Method Article

Formulation of PLGA nanoparticles containing short cationic peptide nucleic acids

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A B S T R A C T

Peptide nucleic acids (PNAs) have emerged as one of the most versatile tools with a wide range of biomedical applications including antisense, antimiR, antigene, as well as site-specific gene editing. The application and potential of PNAs has been limited due to low solubility and poor cellular uptake. Several strategies have been employed to overcome the aforementioned challenges like conjugation to cationic peptides or nanotechnology to achieve superior transfection efficiency ex vivo and in vivo. Here, we report a detailed procedure optimized in our lab for synthesis of short cationic PNA probes, which exhibit high purity and yield in comparison to full-length PNA oligomers. We also provide step-by-step details of encapsulating short cationic PNA probes in poly(lactic-co-glycolic acid) nanoparticles by double emulsion solvent evaporation technique.

1. Detailed procedure for synthesis of short cationic PNAs with or without fluorophore (dye) conjugation while ensuring high yield and purity.
2. Step-by-step details for encapsulation of short cationic PNAs in PLGA nanoparticles via double emulsion solvent evaporation technique.

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**Peptide Nucleic Acids (PNAs)**

PNAs were first discovered in 1991 by Nielsen, Egholm, Berg and Buchardt at the University of Copenhagen [1]. PNAs are synthetic nucleic acid analogues where the phosphodiester backbone of DNA is replaced by N-(2-aminoethyl)-glycine repeating units imparting several advantageous properties [2]. The pseudopeptide backbone is charge neutral which imparts resistance to enzymatic degradation [3] and results in superior binding affinity to DNA/RNA due to reduced electrostatic repulsion [4]. PNAs bind to the target DNA/RNA via complementary Watson and Crick or Hoogsteen base pairing rules resulting in high specificity towards their target [5].

Since their discovery, PNAs have been established as a versatile tool with a wide range of therapeutic and diagnostic applications. Multiple studies have successfully utilized PNAs for targeting mRNAs [6], non-coding RNAs (microRNAs) [7,8,9,10] and genomic double stranded (ds)DNA [11,12,13] for regulating gene expression [14,15]. In addition to anti-sense and gene editing applications, PNAs have also been used for DNA barcoding [16], biosensors [17] and as an anti-infective agent [18]. However, the actual potential of PNAs has not been fully realized yet due to poor intracellular delivery and low aqueous solubility [15]. Among several approaches to overcome these challenges, one of the most unique approaches is to conjugate cationic residues onto the PNAs to improve its aqueous solubility and intracellular delivery [19]. Additionally, nanotechnology has paved the way to further increase the delivery of PNAs to the target site in the cytoplasm [20]. miRs are small non-coding RNAs (~22–25 nucleotide length) which negatively regulates the expression of several mRNAs [21]. Dysregulation of miRs has been reported to be associated with pathogenesis of cancer, cardiovascular, muscle as well as neurodegenerative disorders [22,23]. These miRs bind to the target mRNAs via a short 8 mer sequence called seed region which is critical for activating the RNA induced silencing complex (RISC) assembly. Prior studies have reported use of full-length PNAs for inhibiting the upregulated miRs, however, we designed short cationic 8 mer PNAs for targeting only the seed region to achieve comparable efficacy [24]. Full-length PNAs are highly hydrophobic in nature and tend to aggregate during the solid-phase synthesis. The folding of long PNA oligomers on the neighboring chains results in higher fraction of impurities and poor yield. In addition, synthesis and purification of full-length PNAs containing purine rich regions is significantly challenging. Further, these limitations pose a major hurdle for clinical translation of full-length PNAs due to the higher cost and time associated with their synthesis. However, the short (8mer) cationic PNAs are non-aggregating and hydrophilic in nature resulting in ease of synthesis with superior yield and minimum impurities. Hence, the scale up process of these short PNAs will be significantly cost-effective for clinical applications.

Here we report a detailed protocol for synthesis of short (8mer) cationic PNAs targeting the seed region of miRs (miR-155) which has been optimized in our lab, and is based on previously reported solid-phase synthesis of PNAs [25]. We provide step-by-step procedure developed in our lab for encapsulation of these short cationic PNAs in poly (lactic-co-glycolic acid) (PLGA), a USFDA approved biodegradable and biocompatible polymer, based nanoparticles (NPs).
1. Solid Phase Synthesis

Peptide shaker with vessel (on right) for synthesis of PNA

2. Purification

RP-HPLC (Shimadzu) with fraction collector

3. Quality Control

Mass Spectroscopy (Matrix Assisted Laser Desorption/ Ionization Time of Flight, MALDI-TOF)

Fig. 1. Major steps for synthesizing peptide nucleic acids (PNAs). PNAs are synthesized on a solid support following solid-phase synthesis (Step 1) protocols. After the completion of PNA synthesis, it is cleaved from the solid support and purified via reverse phase high performance liquid chromatography (RP-HPLC) (Step 2) and the mass of the purified PNA (Step 3) is determined via Matrix Assisted Laser Desorption/ Ionization Time of Flight (MALDI-TOF) spectroscopy.

