Chapter 20

Constraining TAT Peptide by γPNA Hairpin for Enhanced Cellular Delivery of Biomolecules

Siddhartha Thennakoon, Rick Postema, and Xiaohong Tan

Abstract

Based on the exceptionally high stability of γPNA (Gamma-modified peptide nucleic acid) duplexes, we designed a peptide/γPNA chimera in which a cell-penetrating TAT (HIV Tat-derived) peptide is flanked by two short complementary γPNA segments. Intramolecular hybridization of the γPNA segments results in a stable hairpin conformation in which the TAT peptide is constrained to form the loop. The TAT/γPNA hairpin (self-cyclized TAT peptide) enters cells at least tenfold more efficiently than its nonhairpin analog in which the two γPNA segments are noncomplementary. Extending one of the γPNA segments in the hairpin results in an overhang that can be used for binding and delivering a variety of nucleic acid-conjugated molecules into cells via hybridization to the overhang. We demonstrated efficient cellular delivery of an anti-telomerase γPNA that specifically reduced telomerase activity of A549 cells by over 97%.

Key words γPNA, TAT peptide, Self-cyclization, Hairpin, Cellular delivery

1 Introduction

The efficient delivery of bioactive molecules into cells is a major challenge in biomedical research [1]. Cell-penetrating peptides (CPPs), such as TAT [2, 3], offer one of the most frequently used means for efficient, intracellular delivery of various cargos including nucleic acids [4–6] or proteins [7–10]. Interestingly, the transduction efficiency of the TAT peptide can be further enhanced by covalent cyclization [11, 12], but current covalent cyclization methods require extra purification steps, and generating the final cyclized products, especially those rich in basic and polar residues, such as TAT, can exhibit low yields (<10%) [13–15]. Therefore it is highly desirable to develop a simple, high-efficiency method for peptide cyclization. Inspired by the design of molecular beacons [16] and our previous work using DNA hairpins to mask fluorescent reporter functionality or aptamer activity [17–19], we reasoned that Watson–Crick base pairing could provide a simple and

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generalizable mechanism for noncovalently constraining peptides into quasi-cyclic conformations. Thus, embedding TAT within the loop of a hairpin-forming nucleic acid oligomer should effectively cyclize the TAT while an overhanging single-stranded region can be used to pick up and deliver molecular cargo functionalized with a complementary nucleic acid. We demonstrated efficient cellular delivery of an anti-telomerase γPNA that specifically reduced telomerase activity of A549 cells by over 97% [20]. To the best of our knowledge, this study is the first to report how to manipulate peptide cyclization as well as assembly through Watson–Crick base pairing for enhancing biomolecular delivery into cells [21].

2 Materials

Ultrapure water is prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at room temperature (RT = 25 °C). Prepare and store all reagents at RT, unless indicated otherwise. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Peptide-γPNA Hybrid Synthesis and Characterization

1. 20% Piperidine; deprotection solution: Measure 20 mL of piperidine by a 100 mL graduated cylinder, and add dry DMF (N,N-Dimethylformamide) to a volume of 100 mL.
2. 5% Acetic anhydride; capping solution: Measure 5 mL of acetic anhydride by a 10 mL graduated cylinder, and transfer it to a 100 mL graduated cylinder and add dry DMF to a volume of 100 mL.
3. 92.5% TFA; cleavage solution: Mix together 925 μL of TFA (trifluoroacetic acid), 25 μL ultrapure water, 25 μL of TIPS (triisopropylsilane), and 25 μL of m-cresol (3-methylphenol). Use it freshly (*see* **Note 1**).
4. UV-Vis spectrophotometer with a temperature controller.
5. Rink Amide MBHA resin.
6. Fmoc Protected Amino Acid Monomers & Derivatives.
7. Fmoc Protected γPNA Monomers.

2.2 RP-HPLC (Reversed Phase High-Performance Liquid Chromatography)

1. 5% CAN (Acetonitrile), 0.05% TFA; HPLC solution A: Mix together 50 mL of ACN, 0.5 mL of TFA, and 949.5 mL ultrapure water.
2. 95% ACN, 0.05% TFA; HPLC solution B: Mix together 950 mL of ACN, 0.5 mL of TFA, and 49.5 mL ultrapure water (*see* **Note 2**).

