DNA origami templated assembly allows controlling the coupling between plasmonic resonances of individual particles and enables tailoring optical properties of the constructed nanostructures. Recently, chiral plasmonic systems attracted a lot of attention due to the strong correlation between the spatial configuration of plasmonic assemblies and their optical responses, e.g., circular dichroism. In this protocol, we describe the whole workflow for generation of DNA origami-based chiral assemblies of gold nanorods (AuNRs). The protocol includes detailed description of design principles and experimental procedures for fabrication DNA origami templates, synthesis of AuNRs, and assembly of origami-AuNRs structures. In addition, characterization of structures using transmission electron microscopy (TEM) and circular dichroism (CD) spectroscopy is included. The described protocol is not limited to chiral configurations and can be adapted for the construction of various plasmonic architectures.

**INTRODUCTION:**

DNA nanostructures, in particular DNA origami, have been widely used to arrange molecules and other nanoscale components, e.g., proteins and nanoparticles (NPs), with nanometer precision into almost arbitrary geometries1–5. The ability to arrange metal NPs on DNA origami templates with high yield and accuracy enables the fabrication of plasmonic structures with novel optical properties6–10. DNA origami technique is especially useful for the generation of chiral plasmonic structures, which require genuinely three-dimensional architectures11–20.

This protocol describes in detail the entire process of the fabrication of DNA origami templated chiral assemblies of gold nanorods (AuNRs). The software used for design21 and structure prediction22,23 of DNA origami is intuitive and freely available. The origami fabrication and AuNRs synthesis uses common biochemistry lab equipment, e.g, thermocyclers, gel electrophoresis, hot plates, centrifuges, etc. The structures are characterized using standard transmission electron microscopy (TEM) and circular dichroism (CD) spectroscopy.

Fabrication of similar plasmonic nanostructures with top-down methods, e.g., electron beam lithography, would require rather complicated and expensive equipment. In addition, DNA origami templates provide possibility to incorporate structural reconfigurability in plasmonic assemblies24–33, which is extremely challenging for structures fabricated with lithography techniques. Compared to other molecular based approaches34–37 DNA origami-based fabrication provides high level of spatial precision and programmability.

**PROTOCOL:**

1. **Design the DNA origami** 
   1. Identify desired relative spatial arrangement of AuNRs and suitable shape of DNA origami template (**Figure 1A**). Estimate structural parameters of the AuNRs and the origami templates. Locate approximate positions of staples that need further modification (**Figure 1B**).
   2. Download and install caDNAno18 to design DNA origami template. In caDNAno, route the scaffold strand according to the desired shape of the template and generate the staple strands (Click **Seq Tool**). Click **Paint Tool** and mark the staple strands that require further modification, i.e., (**Figure 1C**).
   3. Click **Export Tool** to export the DNA staple sequences (**Figure 1C**) to csv file.
   4. Design double-stranded locks to fix the angle *Θ* between the two origami bundles. Depending on the relative orientation of the two bundles, the origami construct can adapt left- or right-handed (LH/RH) chiral spatial configuration (**Figure 1B**).
   5. Import the staples csv file in a spreadsheet application. Add polyA10 sequence at the end of the staples used for AuNRs assembly (handles). Modify the staple strands on the designed lock sites with lock sequences.
2. **Assembly of the DNA origami templates**
   1. Prepare working stock of staple stands (SM), including strands with handles and locks, by mixing equal amounts of concentration-normalized staple oligonucleotides, e.g.*,* 100 μM).

Note: origami structures usually contain several hundreds of staple strands. Staples are typically purchased from vendors specializing in chemical synthesis of DNA oligonucleotides in multiwell, e.g., 96-well-plates.

* 1. For 500 µL of 10 nM origami, mix 50 µL TE (10X), 100 µL MgCl2 (100 mM), 25 µL NaCl (100 mM), 170 µL H2O, 100 µL SM (0.5 µM), 5 µL lock strands (5 µM) and 50 µL scaffold (100 nM).
  2. Anneal the mixture in a thermocycler from 80 °C to 20 °C through process in **Table 1**.

1. **DNA origami purification**

Note: this section describes protocol for agarose gel purification. DNA origami templates can also be purified using alternative approaches38,39.