Synthesis of cationic PNAs

The steps involved in obtaining a pure fraction of desired PNA include solid-phase synthesis of the PNA oligomer, purification, and quality control (Fig. 1). The list of chemicals and equipment used for PNA synthesis are provided below (Table 1). We recommend using lab-coat, gloves and eye goggles as personal protective equipments while performing the synthesis of PNA. The solid-phase synthesis and handling of all chemicals should be performed under the chemical hood.

Steps in solid-phase synthesis of PNA:

Below are steps involved in synthesis of cationic PNAs:

a. Amino acid loading of 4-Methylbenzyldihydrylamine (MBHA) resin
b. Synthesis of PNA
c. Amino acid conjugation
d. Cleavage

The details of each step involved in PNA synthesis (Fig. 2) is provided below.

Amino acid loading on MBHA resin

The synthesis of PNA starts with loading amino acids on the resin which acts as a solid support for oligomer synthesis. It is recommended to incorporate one or more amino acid residues on PNAs to prevent self-aggregation and improve the handling and solubility of PNAs [19]. Resin can be loaded either at the scale of 100 mg or 1 g with the amino acid based on the need. In general, it is advisable to prepare 1 g of resin loaded with the amino acid because the same amino acid loaded resin can be used for synthesizing multiple PNA sequences in the future. Here we have explained the procedure for preparing 1 g of amino acid loaded MBHA resin.

All the reagents including MBHA resin, Boc-amino acids (Boc-Arg(Tos)-OH, Boc-Lys(Cl-Z)-OH) and PNA monomers are vacuum dried overnight to remove any residual moisture (Important note: All the reagents are paraflimed and stored in refrigerator (4–8°C) for long term storage). Meanwhile peptide solid-phase synthesis vessel is thoroughly washed with water and methanol followed by drying in the oven.

1. Weigh the vacuum dried MBHA resin (1 g) and transfer to the dried peptide synthesis vessel. Add dichloromethane (DCM) to the resin and ensure that resin bed is completely soaked in the DCM. Allow the resin to be soaked for at least 2 h before starting the amino acid coupling step.

2. Preparation of coupling solutions for 1 g MBHA resin:

0.2M Boc-Arg(Tos)-OH or Boc-Lys(Cl-Z)-OH: Weigh 45 mg of Boc-Arg(Tos)-OH or Boc-Lys(Cl-Z)-OH and dissolve in 500 μl of N-methyl-2-pyrrolidine (NMP).

0.5M DIEA: Add 87 μl of DIEA to 913 μl of pyridine.
0.2M HATU: Weight 46 mg of HATU and dissolve in 600 μl of NMP.

3. After the resin has been soaked in DCM for more than 2 h, wash the resin with DCM (1–2 mL) 4 times. The vessel used for PNA synthesis has a porous bed and a knob, hence we can apply air pressure from top of the vessel while keeping the knob open to remove the DCM without any loss of resin.

4. Transfer 450 μl of 0.2 M Boc-Arg(Tos)-OH or Boc-Lys(Cl-Z)-OH and 460 μl of 0.5 M DIEA to another tube containing 1590 μl NMP (Solution A).

5. Dilute 0.2 M HATU by adding 2 mL of NMP (Solution B).

6. Combine solution A and solution B in 15 mL falcon tube and vortex vigorously to allow the activation of Boc-Arg(Tos)-OH or Boc-Lys(Cl-Z)-OH. Let it stand for 3 min to obtain the final coupling solution.

7. Remove DCM from the resin and add the coupling solution to the resin bed. Ensure that resin is completely dispersed in the coupling solution using a spatula and allow the vessel to rock at intermediate speed (for Kamush® peptide shaker, a speed of 4-5 with an elevation of 30°–45° is suitable) overnight.

8. Wash the resin with DMF at least 4 times followed by washing with DCM (6X) by completely dispersing the resin in DCM every time.

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Table 1
List of chemicals and equipments used in PNA synthesis

| Chemical                                                      | Source                        | Catalog No. |
|---------------------------------------------------------------|-------------------------------|-------------|
| 4-Methylbenzhydrylamine (MBHA) Resin (100–200 mesh) (0.59 meq/g) | Peptides International       | 2402141     |
| HATU                                                          | Peptides International        | 005424D     |
| Boc-Arg(Tos)-OH                                               | Sigma Aldrich                 | 15506       |
| (N-α-t-Butyloxy carbonyl-Nα-Tosyl-L-Arginine)                 | Peptides International        | BLK-2135    |
| Boc-Lys(Cl-Z)-OH                                              | Sigma Aldrich                 |             |
| (N-α-t-Butyloxy carbonyl-Nγ-2-Chlorobenzoyloxycarbonyl-L-Lysine) | ASM Chemicals and Research   |             |
| Boc-PNA-Monomers (regular)                                    | Peptides International        |             |
| 1. BOC-PNA-T-OH                                               | Sigma Aldrich                 |             |
| 2. BOC-PNA-G(Cbz)-OH                                          | Sigma Aldrich                 |             |
| 3. BOC-PNA-A(Cbz)-OH                                          | Sigma Aldrich                 |             |
| 4. BOC-PNA-C(Cbz)-OH                                          | Sigma Aldrich                 |             |
| Boc-MiniPEG-3                                                 | Peptides International        | BXX-5523-PI |
| 5-Carboxytetramethylrhodamine (TAMRA)                         | VWR International             | 91809-66-4  |
| Dimethylformamide (DMF)                                       | Sigma Aldrich                 | 319337      |
| Dichloromethane (DCM)                                         | Sigma Aldrich                 | D65100      |
| N-methyl-2-pyrolidine (NMP)                                   | Sigma Aldrich                 | 328634      |
| Trifluoroacetic acid (TFA)                                    | Alfa Aesar                    | L06374      |
| Acetic anhydride                                              | Sigma Aldrich                 | 242845      |
| Pyridine                                                     | Sigma Aldrich                 | 270970      |
| Potassium cyanide (KCN)                                       | Alfa Aesar                    | 12136       |
| Ninhydrin                                                    | Alfa Aesar                    | 43846       |
| N, N-Diisopropylethylamine (DIEA)                             | TCI                           | D1599       |