2.3 Cell Culture and Incubation

1. A549 cells.
2. Trypsin-EDTA.

3. DMEM (Dulbecco's modified Eagle medium) culture medium: Add 50 mL of FBS, 50 mg streptomycin, and 50,000 units of penicillin into 500 mL of DMEM. Store at 4 °C.
4. CO_2 incubator.
5. Cell culture dish (60 × 15 mm).
6. 96-well cell culture plate.
7. Inverted microscope.
8. Fluorescence microscope.
9. Thermocycler.
10. TRAPeze Telomerase Detection Kit.

2.4 Polyacrylamide Gel

1. 1 × TBE buffer: Prepared from TBE Buffer (Tris-borate-EDTA) (10×) (*see* **Note 3**).
2. Thirty percent acrylamide/Bis solution (29.2:0.8). Store at 4 °C.
3. Ammonium persulfate: 10% solution in water. Store at −20 °C.
4. TEMED (*N,N,N′,N′*-tetramethyl ethylenediamine). Store at 4 °C.
5. EB (ethidium bromide) solution: Dissolve 0.05 mg EB in 100 mL water (*see* **Note 4**).

3 Methods

Carry out all procedures at RT unless otherwise specified.

3.1 Peptide-γPNA Synthesis

1. Weigh 50–500 mg of RAM resin (Rink Amide MBHA resin) and transfer it into a glass reaction vessel. Swell the resin in dry DMF for 1 h (*see* **Note 5**).
2. Incubate the resin with 1 mL of deprotection solution by shaking for 2 min. Drain the resin and add another 1 mL of deprotection solution for 20 min with shaking. Drain the resin and wash it by DMF (6×) (*see* **Note 6**).
3. According to Fmoc-monomer: DIEA: COMU: resin coupling capacity = 4: 8: 4: 1, weigh each Fmoc-monomer and COMU ((1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate) and dissolve them together by 1 mL of dry DMF, then add DIEA (N, N-diisopropylethylamine) and allow a 2-min pre-activation step.
4. Add coupling reagents to the deprotected resin and incubate for 60 min with shaking. Wash it by DMF (3×), DCM (dichloromethane) (2×), DMF (3×) (*see* **Note 7**).

5. After each coupling, the resin is incubated with the 1 mL of capping solution for 5 min. Then the resin is washed by DMF (3×), DCM (2×), DMF (3×).
6. Repeat above deprotection, coupling, and capping steps to finish extension of each indicated polymer sequence.
7. Incubate the resin with the deprotection solution for 20 min (2×). The resin is washed by DMF (8×) and MEOH (methanol) (6×). Dry the resin under vacuum for at least 2 h (*see* **Note 8**).
8. Incubate the resin with 1 mL of cleavage solution for 3 h with shaking. Collect the solution into a 50 mL conical tube (*see* **Note 9**), and treat the resin again with another 1 mL of cleavage solution. Combine the solution with the previous one.
9. Mix the total 2 mL of cleavage solution with 20 mL of pre-cold ether (− 20 °C) and put at −20 °C for at least 1 h (*see* **Note 8**). The tube is centrifuged for 3000 × *g* × 10 min at 4 °C. Wash the pellet by pre-cold ether (2×) and dry it in air (*see* **Note 10**).

3.2 Peptide-γPNA Purification and Characterization

1. Dissolve the crude peptide-γPNA by 20% ACN and the sample is passed through a 0.2-μm syringe filter (*see* **Note 11**).
2. Load the sample into a RP-HPLC system, using a C-18 semi-preparative column.
3. Prepare saturated matrix solution of CHCA (α-cyano-4-hydroxycinnamic acid) in a solvent mixture containing 50% ACN and 0.1% TFA.
4. Deposit 0.5–1 μL of the HPLC sample solution onto each MALDI target plate position and allow to dry. Then deposit 0.5 μL of the matrix solution onto each sample spot and allow to dry.
5. The HPLC peaks are collected and characterized by using MALDI-TOF-MS (matrix assisted laser desorption ionization-time of flight mass spectrometry).
6. Dry peptide-γPNA products by lyophilization.

3.3 Cyclized peptide Formation and Characterization

1. γPNAs with self-complementary termini are capable of folding into intramolecular hairpins, consistent with the design shown in Fig. 1. Hairpin formation through base pairing between the two complementary segments confines the TAT peptide within the loop, creating a quasi-cyclic conformation.
2. Dissolve each purified peptide-γPNA in PBS (phosphate buffered saline) to reach a final concentration of 5 μM. The samples are heated in a heating block at 95 °C for 5 min and immediately put on ice for 5 min, to form cyclized peptides (*see* **Note 12**).

