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Polymer Nanoarray Approach for the Characterization of Biomolecular Interactions

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Abstract

Pair-wise interactions at the single-molecule level can be done with nanoprobings techniques, such as AFM force spectroscopy, optical tweezers, and magnetic tweezers. These techniques can be used to probe interactions between well-characterized assemblies of biomolecules, such as monomer-dimer, dimer-dimer, and trimer-monomer. An important step of these techniques is the proper assembly of dimers, trimers, and higher oligomers to enable the interactions to be probed. We have developed a novel approach in which a defined number of peptides are assembled along a flexible polymeric molecule that serves as a linear matrix, termed as *flexible nanoarray (FNA)*. The construct is synthesized with the use of phosphoramidite chemistry (PA), in which non-nucleoside PA spacers and standard oligonucleotide synthesis are used to grow the polymeric chain with the desired length. The reactive sites are incorporated during FNA synthesis. As a result, the FNA polymer contains a set of predesigned reactive sites to which the peptides are covalently conjugated. We describe the protocol for the synthesis of FNA and the application of this methodology to measure the molecular interactions between amyloid peptides of monomer-monomer, monomer-dimer, and dimer-dimer.

Keywords

Protein-protein interactions; Nanoprobings; AFM; Force spectroscopy; Amyloid oligomers; Click chemistry

1 Introduction

All biological dynamics are controlled by intermolecular interactions. Molecular biophysicists measure these interactions to increase our understanding of the molecular mechanisms of biological processes. A breakthrough in the techniques used to study these interactions was made in the last two decades with the invention of single-molecule methods that enable intermolecular interactions between numerous biological molecules to be probed, as described in recent reviews [1–4]. These techniques primarily include optical trapping or tweezers (OT), force spectroscopy studies with atomic force microscopy (AFM), and magnetic tweezers (MT). These techniques enable one to measure interactions in the range

of hydrogen bonds (primarily OT and MT methods) up to interactions approaching the strength of a covalent bond (AFM force spectroscopy) [5]. Many biological systems consist of several biological molecules; therefore, nanoprobng approaches need to be able to measure the interactions among these molecules within the systems. One example is the characterization of amyloid protein assembly from monomers into oligomers. The self-assembly of amyloid protein into nano-aggregates is a hallmark of diseases such as Alzheimer's and Parkinson's diseases. Characterizing this self-assembly process will improve our understanding of the molecular mechanisms leading to the development of these devastating diseases ([6–9]). We have recently introduced an approach that allowed us to characterize dimers formed by different amyloidogenic polypeptides [10]. The use of a flexible polymeric platform that contained the assembled oligomers allowed us to probe oligomers larger than dimers [11]. The flexible polymer is synthesized from the non-nucleoside PA spacers using a commercial DNA synthesizer. An important feature of this approach is that phosphoramidites with reactive groups can be incorporated at defined stages during the synthesis process, leading to the incorporation of polymers at the reactive sites at predetermined positions. We termed this linear polymeric template flexible nanoarray (FNA, [12, 13]).

This chapter describes protocols for the synthesis of FNA, with an emphasis on the incorporation of reactive groups inside the FNA, and modifications of the FNA ends to immobilize the tether onto the AFM tip and surface. This approach is illustrated by experimental data from AFM probing of dimers, trimers, and tetramers of A β (14-23) peptides, as described in [11, 13].

2 Materials

2.1 Reagents

1. Biotin-conjugated controlled pore glass (*3'-Protected* Biotin Serinol CPG; Glen Research, Sterling, VA).
2. Oxidizer: 0.02 M I₂ in THF/pyridine/H₂O (Glen Research, Sterling, VA).
3. Capping reagent A: THF/2,6-lutidine/Ac₂O (Glen Research, Sterling, VA).
4. Capping reagent B: 16% 1-MeIm in THF (Glen Research, Sterling, VA).
5. Deblocking reagent: 3% dichloroacetic acid in dichloromethane (Glen Research, Sterling, VA).
6. Activator: 0.25 M 5-ethylthio-1H-tetrazole in acetonitrile (Glen Research, Sterling, VA).
7. Spacer 18 phosphoramidite (18-*O*-dimethoxytritylhexaethyle-neglycol, 1-[(2-cyanoethyl)-(N, N-diisopropyl)]-phosphoramidite) (Glen Research, Sterling, VA).
8. DBCO-dT-CE phosphoramidite (5'-dimethoxytrityl-5-[(6-oxo-6-(dibenzo[b, f]azacyclooct-4-yn-1-yl)-capramido-*N*-hex-6-yl)-3-acrylimido]-2'-deoxyuridine,