Equipment

| Source                        | Catalog No. |
|-------------------------------|-------------|
| Peptide Shaker                | LP360AMPk(s) |
| Synthesis Vessels             | CG-1860-02  |
| RP-HPLC                       | Autosampler: 56398, Chromatograph: 59492, Degassing Unit: 68623, Communication Module: 57371, Diode Array Detector: 51751, Fraction Collector: 07534 |
Fig. 2. (A) Chemical structure of PNA monomers including Adenine (A), Guanine (G), Cytosine (C) and Thymine (T) used in PNA synthesis. (B) Work flow representing the steps in PNA synthesis. Here, MBHA resin acts as the solid support for PNA synthesis, and first conjugated with an amino acid, Arginine (R) or lysine (K) (Step 1). After conjugation of amino acid to the resin, PNA monomers are conjugated based on the desired sequence (Step 2). This step is divided into three parts including deprotection, coupling, and capping reactions where Kaiser test is performed twice for each monomer addition to confirm the completion of reactions. During deprotection, protecting group (Boc) is cleaved resulting in exposure of free amines and is indicated by a blue kaiser test. Next, coupling solution containing the PNA monomer is added and reaction is allowed to continue for 2 h. The completion of coupling reaction is confirmed by a yellow kaiser test, and if the kaiser is blue, coupling reaction is performed again until the kaiser shows yellow. After the completion of coupling, capping is performed for 2 min and synthesis proceeds to deprotection reaction to add the next PNA monomer. These steps of deprotection, coupling and capping are repeated until the entire length of PNA is synthesized. Next, the PNA sequence synthesized is cleaved from the resin (Step 3) and purified by reverse phase HPLC (RP-HPLC) for further use.

9. freshly prepare capping solution by mixing 4 mL NMP, 4 mL pyridine and 2 mL of acetic anhydride in a falcon tube.

10. Remove the DCM from resin and add 5 mL of capping solution to disperse resin bed in the vessel. Allow the vessel to rock for at least 2 h.

11. After 2 h, wash the resin with DCM (6X). Add the rest of the capping solution (5 mL) to the resin bed again and allow to rock for another 2 h.

12. Wash the resin with DCM (8X) to completely remove the capping solution.

13. Preparation of Kaiser test reagents:

Potassium cyanide (KCN) working stock solution: Weigh 3.25 mg KCN and dissolve in 50 mL of deionized (DI) water to prepare the main stock. To prepare the working stock, dilute 2 mL of KCN main stock with 98 mL of deionized (DI) water.

Phenol: Ethanol (4:1) stock: Prepare a working stock by mixing 4 mL of phenol with 1 mL of ethanol.

Kaiser A: To prepare Kaiser A, take 980 μl pyridine and add 100 μl of phenol:ethanol (4:1) stock and 20 μl of KCN working stock solution.
Kaiser B: Weigh 5 g of ninhydrin and dissolve in 100 mL of ethanol. (Important note: To protect from light, cover the Kaiser B solution containing tube with the aluminum foil)

14. Kaiser test: After washing the resin with DCM, take out sufficient amount of resin from the bottom of vessel using a spatula and transfer to a 1.5 mL tube. Add enough DCM to the glass vessel to immerse the resin during the Kaiser test.

Add approximately 50 µl of Kaiser A and 50 µl of Kaiser B to 1.5 mL tube to immerse the resin. Close the tube and keep in the heating block at 90°C for 2 min. After 2 min, check the color of resin for presence of any blue tinge.

15. If the resin as well as Kaiser solution looks yellow, it indicates that the capping process was completed and no free -NH₂ groups are present on the resin. However, any blue tinge in the resin beads indicates that there are still free NH₂ groups on the resin and it is not completely capped.

16. If the Kaiser solution is blue, next step is to repeat the capping. Wash the resin again with DCM (4X) and add capping solution (5 mL) and allow to shake for 2 h. Then repeat the Kaiser test which should show complete yellow color for the resin beads.

17. After the Kaiser test is yellow indicating completion of capping process, wash the resin with DCM (4X) again and allow to vacuum dry overnight. For vacuum drying, open the valve of the synthesis glass vessel and attach the outlet of vessel with the vacuum tube. Properly close the top of the vessel with the cap and open the vacuum valve to ensure that vacuum is applied to the vessel.

18. Check that the resin is completely dry using a spatula, if not, then let it dry for another 24 h. After complete drying, transfer the amino acid loaded resin to a clean and completely dry glass vial and seal the vial using parafilm.