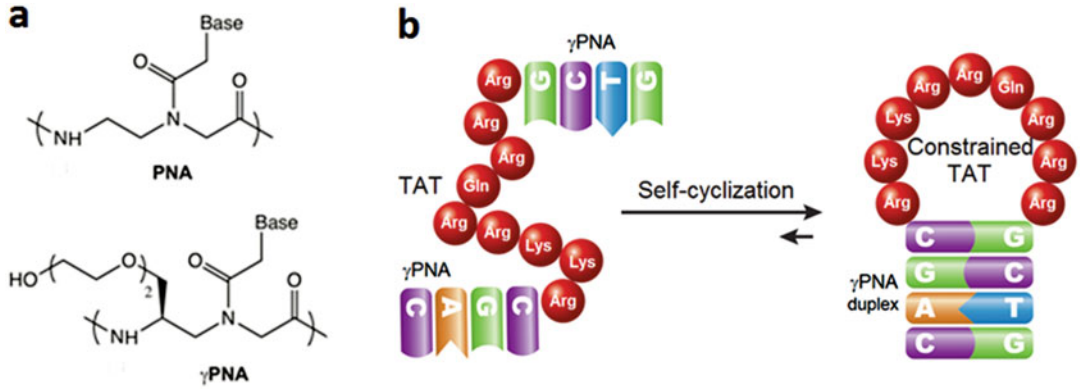

Fig. 1 (**a**) Chemical structures of PNA and γPNA. (**b**) Assembly of a γPNA-TAT peptide chimera into a hairpin conformation stabilized by Watson–Crick base pairing. (Reprinted (adapted) with permission from ref. 21. Copyright (2018) American Chemical Society)

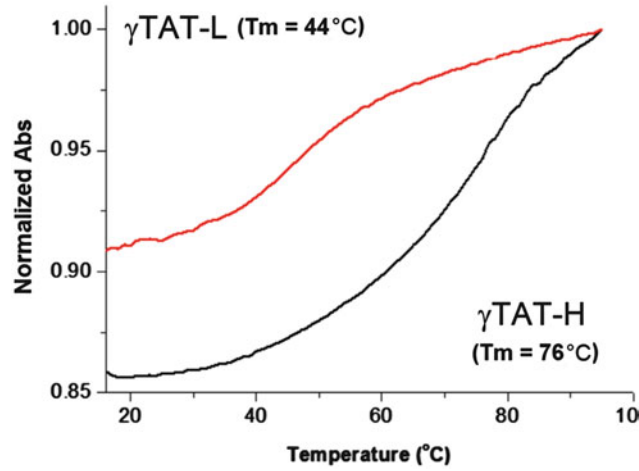

Fig. 2 Characterization of difference between melting curves of γTAT-H (CAGC-ArgLysLysArgArgGlnArgArgArg-GCTG) and γTAT-L (AAAA-ArgLysLysArgArgGlnArgArgArg-GCTG) (the absorbance intensity at 260 nm collected at 2 μM of probe). (Reprinted (adapted) with permission from ref. 21. Copyright (2018) American Chemical Society)

3. Add 400 μL of 5 μM of peptide-γPNA into 600 μL of PBS to prepare 1 mL of peptide-γPNA (2 μM). Load samples in a UV-Vis spectrophotometer with a temperature controller to measure UV-melting curves. UV-Vis absorbance at 260 nm is recorded at the rate of 1 °C /min for both the heating and cooling runs. Determine the Tm (melting temperature) values by taking the first derivative of the absorbance vs temperature profile, as shown in Fig. 2.