- 3'-[(2-cyanoethyl)-(N, N-diisopropyl)]-phosphoramidite (Glen Research, Sterling, VA).
9. Thiol modifier C6 S-S (1-*O*-dimethoxytrityl-hexyl-disulfide, 1'-[(2-cyanoethyl)-(N, N-diisopropyl)]-phosphoramidite) (Glen Research, Sterling, VA).
 10. Azide- terminated A β (14-23) peptides (e.g., Peptide 2.0 Inc, VA, USA).
 11. Tris(2-carboxyethyl) phosphine (TCEP) hydrochloride (Hampton Research, Aliso Viejo, CA).
 12. Heterobifunctional MAL-PEG-SVA (M.wt 3400 g/mol) (Laysan Bio, Arab, AL).
 13. *N*-(g-Maleimidobutyryloxysuccinimide ester) GMBS (Pierce Biotechnology, Grand Island, NY).
 14. Streptavidin thiol (SAVT) (Protein Mods, Madison, WI).
 15. 1-(3-Aminopropyl) silatrane (APS), use the previously described protocol [14] for synthesis.
 16. Mica sheet (Asheville-Schoonmaker Mica Co., Newport News, VA).
 17. MSNL-10 AFM probe (Bruker, Camarillo, CA).
 18. Epoxy glue (EPOTEC 301, Epoxy Technology, Inc., Billerica, MA).
 19. 0.22 μ m syringe filter (Millipore express PES membrane, Sigma-Aldrich, USA).
 20. Triethylammonium bicarbonate buffer, pH 8.0 (Sigma-Aldrich, St. Louis, MO).

2.2 Instruments

1. MerMade 12 (Bioautomation, Irving, Texas) DNA synthesizer.
2. UV chamber with UV lamp (366 nm wavelength) (Homemade).
3. Eppendorf Vacufuge, model 5301 (Hamburg, Germany).
4. Water purification system (aquaMAX-Ultra, YL Instruments Co., Ltd., Gyeonggi-do, Republic of Korea).
5. MFP-3D AFM instrument (Asylum Research, Santa Barbara, CA).
6. HPLC system (Agilent HPLC 1100, Agilent Technologies, Waldbronn, Germany).
7. Phenomenex Gemini C18, 5 μ , 250 $\times$ 4.6 mm (Phenomenex, Torrance, CA).
8. Nanodrop UV-visible spectrometer (ND-1000, Thermo Scientific, Grand Island, NY).
9. Electrospray ionization (ESI) mass spectrometer (Thermo Scientific LTQ LCMS system, Grand Island, NY).

2.3 Buffers and Solutions

1. 0.1 M spacer 18 phosphoramidite solution: Dissolve 0.25 g of the reagent in 3.18 mL of anhydrous acetonitrile (*see* Note 1).
2. 0.1 M DBCO-dT-CE phosphoramidite solution: Dissolve 0.25 g of reagent in 2.06 mL of anhydrous acetonitrile/dichloromethane solvent (*see* Note 1).
3. 0.1 M thiol modifier C6 S-S solution: Dissolve 0.25 g of reagent in 3.25 mL of anhydrous acetonitrile solvent (*see* Note 1).
4. 10 mM sodium phosphate buffer, pH 7.0: Dissolve 0.142 g of sodium phosphate monobasic (NaH_2PO_4) in 90 mL DI water. The pH should be adjusted to 7 with 1 M sodium hydroxide or 10% HCl, and the volume should be made up to 100 mL.
5. 50 mM GMBS cross-linker solution: 50 mM of stock solution of GMBS is prepared by dissolving 1.4 mg of GMBS (M.wt 280.23 g/mol) in 100 μL of DMSO. Dilute the solution to the required concentration with DMSO.
6. 10 mM β -mercaptoethanol: 7 μL of β -mercaptoethanol (M.wt 78.13 g/mol, density 1.114 g/cm³) is dissolved in 10 mL of 10 mM phosphate buffer (pH 7.0) (*see* Note 2).
7. 0.1 M TCEP solution: 140 mg of TCEP-HCl (M.wt 286.65 g/mol) is dissolved in 5 mL 10 mM phosphate buffer (pH 7.0), and then pH is adjusted to 7.0 by strong sodium hydroxide solution. The solution is diluted as required in the experiments (*see* Note 3).
8. 167 μM APS solution: 44 mg of solid APS powder is dissolved in 3.78 mL of DI water to make 50 mM of stock solution. The solution is then filtered with a 0.22 μm syringe filter to remove any undissolved substances. To make 167 μM solution, 1 μL of stock solution is diluted with 299 μL of DI water.
9. HPLC solvents: Solvent A (100% acetonitrile HPLC grade) and Solvent B: 0.1 M triethylammonium bicarbonate (pH 7.5), which is prepared from commercially available 1 M triethylammonium bicarbonate by diluting 10 times with DI water.