* 1. For 1% gel, dissolve 1 g agarose in 100 mL TBE (0.5X) by heating the mixture in a microwave oven. Add 10000 µL of 10X DNA stain according to the stain specification. To minimize the exposure to UV light at extraction step (step 3.6), use DNA stain that can be visualized under blue excitation.
  2. Cool the solution to approximately 40 °C and slowly add 1 mL of MgCl2 (1.3 M) with shaking. Cast gel and incubate for 30 min at room temperature.
  3. Set the electrophoresis devise and pour cold (4 °C) running buffer (0.5X TBE with 11 mM MgCl2) in the gel box. Place the gel box in an ice water bath.
  4. Add loading buffer to the origami samples (6X loading buffer contains: 15% polysucrose 400 and 0.25% bromophenol blue in water). Load the samples into the wells with proper volume according to the comb used, e.g.*,* 50 µL for an 8-well comb of 1.5 mm thickness.
  5. Run the electrophoresis for 2 h at 80 V.

Note: to characterize the origami and separate the open and closed structure, use 2% gel instead of 1% and prolong the running time to 4 h.

* 1. Image the gel with the gel imager (**Figure 2**). Use blue light transilluminator to visualize the bands, cut the origami band, smash the gel on a parafilm and extract the liquid. The recovery yield is approximately 40%.
  2. Pipette the liquid into a centrifugal filter unit and spin at 3000 x g for 5 min. Measure the absorption of the origami solution at 260 nm with a UV-VIS spectrometer. Estimate the concentration of origami using extinction coefficient of 1.3x108 M-1 cm-1.

Note: typical concentration of origami solution after agarose gel purification is 1 - 2 nM.

* 1. Store the purified origami templates at 4 °C for later use.

1. **Synthesis of gold nanorods**

Note: the protocol for AuNRs synthesis is adapted from previous literature40 with minor modifications.

* 1. Wash all glassware with aqua regia for 5 min, rinse with water, sonicate with ultrapure water, and dry before use.
  2. Prepare 0.2 M hexadecyltrimethylammonium bromide (CTAB), 1 mM HAuCl4, 4 mM AgNO3, 64 mM L(+)-ascorbic acid, and 6 mM NaBH4. Use cold water (4 °C) to dissolve NaBH4, and keep it in fridge at 4 °C. Ascorbic acid solution has to be freshly prepared.

CAUTION: CTAB is hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation. Wear suitable protective clothing. In case of insufficient ventilation, wear suitable respiratory equipment.

CAUTION: NaBH4 is extremely hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation. Wear splash goggles, lab coat, gloves, vapor and dust respirator. Be sure to use an approved/certified respirator or equivalent.

* 1. Prepare Au seeds
     1. Add 500 µL CTAB (0.2 M), 250 µL ultrapure water, and 250 µL HAuCl4 (1 mM) into a glass vial. Stir at 450 rpm at room temperature for 5 min.
     2. Increase the stirring rate to 1200 rpm. Add 100 µL cold NaBH4 solution (6 mM, 4 °C). After 2 min, stop the stirring and incubate the solution in a water bath at 30 °C for 30 min before use.
  2. Prepare AuNRs
     1. Dissolve 0.55 g CTAB and 0.037 g 2,6-Dihydroxybenzoic acid in 15 mL warm water (60 - 65 °C) into a round bottom flask. Cool down the solution to 30 °C, add 600 µL AgNO3 (4 mM) and stir at 450 rpm for 2 min. Then leave the solution undisturbed for 15 min at 30 °C.
     2. Add 15 mL HAuCl4 (1 mM) to the solution, and stir at 450 rpm for 15 min. Add 120 µL L(+)-Ascorbic acid (64 mM), then immediately, stir at 1200 rpm for 30 s. Add 12 µL Au seeds, and keep stirring at 1200 rpm for 30 s.
     3. Incubate the solution in a water bath at 30 °C for 18 h. Do not disturb, and use a cap to close the flask.
     4. Transfer the resultant solution to centrifuge tubes, and centrifuge at 9500 x g for 12 min at 20°C. Discard the supernatant, disperse the pellet in 20 mL ultrapure water and perform one more centrifugation step.
     5. Disperse the final pellet in 3.0 mL distilled water. Estimate the concentration of AuNRs from UV-VIS absorption measurement using extinction coefficient for the longitudinal plasmon resonance. The extinction coefficient can be predicted using AuNRs shape parameters41. Store the AuNRs at 4 °C for further use.

1. **Functionalize gold nanorods with single stranded-DNA**

Note: this section describes the protocol for AuNRs functionalization with single stranded-DNA (ssDNA) following the so-called low pH route adapted from a previous literature42. The AuNRs covered with DNA are purified by centrifugation; alternatively, the purification can be performed using agarose gel electrophoresis.

* 1. Incubate 20 µL thiol-functionalized polyT DNA strands (1 mM) with 20 µL freshly prepared tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 14 mM) for 1 h to reduce disulfide bonds.

Note: the thiol groups form bonds with AuNRs and the polyT sequence hybridize with the polyA10 handle on the origami, in which too many or too few base pairs may lead to malfunction or unstable assembly.