19. This amino acid (lysine or arginine) loaded MBHA resin can be used for synthesis of PNA sequences. It is essential to protect it from moisture by parafilming the glass vial and can be stored at room temperature for 6 months.

PNA synthesis

PNA synthesis starts with finalizing the PNA sequence based on the target and is outlined in Table 2 from C terminus (3’ end) to N terminus (5’ end). It is imperative to vacuum dry the monomers, HBTU and amino acid loaded MBHA resin at least overnight to remove any residual moisture.

Reagent Preparation:

0.39M HBTU: After overnight vacuum drying, weigh 740 mg HBTU and dissolve in 5 mL of anhydrous DMF in a labeled amber glass vial or regular glass bottle covered with aluminum foil to protect from light.

0.52M DIEA: Dissolve 362 µl DIEA in 6.4 mL of anhydrous DMF in labeled amber glass bottle or regular glass bottle covered with aluminum foil.

Deprotection Solution: Take 95 mL of TFA in measuring cylinder and add 5 mL of m-cresol. Transfer the solution to a labeled amber-colored glass bottle.

Capping solution: Transfer 24 mL of pyridine, 24 mL of NMP, and 1 mL of acetic anhydride to a labeled amber-colored glass bottle.

Monomer coupling solution: Boc-PNA monomer stocks are stored in -20°C. Transfer the monomers required for PNA synthesis to labeled glass vials and vacuum dry overnight before the start of synthesis. Prepare monomer solution by dissolving below mentioned monomers in 375 µl of NMP + 190 µl of 0.39 M HBTU + 190 µl of 0.52 M DIEA and activate by vortexing for 2–3 min

Boc-PNA-T-OH: 29 mg
Boc-PNA-G(Cbz)-OH: 42 mg
Boc-PNA-A(Cbz)-OH: 40 mg
Boc-PNA-C(Cbz)-OH: 38 mg

Synthesis Steps:

1. For synthesis of PNA, weigh 100 mg of amino acid loaded MBHA resin which has been vacuum dried overnight.

2. Transfer the weighed amino-acid loaded resin to a clean and dry peptide synthesis vessel mounted on a shaker while minimizing the loss of resin during the transfer.
### Table 2

**PNA synthesis**

| Date: | PNA synthesis form (100 or 50 mg) | PNA: |
|-------|---------------------------------|------|
| **PNA sequence** | | |
| (3' → 5') | | |
| **TFA: m-cresol (95:5)** | | |
| (5 mins) | | |
| **TFA: m-cresol (95:5)** | | |
| (5 mins) | | |
| **TFA: m-cresol (95:5)** | | |
| (5 mins) | | |
| **DCM (2x)** | | |
| **DMF (2x)** | | |
| **DCM (2x)** | | |
| **Pyridine (1 min)** | | |
| **Kaiser Test (Blue)** | | |
| **Coupling solution (1 hr)** | | |
| **DMF (4x)** | | |
| **DCM (2x)** | | |
| **Kaiser Test (Yellow)** | | |
| **Capping solution** | | |
| (2 mins) | | |
| **DCM (4x)** | | |

3. Add DCM to the vessel to soak the amino acid loaded resin and close the vessel top. Leave the vessel inside the fume hood for at least 2 h. *(Important note: Ensure all resin is soaked in DCM, use spatula to bring the resin on walls to the bottom of vessel. Do not shake or rock the shaker.)*

4. Deprotection step: After two hours, remove the DCM and wash the resin again with DCM (2x) bringing the resin to bottom of vessel. Add enough deprotection solution (TFA:m-cresol) to cover the resin bed and let the vessel shake for 5 min.

5. Remove the deprotection solution from the vessel and add fresh deprotection solution and shake the vessel for another 5 min. Repeat this step one more time to perform deprotection for a total of three times. The multiple deprotection steps ensure cleavage of the Boc (tert-butyloxycarbonyl) from the amine of last monomer conjugated to the PNA oligomer and exposes the free amine for the next coupling reaction.

6. After third deprotection step, wash the resin with DCM twice followed by washing with DMF (2X). Wash the resin again with DCM (2X) and add pyridine to cover the resin bed and leave for 1 min.

7. Drain off the pyridine and take a small speck of resin using a spatula and transfer to a 1.5 mL tube for Kaiser test. Add pyridine in the vessel in order to keep resin soaked during the Kaiser test.

8. Kaiser test (Blue): Add 20 μl or two drops of Kaiser A and 20 μl or 2 drops of Kaiser B to the tube containing the small resin speck. Keep the tube in heating block at 90°C for 2 min. After 2 min, if the resin looks blue, indicating the removal of Boc protecting group from the amino acids loaded on the resin, move forward with the next steps of PNA synthesis.

   However, if the resin is yellow, it indicates that the deprotection step has not been completed yet. Hence, wash the resin with DCM (4X) and perform deprotection one more time for 5 min and repeat
the Kaiser test to obtain blue color. (Important note: In case Kaiser test still shows yellow color after deprotection steps, it indicates that resin has not been properly loaded with the amino acid, hence it is advisable to prepare the amino acid loaded resin again.)