3 Methods

The schematics for the assembly of FNA with the conjugated peptide A β (14-23) are shown in Fig. 1. The FNA with two DBCO groups separated with six PA units is synthesized in the DNA synthesizer (Fig. 1a). The product is reacted with azide-terminated A β (14-23) to

¹Phosphoramidite solutions should be prepared as fresh as possible with the use of anhydrous solvents because added moisture can lower the yield. The phosphoramidite solution should be used within 2–3 days because it degrades in solution rapidly.

² β -Mercaptoethanol is a sulfur-containing compound with an unpleasant smell. This solution should be prepared inside a safety hood. The handler should wear proper protecting eyeglasses, hand gloves, and a lab coat.

³When TCEP is dissolved in 10 mM sodium phosphate buffer (pH 7.0), the solution becomes acidic. The pH should be adjusted to 7.0 by adding a few microliters of a 1 M NaOH solution. TCEP should be prepared fresh to maintain its reactivity.

obtain the final product (Fig. 1b). The assembly of the final product is verified by ESI mass spectroscopy.

3.1 FNA Synthesis

1. Load 20 mg of biotin CPG in the reaction column.
2. Connect all the reagent and solvent bottles to the proper channels in the DNA synthesizer.
3. Program the sequence in Mermade 12 software.
4. Each phosphoramidite conjugation requires five key steps (DMT deprotection-coupling-capping-oxidation-capping). After each step, wash two times with acetonitrile.
5. Deprotect the DMT group from biotin-connected linkers to form free –OH groups with 3% dichloroacetic acid in dichloromethane.
6. Couple the 18 phosphoramidite (S18) spacer using 0.25 M 5-ethylthio-1H-tetrazole as an activator. The coupling time should be extended to 6 min to maximize the yield.
7. Cap the unreacted OH groups with a mixture of cap A and B reagent.
8. Oxidation is performed with 0.02 M iodine in water/pyridine/THF (1:2:7) to obtain a stable phosphate linkage.
9. Conjugate phosphoramidite monomers to assemble FNA in the following order: (1) four units of S18 spacer, (2) DBCO phosphoramidite, (3) six units of S18 spacer, (4) second DBCO phosphoramidite, and (5) four units of S18 spacer (*see* Note 4).
10. Add a thiol modifier at the end of synthesis, and keep the DMT group ON.
11. Cleave the product from CPG by treating with 30% ammonium hydroxide for 16 h at room temperature.
12. Add Trizma base to prevent DMT removal and evaporate the ammonia with the vacuum concentrator.
13. Filter the solution through a 0.22 μ m syringe filter and discard the precipitate.
14. Collect the filtrate part.
15. Purify the product using reversed-phase high-performance liquid chromatography (RP-HPLC) (acetonitrile gradient in 0.1 M triethylammonium bicarbonate, pH 7.5 buffer, gradient 20–45% in 40 min, Phenomenex Gemini C18 column, 5 μ , 250 $\times$ 4.6 mm).

⁴According to <http://www.glenresearch.com/GlenReports/GR27-17.html>, DBCO undergoes oxidation by I₂ oxidizer if the number of oxidation steps is extensive. As an alternative, CSO ((1S)-(+)-(10-camphorsulfonyl)-oxaziridine) in acetonitrile can be used instead of iodine. In our case, we had only ten oxidation steps from the first DBCO and four steps from the second DBCO, and we were able to use a regular iodine oxidizer.

16. The final structure of the linker (Fig. 1a) is as follows: DMT-O-(CH₂)₆-S-S-(CH₂)₆-(S18)₄-(DBCO-dT)-(S18)₆-(DBCO-dT)-(S18)₄-biotin.
17. Measure the concentration of the product using the Nanodrop instrument. The extinction coefficient of DBCO at 308 nm is 12,000 M⁻¹ cm⁻¹.

3.2 Conjugation of the Azide-Modified Peptide Using Click Chemistry (Fig. 1)

1. Dilute the FNA construct to 50 μM with 10 mM sodium phosphate buffer.
2. Prepare a 1 M stock solution of azide-labeled Aβ(14-23) in DMSO, and dilute the solution to 200 μM with 10 mM sodium phosphate buffer.
3. Mix 100 μL of FNA solution (at 50 μM concentration) with 100 μL peptide solution (at 200 μM concentration), and vortex.
4. Incubate this mixture for 24 h at room temperature.
5. Evaporate the solvent in a vacuum.
6. Purify the product (Fig. 1b) with RP-HPLC (acetonitrile gradient in 0.1 M triethylammonium bicarbonate, pH 7.5 buffer, gradient 20–45% in 40 min, Phenomenex Gemini C18 column, 5μ, 250 × 4.6 mm).
7. Confirm the final product with mass spectroscopy.