CAUTION: TCEP can cause severe skin burns and eye damage. Wear protective gloves/ protective clothing/ eye protection/ face protection.

* 1. Mix 150 µL AuNR (10 nM) and 40 µL TCEP treated thiol-DNA (0.5 mM). Add 1% sodium dodecyl sulfate (SDS) to AuNRs solution to reach a final SDS concentration of 0.05%. Adjust the pH to 2.5 - 3 with ~1 µL HCl (1 M).
  2. Add 40 µL TCEP treated thiol-DNA (0.5 mM) to AuNRs solution. Incubate for 2 h with shaking at 70 rpm.

Note: the AuNRs to DNA ratio should be in the order of 1:5000-15000 depending on the size of the rods. For the AuNRs (70 x 30 nm) prepared following the protocol described in the section 4, 13000 excess of thiol-DNA is recommended.

* 1. Add NaCl to reach a final NaCl concertation of 0.5 M and incubate for 4 h at room temperature with shaking at 70 rpm.

Note: a color change at this step may indicate a failed DNA functionalization.

* 1. Adjust the pH to ~8.5 with TBE buffer (10X) and incubate overnight.
  2. Wash the DNA-AuNRs for 4 times by mixing the samples with 1000 mL washing buffer (0.5X TBE with 0.1% SDS) and centrifuge at 7000 x g for 30 min. Remove the supernatant and resuspend the DNA-AuNRs in the remaining liquid (~40 µL). Estimate the concentration of DNA-AuNRs from UV-VIS absorption measurement as in step 4.4.5.

Note: solution might become slightly ‘cloudy’ at steps 5.3 - 5.4 due to CTAB replacement from the surface of the AuNRs by thiol-DNA. The solution should become clear upon warming up to ~35 °C for 5 min.

1. **Assembly of gold nanorods on DNA origami templates**
   1. Add MgCl2 to the solution of purified DNA-AuNRs to a final concentration of 10 mM. Mix purified DNA-AuNRs and origami with 10:1 ratio.

Note: lower ratio may decrease the product yield43.

* 1. Anneal the mixture in a mixer with temperature control from 40 °C to 20 °C while shaking at 400 rpm with the procedure in **Table 2**.

Note: for CD characterization, the sample can be measured after this step without further purification.

* 1. Use 0.7% agarose gel electrophoresis (3.5 h at 80 V) to purify the final origami-AuNRs structures.
  2. Use white light transilluminator for imaging. Cut the product band (origami-AuNRs dimer) (**Figure 3**), smash the gel on a parafilm and extract the liquid. Pipette the liquid into a centrifugal filter unit and spin at 3000 x g for 5 min. Resuspend the origami-AuNRs in the solution. The recovery yield from the gel is approximately 50%.
  3. Estimate the concentration of the origami-AuNRs structures from UV-VIS absorption measurement as in step 4.4.5.

1. **Transmission electron microscopy imaging**

Note: uranyl formate (UFo) staning protocol is adapted from previous literature44.

* 1. Mix 200 µL UFo solution (0.75%) and 1 µL NaOH (5 M) and vortex immediately for 2 - 3 min. Centrifuge the stain solution for 3 - 4 min at 14000 x g. Protect the stain from light exposure, e.g., by wrapping in aluminum foil.

CAUTION: UFo is toxic if inhaled or swallowed and can cause eye irritation. In case of brief exposure or low pollution use respiratory filter device. In case of intensive or longer exposure use self-contained respiratory protective device. Wear gloves. The glove material has to be impermeable and resistant to UFo and its solutions. Wear tightly sealed goggles.

* 1. Glow discharge carbon/formvar coated TEM grids for 6 s just before use to increase hydrophilicity and promote sticking of the structures. Pipette 5 µL sample drops on the TEM grid, incubate for 5-8 min and remove the drop by gently touching a filter paper with the edge of the grid.
  2. Pipette one big (~20 µL) and one small (~10 µL) drop of the stain solution on a parafilm. Put the grid on the small stain solution drop and dry immediately by touching the filter paper with the edge of the grid. Then put it on the big stain solution drop for 30 s.
  3. Remove the liquid on the grid by touching the filter paper with the edge of the grid. Place the grid in the grid holder. Wait for the grid to dry for at least 10 min.
  4. Characterize the samples of origami (**Figure 4**), AuNRs (**Figure 5**), and origami-AuNRs (**Figure 6**) by TEM.

1. **Circular dichroism measurement**
   1. Purge the CD spectrometer with N2 for 20 min.

Note: most of the CD spectrometers require purging with N2 before lamp ignition. Check the CD spectrometer manual.

* 1. Set the bandwidth, scanning range, and acquisition step.