**Alternative tests to detect the completion of coupling step:** i) Bromophenol blue test

Add 2–3 drops of 3% w/v solution of bromophenol in DMF to a small amount of resin beads and observe the change at room temperature. The presence of blue-green color indicates presence of amines and incompletion of coupling step. However, yellow color with only slight green tinge indicates completion of the coupling step.

ii) 2,4,6-Trinitrobenzene sulfonic acid test
Reagent A: 1% 2,4,6-Trinitrobenzene sulfonic acid in DMF
Reagent B: 10% DIPEA (N, N-Diisopropylethylamine) in DMF

**Steps:** Add one drop of reagent A and one drop of reagent B in a tube containing few resin beads. Keep the beads at room temperature for 5 min and observe for any change in color. The presence of red color indicates presence of free amines and incomplete coupling reaction.

9. Based on the PNA sequence, weigh the Boc-PNA monomer as mentioned above and transfer to a 1.5 mL tube. Add 375 μl of NMP and 190 μl of 0.39 M HBTU and 190 μl of 0.52 M DIEA to the tube containing the monomer followed by vortexing for about 3 min to obtain a clear yellow solution. (Tip: The monomer solution of Boc-PNA-G(Cbz)-OH takes about 5–10 min for complete solubilization and to become clear yellow due to its hydrophobic nature). It is advisable to prepare the monomer coupling solutions during the steps of deprotection.

10. Remove the pyridine from the vessel and transfer the activated monomer coupling solution to the vessel. Use a pipette to transfer the entire 755 μl of coupling solution and add it to the top of the resin bed. Let the vessel shake and ensure that entire resin is being agitated and is in contact with the coupling solution. If some resin is stuck to the walls of resin, use a spatula to bring the resin closer to the bottom and coupling solution.

11. Coupling steps can take about 1.5 h. Addition of G monomer to the PNA sequence needs maximum time (~2 h).

12. After 1.5 h, wash the resin bed with DMF at least 4 times to remove the coupling solution followed by washing with DCM (2X).

13. Transfer small speck of resin from the vessel to a 1.5 mL tube. Further, add DCM to the vessel in order to keep resin soaked during the Kaiser test.

14. Kaiser test (Yellow): Add 20 μl or two drops of Kaiser A and 20 μl or 2 drops of Kaiser B to the tube containing the small speck of resin. Keep the tube in heating block at 90°C for 2 min. If the resin is yellow after 2 min, it indicates the successful completion of coupling step and proceed with the next step of PNA synthesis.

However, if the resin has any blue speck, it indicates monomer coupling was not completed. In that case, prepare the same monomer coupling solution again. Once the coupling solution is ready, wash the resin with DCM (2X) and add the coupling solution to the resin bed and allow it to shake for another 1 h. After 1 h, repeat the same steps of washing with DMF and DCM followed by Kaiser test. The Kaiser test must show complete yellow color before moving to the next steps of PNA synthesis.

15. Once the Kaiser test is yellow, remove the DCM from the resin bed and add capping solution just enough to cover the resin bed. Allow the vessel to shake for 2 min and wash the resin with DCM (4X) to remove the capping solution.

16. Once the coupling for first PNA monomer is complete, follow the PNA synthesis sheet (Table 2) to continue the monomer additions based on the sequence of PNA.

**Amino acid conjugation**

Multiple amino acids can be conjugated on 5’ end of PNA to synthesize cationic PNAs. The coupling steps are the same as described above under PNA synthesis. It is essential to vacuum dry the amino acid monomers (Boc-Arg(Tos)-OH or Boc-Lys(Cl-Z)-OH) overnight. After completion of last PNA monomer conjugation, continue with deprotection step three time (5 min each) followed by washing and Kaiser test (Blue). Weigh the amino acid monomer (32 mg of Boc-Arg(Tos)-OH or 30 mg of Boc-Lys(Cl-Z)-OH) and dissolve in 375 μl NMP + 190 μl 0.39 M HBTU + 190 μl 0.52 M DIEA to obtain
a clear coupling solution. After the Kaiser is blue, add the amino acid coupling solution and let the vessel shake for 2 h. After 2 h, wash the resin (4X DMF and 2X DCM) followed by Kaiser test (Yellow) to check the completion of reaction/conjugation. If the Kaiser test is yellow, follow the next steps of synthesis otherwise repeat the coupling step again. Perform amino acid coupling three times to conjugate three cationic residues to the PNA sequence.

**Fluorophore conjugation**

Conjugation of fluorescence dye is essential to study the cellular uptake and biodistribution of PNAs. Free acid form of 5-carboxytetraethylrhodamine (TAMRA) dye, single isomer is used for conjugation to PNAs. Before conjugation of TAMRA, mini-polyethylene glycol (Mini-PEG) unit is conjugated to the PNA which acts as a flexible linker between the PNA oligomer and TAMRA. Boc-Mini-PEG-3 has a molecular weight of 307.35 Da. It has three ethylene glycol units where NH₂ is protected by boc group and has a free carboxylic acid group on the other end which conjugates with the PNA oligomer via an amide bond.