3.4 Cell Culture

1. Culture A549 in the 60 mm × 15 mm dish with DMEM containing 10% FBS (fetal bovine serum), penicillin, and streptomycin in a humidified incubator at 37 °C, 5% CO_2.
2. When cells reach 70% ~ 80% confluence, aspirate the growth medium from the cells and wash the cells with PBS. Aspirate PBS, then add 500 μL of trypsin-EDTA (ethylenediaminetetraacetic acid) solution and incubate cells for several minutes at 37 °C, checking the culture with inverted microscope to make sure that the cells are rounded up and detached from the surface.
3. Add 2 mL of fresh culture medium into the dish, then pipet all adherent cells into cell suspension and pipet up and down. Transfer the cell suspension into a 15 mL centrifuge tube and centrifuge at 100 *g* for 5 min. Aspirate the supernatant and add 5 mL of fresh medium to resuspend the cells. Mix 500 μL of cell suspension with 4.5 mL of fresh medium and load it into a new 60 mm × 15 mm dish with appropriate label. Culture in a humidified incubator at 37 °C, 5% CO_2.

3.5 Cellular delivery and Cell Imaging

1. TARMA (5-carboxytetramethylrhodamine)-labeled antisense γPNA (TAGGGTTAGACAA) and FAM (6-carboxyfluorescein)-labeled Cyc-TAT (CAGC-ArgLysLysArgArgGlnArgArgArg- GCTGTTGTC) are dissolved in PBS-Mg (containing 1 mM $MgCl_2$) to reach 3 μM concentration for each of them.
2. Heat above solution at 95 °C for 5 min and allow it to slowly cool down to RT around 1 h. The 3 μM of pre-annealed Cyc-TAT/antisense γPNA complex (*see* **Note 13**), as shown in Fig. 3a, is incubated with cells in a humidified incubator at 37 °C, 5% CO_2 for 2 h or more.
3. Wash cells by 0.2 mL of PBS-Mg x 3 and imaged under a fluorescence microscope (*see* **Note 14**), using filter combinations designed for FAM and TAMRA, as shown in Fig. 3b.

3.6 TRAP (Telomere Repeat Amplification Protocol) Assay

1. Detect telomerase activity from cells by using the TRAPeze telomerase detection kit according to the manufacturer's instructions.
2. Incubate A549 cells in a 96-well plate. After treatment, homogenize cells in provided CHAPS lysis buffer (50 μL) on ice. Incubate the homogenate on ice for 30 min, centrifuge it for 20 min at 12000 × *g* and 4 °C.
3. Transfer the extract (20 μL) to a PCR tube and mix it with 5 μL of 5× TRAPEZE® RT Reaction Mix. Place each reaction mixture in the thermocycler and heated to 30 °C for 30 min. Then 30 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min are performed.