3.3 Probing of Monomer-Monomer Interactions

3.3.1 Mica Surface Functionalization

1. Cut a piece of mica (~200 μm thickness) into a ~1.5 × 1.5 cm square, and glue it to a glass slide using epoxy glue (*see* Note 5).
2. Cleave the mica with scotch tape (*see* Note 6).
3. Place 150 μL of 167 μM APS solution on the mica, allow it to react for 30 min, and then wash the surface with DI water.
4. Add 150 μL of 167 μM MAL-PEG-SVA (M.wt. 3400 g/mol) in DMSO and incubate for 3 h, and then wash the surface with DMSO and DI water.
5. Prepare 10 nM streptavidin thiol (SAVT) in 10 μM TCEP in sodium phosphate buffer, and incubate for 30 min.
6. Add the protein solution onto the surface, and incubate overnight at 4 °C.
7. Wash the surface with DI water, add 100 μL of a 10 mM solution of β-mercaptoethanol, and keep for 15 min.
8. Wash the surface with DI water and store at 4 °C.

⁵It is important to pay attention while gluing the mica sheet to a glass slide using EPOTEC 301 glue. To work properly, at least 4 g part A of the glue should be mixed with 1 g part B, followed by a thorough mixing. A little amount of this mixture should be applied as a thin layer onto the glass surface where the mica sheet will be placed. Then, the whole system should be cured at 70 °C for 2 h inside a vacuum chamber. A vacuum would help to remove air bubbles between the mica and glass. The surface should be cooled before being used.

⁶To get a uniform mica surface, perform cleaving with scotch tape until a uniform layer of cleaved mica is clearly seen on the tape.

3.3.2 AFM Tip Functionalization

1. Clean the AFM tip by immersing it into ethanol for 15 min, rinsing with water, and drying with gentle argon flow (*see* Note 7).
2. Place the AFM tip in the UV chamber, and expose it with UV light (366 nm wavelength) for 45 min.
3. Immerse the tip in 200 μ L of a 1 μ M APS solution for 30 min, and rinse it with DI water.
4. Incubate the tip in 200 μ L of 1 μ M *N*-(γ -maleimidobutyryloxysuccinimide ester) (GMBS) solution for 1 h, and rinse it with water.
5. Prepare a 10 nM solution of FNA A β (14-23) dimer (Fig. 2a) in 10 mM sodium phosphate buffer (pH 7.0). Activate the thiol group by treating the FNA construct with 10 μ M Tris (2-carboxyethyl) phosphine (TCEP), and incubate it for 30 min.
6. Place a droplet of FNA solution (50 μ L) onto a piece of parafilm, and incubate the tip in that solution overnight at 4 $^{\circ}$ C.
7. Wash the tip with water and quench the unreacted maleimide groups by treating the tips with a 10 mM solution of β -mercaptoethanol for 10 minutes (*see* Note 2).
8. Wash the tip in water and store at 4 $^{\circ}$ C.

3.3.3 Force Measurements—Force-distance (F-D) measurements were performed on the Asylum MFP 3D AFM instrument. A β (14-23) oligomers were probed in 10 mM sodium phosphate buffer (7.0) at room temperature.

1. Mount the functionalized AFM tip in the tip holder, and adjust the laser and photodiode accordingly.
2. Place the functionalized mica surface on the AFM stage; add 200 μ L of probing buffer. Manually move the tip toward the surface so that the tip remains immersed in the water layer. Adjust the laser deflection.
3. Approach the AFM tip to the surface.
4. Calculate the actual spring constant of the cantilever by the thermal noise analysis method as recommended by the manufacturer. The calculated spring constant for MSNL-10 D probes is in the range of 20–30 pN/nm.
5. During measurement, set the trigger force at 100 pN for 0.5 s, and perform the approach-retract cycle at a speed of 500 nm/s.
6. Collect several thousand force curves to obtain sufficient statistics.

⁷The AFM tip should be handled very carefully to avoid breaking the cantilevers. During tip washing, the tip should be immersed into ethanol for 10 min, and then transferred to pure water for another 10 min. Before putting the tip into the UV chamber, the tip should be dried with a gentle flow of argon gas.