1. After completion of amino acid conjugation, perform capping step twice to ensure all free NH₂ groups are capped. Next, perform deprotection (3X) and ensure the completion of deprotection by blue Kaiser test.
2. For Boc-Mini-PEG-3 conjugation, weigh 22.5 mg of Boc-Mini-PEG-3 and dissolve in 375 μl of NMP and 190 μl of 0.39 M HBTU and 190 μl of 0.52 M DIEA to obtain a clear solution.
3. Add the Boc-Mini-PEG-3 coupling solution to the resin and allow the vessel to shake for 2 h. After washing the resin, check the completion of conjugation with yellow Kaiser test.
4. Perform capping of the resin twice before conjugation of TAMRA dye.
5. For 100 mg scale of PNA synthesis, dissolve 30 mg of TAMRA in 350 μl NMP and 175 μl of each 0.39 M HBTU and 0.52 M DIEA and vortex aggressively for about 5 min to ensure that TAMRA is completely dissolved to obtain bright red coupling solution.
6. Add the TAMRA dye coupling solution on top of the resin inside the vessel and cover the entire vessel with aluminum foil. Let the vessel shake for about 18 to 24 h.
   (Important note: Since we cannot perform Kaiser test after TAMRA conjugation to check the completion of reaction, it is essential that all the reagents are used accurately and conjugation is allowed to continue as long as possible.)
7. After 24 h, wash the resin multiple times with DMF (4X) followed by washing with DCM (4X) before proceeding to the cleavage of PNA.

**Cleavage of PNA oligomer**

1. To cleave the PNA oligomer from the MBHA resin, add deprotection solution (TFA:m-cresol) and allow to shake for 5 min. Repeat this step twice.
2. Meanwhile prepare the cleavage cocktail by mixing 600 μl TFA + 300 μl Trifluoromethanesulfonic acid (TFMSA) + 150 μl m-cresol + 150 μl thioanisole in a 1.5 mL tube. Transfer the cleavage cocktail to -20°C for about 3 min until the completion of deprotection step. (Important: TFMSA is extremely corrosive, handle the cleavage cocktail with extreme caution.)
3. Take out the cleavage cocktail from the freezer and add onto the top of resin. If TAMRA is conjugated to the PNA, cover the vessel with aluminum foil and allow the vessel to shake for 1.25 h.
4. Label two 15 mL falcon tubes. Open the valve of synthesis vessel and collect half of the cleavage mixture in one tube and rest half in another falcon tube. Add 500 μl of TFA to the resin in the vessel and collect the mixture in the second falcon tube.
5. Add diethyl ether to each falcon tube up to 14 mL and vortex to precipitate the PNA (Important note: Red color precipitate will be visible for TAMRA PNA and white precipitate for regular PNA).
6. Keep the tubes in -20°C freezer for 5 minutes followed by centrifugation at 5000 rpm for 4 min at 4°C (pre-cool the centrifuge to 4°C) to sediment the precipitated PNA.
7. After centrifugation, decant the supernatant diethyl ether from both tubes and add fresh diethyl ether till 14 mL followed by vortexing to disperse the pellet.
Fig. 3. HPLC chromatograms of short cationic crude PNAs. PNA2 is lysine (K) conjugated short PNA and PNA7 is TAMRA conjugated version of PNA2. PNA3 is arginine (R) conjugated short PNA and PNA8 is TAMRA conjugated version of PNA3.

8. Transfer the tubes to -20°C freezer for 5 min followed by centrifugation at 5000 rpm for 4 min to sediment the precipitated PNA. Repeat the step one more time to obtain the final pellet of crude PNA.

9. Allow the crude PNA in both tubes to vacuum dry for at least 6 h.

**Purification of PNAs**

1. Reconstitute the crude PNAs by adding 100 μl of acetonitrile (ACN) and 200 μl water in each falcon tube. Vortex the tubes to completely dissolve the crude PNAs. (*Add 50 μl more ACN in case the crude PNA does not dissolve*).

2. Combine the dissolved crude PNAs in 1.5 mL tube and centrifuge the tube at 10,000 rpm for 5 min. Start the purification of crude PNA using Reverse Phase High Performance Liquid Chromatography (RP-HPLC, Shimadzu) with automatic fraction collector and XBridge® BEH C18 OBD™ Prep Column.

3. The gradient mobile phase composition is as below:

| Time   | Mobile Phase | % |
|--------|--------------|---|
| 15 min | Solvent B    | 20|
| 17.5 min | Solvent B | 30|
| 20.5 min | Solvent B | 90|
| 22.5 min | Solvent B | 0 |
| 23 min   | Stop         |   |

4. Short cationic PNAs show high yield with low impurity content as evident from the chromatogram of crude PNAs (*Fig. 3*).

PNA2: 5’KKK-AGCATTAA-K-3’
PNA3: 5’RRR-AGCATTAA-R-3’
PNA7: 5’-TAMRA-OOO-KKK-AGCATTAA-K-3’
PNA8: 5’-TAMRA-OOO-RRR-AGCATTAA-R-3’
Both PNA2 and PNA3 crude PNAs when separated by RP-HPLC showed high purity with low impurity content. In Fig. 3, peak 3 in the chromatogram of PNA2 is the complete sequence of PNA with lysine residues, however peak 1 is small fragment of PNA which did not continue, hence peak 3 was collected as the pure product. Similarly, for PNA3, peak 7 is the pure fraction of PNA which was collected. Further, TAMRA conjugated PNAs are more hydrophobic and elute later in the chromatogram. As evident in chromatogram of PNA7, there are two major products, peak 11 is TAMRA conjugated PNA and peak 5, 7 are similarly non TAMRA PNAs.