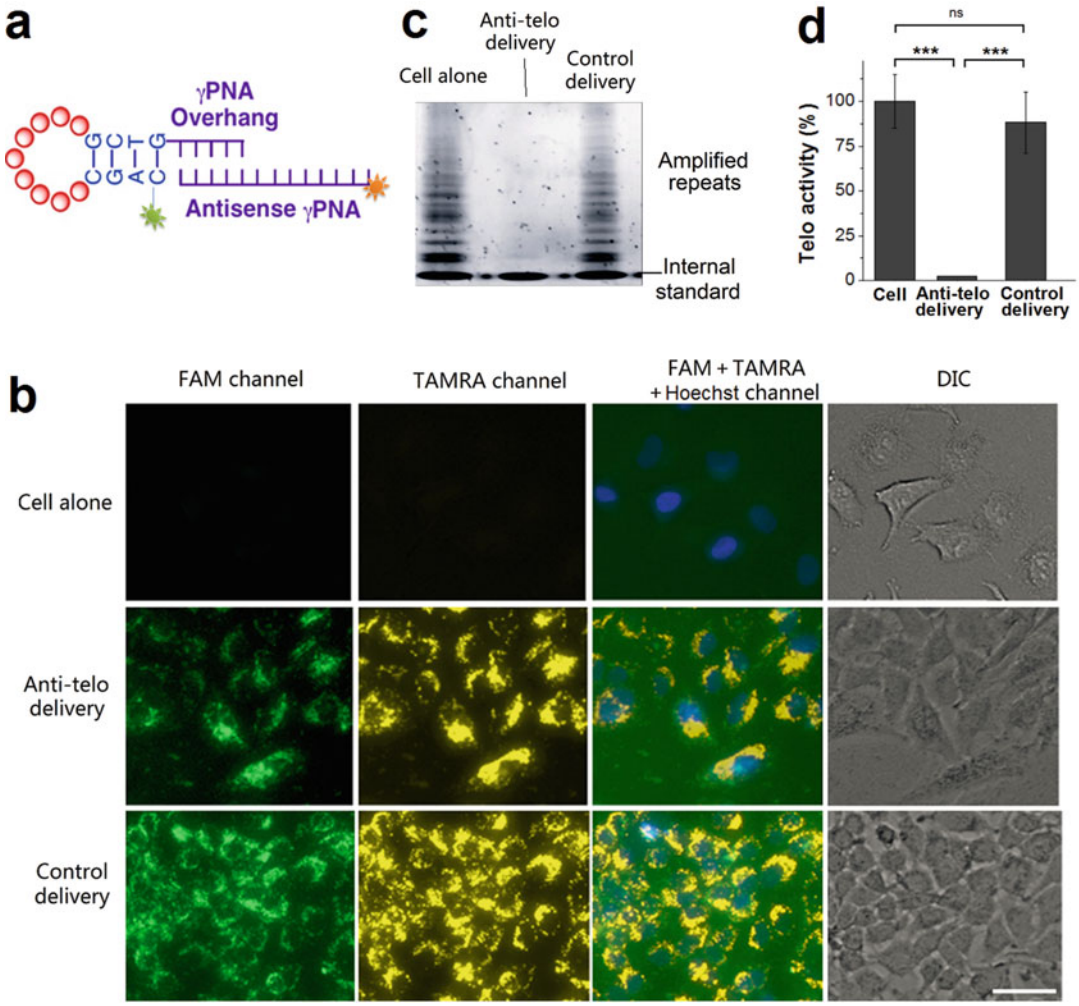

Fig. 3 Inhibition of telomerase of A549 cells. (**a**) Cartoon of the overhang Cyc-TAT-mediated delivery of template-directed anti-telo γPNA. (**b**) Fluorescence microscopy of cells with various treatment (for each probe the concentration is 3 μM). Scale bar: 50 mm. (**c**) Telomere repeat amplification protocol (TRAP) gels showing the inhibition of telomerase as a function of the Cyc-TAT/anti-telo γPNA complex but not for Cyc-TAT/control γPNA complex. (**d**) TRAP gel bands are quantified by using ImageJ software. The *p* values calculated from student *T*-tests are ns > 0.05 and $^{***}p \leq 0.001$ ($n = 3$). (Reprinted (adapted) with permission from ref. 20. Copyright (2019) Wiley-VCH)

4. For TRAP gel, mix 2.5 mL of 1× TBE buffer, 3.33 mL of acrylamide mixture, and 4 mL water in a 50 mL conical flask. Add 80 μL of ammonium persulfate and 10 μL of TEMED and cast gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette (*see* **Note 15**). Insert a 10-well gel comb immediately without introducing air bubbles.
5. Mix PCR samples with 5× sample loading solution and load them onto the 10% native polyacrylamide gel and stained with EB solution before scanning (Fig. 3c). Quantify TRAP gel bands by using ImageJ software (Fig. 3d).

4 Notes

1. We always prepare fresh cleavage solution.
2. Having ~5% water in solution B can increase solution hydrophilicity, which can facilitate the HPLC purification of peptides.
3. 10× TBS is commercially available.
4. EB is toxic. Protective equipment including a lab coat, chemically resistant gloves, and safety goggles should be worn.
5. There is no need to shake the vessel for swelling resin.
6. We observe that resin, after deprotection, becomes very sticky to attach the glass if DCM is used as wash solution. Consequently we only use DMF wash after deprotection step.
7. After coupling, resin is not sticky to attach the glass, therefore DCM is also used for wash.
8. We prefer overnight treatment.
9. We directly use the filter in the glass reaction vessel to collect the cleavage solution under some air pressure.
10. Generally it takes around 20 min to completely dry the sample.
11. Sometimes the peptide-γPNA is hard to be completely dissolved, and we generally increase the ACN concentration to 30%. It is very important to remove any insoluble samples before HPLC loading.
12. This is called self-cyclization. For the peptide-γPNA with self-complementary termini, the hairpin formation can cyclize the peptide in the middle.
13. Generally this annealing step is good enough to form the cyclized TAT as well as the duplex PNA together. You can also keep the sample at RT for overnight to get maximum yields.
14. A549 cells attach the well very tightly. If you use other easy-to-detach cell lines such as HEK293, wash them gently to avoid losing cells.
15. Don't vortex the gel solution. Avoid air bubbles and cast the gel immediately after gentle mix.

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