3.4 Probing of Monomer-Dimer Interactions

The FNA approach for the characterization of A β (14-23) trimers [11] (Fig. 2b).

1. Modify the mica surface using the same protocol as described in Subheading 3.3.1, except at **step 5**; inject 40 nM A β (14-23) pretreated with TCEP.
2. Functionalize the AFM tip using the same method as discussed in Subheading 3.3.2, except using 167 μ M of Mal-PEG-SVA (M.wt 3400 g/mol) instead of GMBS.
3. Perform force measurements with the functionalized mica surface and AFM tip as described in Subheading 3.3.3.

3.5 Probing of Dimer-Dimer Interactions (Fig. 2c)

1. Modify the mica surface using the same protocol as described in Subheading 3.3.1, except at **step 5**; inject 40 nM FNA A β (14-23) dimer (pretreated with TCEP).
2. Functionalize the AFM tip using the same method as discussed in Subheading 3.3.2, except using 167 μ M of Mal-PEG-SVA (M.wt. 3400 g/mol) instead of GMBS.
3. Perform force measurements with the functionalized mica surface and AFM tip as described in Subheading 3.3.3.

We illustrate below how the FNA-based methodology was used to characterize the interactions within self-assembled amyloid dimers. The FNA construct with two A β (14-23) peptides terminated with biotin is immobilized onto the AFM tip (Fig. 3). Streptavidin is tethered to the mica surface via a flexible PEG tether. As the tip approaches the surface to make biotin-streptavidin contact, the peptides spontaneously assemble into a dimer, as shown in Fig. 3a(i). Upon retracting the tip from the surface, the PEG tether stretches, as shown in Fig. 3a(ii). Further retraction of the tip can lead to dimer rupture and stretching of the FNA tether (Fig. 3a(iii)). Finally, at a highly applied force, the biotin-streptavidin link ruptures, as shown in Fig. 3a(iv). All of these events are identified in the force-distance curve shown in Fig. 3b. The arrows between frames (a) and (b) illustrate the appearance of each event on the force-distance curve, and the rupture forces for each event are measured ($F_1 = 50$ pN and $F_2 = 62$ pN). Each event is validated by measuring the contour lengths L_{c1} and L_{c2} , based on the known lengths of the tethers. The details for this type of analysis can be found in reference [13]. Specifically, the distance between the two peaks in Fig. 3b is ~ 12 nm, which is consistent with the distance between the DBCO units [13]. Additional analysis of the rupture process, as performed in [12] for A β (14-23) dimer, can reveal interesting properties of dimer assembly. The FNA approach was critical in probing interactions between A β (14-23) dimer and monomer and dimer-dimer interactions [11]. This suggests that FNA can be used to probe higher order oligomers with three or more conjugated monomer units. Although the examples above described probing interactions between peptides, any other interacting partners can be probed after conjugating to an appropriately designed FNA template.

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Fig. 1.

Schematic presentation of the FNA structure. **(a)** The FNA polymer is synthesized in a DNA synthesizer, in which two DBCO groups are incorporated into the polymer chain at specified positions. **(b)** The FNA construct with two $A\beta(14-23)$ peptides conjugated to DBCO groups is further synthesized using a metal-free “click reaction”

Fig. 2. Schematic for AFM force spectroscopy studies of FNA-Aβ(14-23) dimer. (a) The spontaneous assembly of Aβ(14-23) dimers within the FNA construct. (b) The interaction between the FNA-Aβ(14-23) dimer and an Aβ(14-23) monomer leading to a trimer. (c) The interaction of two FNA-Aβ(14-23) dimers leading to a tetramer

Fig. 3. AFM force spectroscopy experiment for probing dimers assembled within the FNA-Aβ(14-23) dimer construct. **(a)** Cartoon for selected stages of the AFM probing experiment: (i) Approaching the FNA-Aβ(14-23) dimer to the surface yields the complex between terminal biotin (red triangle) and the surface-immobilized streptavidin (green four-leaf construct illustrating the tetramer structure of the protein), (ii) retracting the tip and stretching the PEG polymer tethered to streptavidin, (iii) rupture of the Aβ(14-23) dimer, and (iv) rupture of the biotin-streptavidin link. **(b)** A typical force vs. distance (F - D) retraction curve for the event in **(a)**. F_1 and F_2 are rupture forces for the dissociation of the Aβ(14-23) dimer and biotin-streptavidin links, respectively. Lc_1 and Lc_2 are the contour lengths corresponding to these two events. The red lines on the graph show the approximation of the extension parts of the force curves according to the wormlike chain model [15]. The arrows point to the events in the force curves that correspond to the cartoons in frame **(a)**