TAMRA conjugation is simply confirmed via the color of PNA fraction collected from peak 11 which is pink and from absorbance curve at 549 nm which shows peak only at position of peak 11.

Quality control

It is essential to collect all the fractions of PNA during purification. For short PNAs, pure fraction is the major peak visible in HPLC chromatogram and easily visible. However, it is essential to confirm the quality of PNA which can be done using below techniques:

- **Mass spectroscopy**: To confirm the molecular weight of pure fraction of PNAs, Mass Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Spectroscopy is used. The molecular weight (MW) of fraction obtained from MALDI is compared against the theoretical MW. *(Approximately 15 μl of 50 μM concentration of PNA fraction is sufficient for MALDI-TOF analysis)*

  *In vitro binding study*: In order to confirm that synthesized PNA sequence is complementary to the target, a gel shift assay can be performed by incubating the PNA sequence with the target DNA/RNA followed by separation on a polyacrylamide gel.

### Encapsulation of PNAs in PLGA NPs

Recently, PLGA based NPs have been used for encapsulating PNAs to achieve higher intracellular delivery for antisense [26] and gene editing applications [27]. The list of reagents and equipment required for formulation of PNA loaded PLGA NPs is provided below (Table 3).

| Reagent Description | Source | Catalog No. |
|---------------------|--------|-------------|
| **Poly (DL-lactide-co-glycolide) polymer, 50:50 (DL:PLG)** | Lactel Absorbable Polymers | B6010-1 |
| Ester terminated Inherent Viscosity: 0.39 dl/g Polyvinyl alcohol (PVA) (87–90% hydrolyzed, MW 30,000 – 70,000) | Sigma Aldrich | P8136 |
| Trehalose dihydrate | Sigma Aldrich | T0167 |

| Equipment Description | Source | Catalog No. |
|-----------------------|--------|-------------|
| Sonics VibraCell | Fisher Scientific | VCX130 |
| Freezone Lyophilizer (2.5 L, -50°C) | Labconco | 700201000 |
| Vortexer | Fisher Scientific | - |
| Glass tubes (15 mm × 85 mm) | Corning | 9945-15 |
| Multi-position stirrer | BT Lab Systems | BT1015 |
| Eppendorf tubes (1.5 mL) | Fisher Scientific | 05-408-129 |

**Table 3**

List of reagents and equipment required for PNA loaded PLGA NPs

**Table 4**

Preparation:

1. Autoclave the beakers, glass tubes (220°F, 30 min), polypropylene/plastic tubes (~50 ml), 1.5 ml tubes (220°F, 15 min).
2. 5% PVA aqueous solution: Dissolve 25 g of PVA in 500 mL of sterile purified water. Allow stirring overnight at room temperature for complete solubilization. Store at 4°C and use within 6 months.
Table 4
Yield of NPs formulated at different scale

| NP batch size | % Yield ± SD |
|---------------|--------------|
| 20 mg         | 61.2 ± 6.4   |
| 40 mg         | 81.5 ± 1.6   |
| 80 mg         | 77.9 ± 5.1   |

**Fig. 4.** Work flow depicting different steps involved in formulation of PLGA nanoparticles (NPs) loaded with PNA. First the PLGA polymer is soaked in dichloromethane (DCM) for more than 6 hours (Step 1). Next, the aqueous PNA solution is added dropwise to the PLGA in DCM followed by sonication (Step 2) to form first water and oil (W/O) emulsion. This emulsion is added to the 5% polyvinyl alcohol (PVA) aqueous solution dropwise and sonicated to form water-in-oil-in-water (W/O/W) double emulsion (Step 3) which is then added to 0.3% PVA aqueous solution (Step 4) and allowed to stir overnight (Step 5). Here rhodamine (TAMRA) dye conjugated PNA which is pink in color was used for encapsulation in PLGA NPs.

3. PLGA polymer (50:50, ester terminated, 0.39 dL/g) is stored in -20°C.
4. PNA stocks: Prepare PNA stocks at concentration of 1 mM in purified water.
5. Trehalose solution: Prepare an aqueous solution of 5 mg/ml trehalose by dissolving 250 mg of trehalose in 50 mL of purified water. Store at room temperature.
6. Personal protective equipment: It is recommended to use the lab-coat, gloves and goggles while handling the nanoparticles.

**Formulation of PLGA NPs**

Although there are multiple techniques reported in the literature for formulation of PLGA based polymeric NPs [28], the double emulsion solvent evaporation method is the most suitable and widely used approach to encapsulate water soluble PNAS at high efficiency [9]. The PNA loaded NPs can be formulated at different scales ranging from 20 mg to 80 mg, however the final yield of NPs is higher for larger scale formulations (Table 4). The procedure detailed below (Fig. 4) is for a 40 mg batch of PNA loaded NPs and the amount/volume of reagents can be scaled up or down based on the batch size.

Steps:
1. Weigh 40 mg of PLGA polymer (solid rectangular shaped flakes at room temperature) on a butter paper and transfer to the autoclaved glass tube. Add 500 μL of DCM and immediately cover the glass tube with aluminum foil followed by parafilm to prevent the evaporation of DCM. Mark the visible level of DCM on the glass tube using a marker and allow the polymer to soak in DCM at least for 6 h or overnight.
2. After soaking the polymer in DCM for 6 h or overnight, start the preparation for formulating the NPs.