Note: the scanning range depends on the optical properties of AuNRs, which depend on the size of the AuNRs.

* 1. Measure blank CD with buffer.
  2. Measure the CD spectra of origami-AuNRs samples (**Figure 7**).

Note: i) use quarts or glass cuvettes for CD measurement. Plastic cuvettes are unsuitable for CD spectroscopy. ii) most of the CD spectrometers allow simultaneous acquisition of absorption and CD data.

**REPRESENTATIVE RESULTS:**

TEM images of DNA origami templates, AuNRs and final origami-AuNRs assemblies are shown in **Figure 4**, **Figure 5** and **Figure 6A** respectively. Due to binding preference to TEM grids origami-AuNRs assemblies are usually seen as parallel origami bundles and rods (**Figure 6A**). Thermal annealing is required for the correct alignment of AuNRs on origami templates (**Figure 6A** and **Figure 6B**). The protocol enables high yields of assembly of AuNRs into chiral metamolecules with strong plasmonic CD responses (**Figure 7**).

**FIGURE AND TABLE LEGENDS:**

**Table 1. Temperatures and rates for thermal annealing of DNA origami templates.**

**Table 2. Temperatures and holding times for annealing of AuNRs and DNA origami templates.** The cooling rate between the steps is set to 0.1 °C/min. The DNA origami- AuNRs samples are annealed while shaking at 400 rpm.

**Table 3. Material for all experiments.**

**Figure 1.** **Design of DNA origami templated chiral metamolecules. A.** Identify desired relative spatial arrangement of gold nanorods (AuNRs) and suitable shape of DNA origami template. **B.** Estimate structural parameters of the AuNRs (*DAuNR, LAuNR*) and origami template (*Worigami, Lorigami*,*Θ*). Locate approximate positions of staples that need further modification. **C.** Design DNA origami templates using caDNAno.

**Figure 2.** **The agarose gel electrophoresis of origami.** **A**. Purification with 1% agarose gel electrophoresis for 2 h at 80 V. **B**. Characterization with 2% agarose gel electrophoresis for 4 h at 80 V.

**Figure 3. The agarose gel electrophoresis purification of origami-AuNRs**. 0.5% gel run for 3.5 h at 80 V of samples prepared following the assembly procedure with different DNA-AuNRs to origami ratio (20:1, 5:1) and samples (10:1 DNA-AuNRs to origami ratio) with/without annealing procedure. For TEM images of samples in bands 1, 2, 3 see **Figure 6**.

**Figure 4. Representative TEM image of the DNA origami templates.** Origami structure consists of two 14-helix bundles (80 nm × 16 nm × 8 nm) linked together by the scaffold strand.

**Figure 5. Representative TEM image of the AuNRs.** The average dimensions of synthesized AuNRs are 70 x 30 nm.

**Figure 6. TEM images of origami-AuNRs assemblies.** **A.** AuNRs dimers on origami after annealing (band 1 in Figure 3). **B.** AuNRs dimers on origami without annealing (band 2 in Figure 3). **C.** Origami-AuNRs aggregates (band 3 in Figure 3).

**Figure 7. CD spectra of the origami-AuNRs assemblies.** The CD spectra of the closed structures (the origami templates fixed by lock strands into a right-handed configuration with 50° between two origami bundles) and the open structure (the origami templates without lock strands).

**DISCUSSION:**

The protocol introduces the whole workflow of design, assembly, purification and characterization of DNA origami-based chiral assemblies of AuNRs. DNA origami templates used in the protocol are particularly suitable for the fabrication of stimuli responsive assemblies. Various types of responses and functionalizes can be incorporated into the lock strands that defines the chiral state of the origami template (**Figure 1B**)24–26,31. For static assemblies, simpler block-shaped templates are often sufficient14,45–47.

For achieving reliable and reproducible optical responses of chiral assemblies, we strongly recommend adapting the protocol for AuNRs synthesis40, since the quality and optical properties of commercial products may vary between batches. Additional annealing (step 6.2) is often crucial for ensuring the correct attachment of AuNRs to DNA origami templates (**Figure 6**).

The DNA origami-based approach to fabrication of plasmonic nanostructure inherits limitations of DNA origami technique48. The size of the origami templates is typically limited by the size of the scaffold strand. The stability of DNA structures is reduced under law salt conditions. The cost of synthetic stable strands remains rather high. However, recent developments in structural DNA nanotechnology are expected to overcome these limitations49-55.

Finally, we would like to mention that the protocol described here is not limited to chiral assemblies. DNA origami provides very flexible platform for the fabrication of complex plasmonic nanostructures.

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**DISCLOSURES:**

The authors have nothing to disclose.

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