- Prepare 0.3% PVA solution from the 5% PVA stock: Dilute 6 mL of 5% PVA stock with 94 mL of sterile purified water. (Tip: It can be stored at 4°C for 6 months)
- Add 10 mL of 0.3% aqueous PVA solution in a 50 mL autoclaved beaker with a stir bar and stir on a multi-position stirrer inside the chemical safety hood.
- Transfer 1 mL of 5% aqueous PVA solution to another autoclaved glass tube.
- Measure the concentration of PNA stock solution right before the formulation of NPs and calculate the volume of PNA stock required for 80 nanomoles of PNA to start with a loading of 2 nanomoles/mg PLGA polymer. For a PNA stock of 1 mM, 80 μl of the stock is needed. (Tip: Volume of PNA stock should not be higher than 120 μl for a 40 mg batch of NPs)
- Wipe off the gloves, pipette, stirrer, vortexer, probe of the sonicator with 70% ethanol to maintain sterility.
- Remove the parafilm and foil from the glass tube containing the PLGA polymer dissolved in DCM. If the level of DCM is lower than the mark, add sufficient volume of DCM to the glass tube to reach the mark. Mix the polymer solution on the vortexer to achieve uniform distribution.

3. Take 80 μl of the PNA stock (1 mM) using a 100 μl pipette and add it dropwise to the glass tube containing PLGA polymer solution in DCM while it is being vortexed at a speed of 8–10. (Tip: Add the PNA stock to the polymer solution in center of the glass tube and avoid touching the tube walls to maximize the loading of PNA in NPs)
4. Sonicate the water-in-oil emulsion (w/o) using the probe sonicator for 30 s (10 s×3, 5 s interval between each cycle) at 38% amplification.
5. Add the first w/o emulsion slowly using a 100 μl pipette to the center of glass tube containing 5% aqueous PVA solution while being vortexed (8–10 speed). (Important note: Avoid touching walls of the tube and add in center of the tube)
6. Next, sonicate this water-in-oil-in-water emulsion (w/o/w) again using the probe sonicator for 30 s at 38% amplification.
7. Add this w/o/w double emulsion slowly to the stirring 0.3% aqueous PVA solution inside the chemical safety hood using a 1000 μl pipette. Cover the beaker with aluminum foil and poke multiple holes in the foil using a syringe to allow the DCM to evaporate. Leave the formulation to stir overnight inside the chemical safety hood.

Washing and lyophilization of PLGA NPs

1. After overnight stirring of the formulation, transfer the formulation to an autoclaved polypropylene/plastic tube. Add ~500 μl of 0.3% PVA to the beaker and add back to plastic tube to retrieve the complete formulation. (Important note: Keep the tube containing formulation on ice at all time during handling) (Fig. 5).
2. In order to remove excess PNA and PVA, centrifuge the tube containing formulation at 9500 rpm for 10 min at 4°C. (Important note: Pre-cool the centrifuge to 4°C to prevent release or degradation of NPs.)
3. Pre-cool the purified water for washing the NPs in ice. After the completion of centrifugation, decant off the supernatant and add 10 ml of cold purified water to the tube. Resuspend the NPs by vortexing and sonication.
4. Perform the centrifugation and washing with cold purified water twice.
5. During the washing steps, label and weigh 14 empty autoclaved 1.5 mL tubes for one 40 mg NP batch and record the weights in an excel sheet.
6. After the third centrifugation cycle, decant the supernatant and resuspend the washed NPs in 2.8 mL of trehalose solution (5 mg/ml) which was pre-cooled in ice.
7. Transfer 210 μl of resuspended NPs to pre-weighed 1.5 mL tubes and parafilm the top of each tube while keeping the cap open. Puncture the parafilm with 2-4 holes on top of the tubes using a syringe.
8. Next, flash freeze the NP formulation in each eppendorf tube in liquid nitrogen by keeping the tubes immersed in liquid nitrogen for 1–2 min. Arrange the flash frozen NPs in paper separators and transfer to the freeze-drying flask. Immediately connect the flask to the freeze dryer and turn on the vacuum. (Tip: Do not let the NPs to thaw during the transfer of tubes to the flask and connecting the flask to the freeze dryer.)

9. Allow the NPs to freeze dry for at least 48 h to ensure complete removal of water from the formulation. After 48 h, remove the flask from the freeze dryer and transfer the NP tubes to a rack. Wipe the gloves with 70% ethanol and remove the parafilm from top of the tubes and close the top.

10. Weigh the NP tubes and record the weight of NP tubes next to the respective empty tube weights. (Tip: Wipe the weighing balance, working area, and laptop with 70% ethanol to prevent any contamination of NPs during handling.)

11. Calculate the exact amount of NPs in each tube using below equation:

   \[ \text{Weight of NPs} = 0.7 \times (\text{Weight of NP tube} - \text{Weight of empty tube}) \]

12. Label tubes with the exact amount of NPs and parafilm the closed tubes while maintaining sterility. Store the NPs at -20°C and use within 6 months.

13. The yield of NPs is calculated by subtracting the weight of trehalose, i.e. 14 mg (2.8 mL × 5 mg/ml) from the total solids (NPs + Trehalose) weight in 14 tubes.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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