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Assembly of Serotonin on DNA Tetrahedron for Application in Treating α-Synucleinopathy

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Bhagyesh Parmar[1], Geethu Prakash[1], Sanjay Kosara[1], Sweny Jain[1], Payal Vaswani[1], Dhiraj Bhatia[1]

[1]Department of Biological Sciences and Engineering, Indian Institute of Technology, Palaj, Gandhinagar 382355, India

IIT Gandhinagar

Dhiraj Bhatia
Indian Institute of Technology Gandhinagar

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Abstract

This study presents a comprehensive protocol for assembling and applying tetrahedral DNA nanostructures (TDNs) as carriers for neurotransmitter delivery, explicitly targeting the treatment of α-synucleinopathies such as Parkinson's disease. TDNs, self-assembled from four single-stranded DNA oligonucleotides, offer a stable, programmable, and biocompatible platform with enhanced nuclease resistance and the ability to traverse the blood-brain barrier (BBB) via catherin-1-mediated transcytosis. The protocol details the synthesis, characterization, and functionalization of TDNs with neurotransmitters-serotonin, epinephrine, and norepinephrine-through electrostatic and hydrogen bonding interactions, exploiting the negative charge of the DNA backbone and the positive charge of protonated neurotransmitter amines. Structural validation is achieved via electrophoretic mobility shift assays, atomic force microscopy, dynamic light scattering, and fluorescence quenching studies. Functional assays in MPTP-induced PC12 cell models demonstrate that TDN-neurotransmitter complexes (TD:NTs) are efficiently internalized, reduce α-synuclein accumulation, and mitigate key pathological features, including oxidative stress, iron buildup, lipid peroxidation, and autophagy. In vivo zebrafish studies confirm high brain tissue accumulation and negligible neurotoxicity. Compared to conventional nanocarriers, TDNs exhibit superior biocompatibility, BBB permeability, and drug loading efficiency, while minimizing immunogenicity and off-target effects. Nonetheless, limitations such as restricted drug payload, rapid systemic clearance, and susceptibility to enzymatic degradation are acknowledged, alongside the need for further research into long-term safety and targeted delivery. This protocol establishes a robust workflow for TDN synthesis, neurotransmitter conjugation, and biological validation, underscoring the potential of TDNs as next-generation nanocarriers for targeted neurotransmitter delivery in neurodegenerative disease therapy.

Guidelines

Neurotransmitters are chemical messengers facilitating communication between neurons and other target cells, such as muscles or glands. These precise chemical signals regulate essential bodily activities, including heart rate, respiratory rate, sleep regulation, muscle control, mood stabilization, cognitive processes, and the maintenance of general physiological equilibrium. Neurotransmitter transport is essential for the functioning of the nervous system and is crucial in treating and managing neurological disorders and neurodegenerative diseases. Neurotransmitter balance disruptions coming from overproduction, receptor failure, together with synaptic damage or enzymatic deficiencies, lead to Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy, and mental disorders. Therefore, advancing neurotransmitter delivery strategies is essential, since they will address contemporary healthcare challenges. DNA nanotechnology started as a revolutionary scientific discipline that Nadrian Seeman introduced during the 1980s. DNA nanotechnology has evolved into a field that enables researchers to build programmable structures with diverse DNA components. DNA nanotechnology has transformed the set of tools that molecular biologists and bioengineers have to work with, allowing the bottom-up assembly of functional nanosystems. Among them, TDNs have the benefits of rigidity, controlled geometry, and functionalization accessibility. The applicability of these DNA-based scaffolds in biomedical research has also broadened enormously, particularly in the fields of regenerative medicine and targeted therapeutics. It has been noted in the past that they are effective in crossing the blood-brain barrier, regulating the immune response, and following certain receptor-mediated endocytosis pathways. The Tetrahedral DNA Nanostructure (TDN) is one of the most widely studied DNA nanostructures. Tetrahedral DNA nanostructures (TDNs) have recently become versatile, programmable nanoscale tools that can be used in stem cell biology and neuroengineering. Their biocompatibility, structural stability, and ability to deliver payloads make them outstanding candidates to guide mesenchymal stem cell (MSC) differentiation and neuroregeneration. Recent developments demonstrate that TDNs, upon conjugation with therapeutic molecules or peptides, can penetrate biological barriers, regulate cellular internalization, and rewire cell behavior in a highly spatial and temporally controlled manner. This manuscript outlines the structural design of TDNs, their interaction with neural environments, and how they can be used to induce stem cell fate decisions, with recent in vitro and in vivo results. TDNs stand out as preferred nanostructures because they demonstrate excellent structural stability and simple assembly methods, predictable geometries, high programmability, and outstanding compatibility with biological systems. TDNs demonstrate excellent characteristics that make them perfect candidates for biological and medical applications. The resistance of TDNs to nucleases enables them to preserve their structure and operational characteristics longer than linear DNA in biological systems and environments. The geometric structure of TDNs decreases electrical resistance while creating asymmetric membrane charges, enabling their cell uptake without requiring external targeting agents. The feature proves advantageous when drugs need to penetrate brain capillary endothelial cells and overcome the blood-brain barrier (BBB), representing a significant challenge in CNS drug delivery. Effective drug carriers must have six vital features: strong biocompatibility, high stability during transport, simple drug loading capabilities, specific targeting mechanisms, rapid cellular uptake, and minimal side effects. Drug carriers must demonstrate penetration capacity through biological barriers, including the BBB. The structural properties of DNA nanostructures, specifically TDNs, show all crucial characteristics, including low immunogenicity, and precise structural customization that boosts their drug carrier potential. The application of TDNs shows great promise for delivering neurotransmitters. Neuroengineering and the reprogramming of stem cells need efficient delivery systems. TDNs engage caveolin- and clathrin-independent endocytosis pathways, and topology (sharpness, curvature) can favor one pathway over the other. Quantum dot-loaded DNA icosahedra have been

tracked in real time in living cells, and demonstrated improved retention and cytoplasmic stability of payloads. Enhanced uptake and retention has been realized further with pH-responsive or lipid-modified DNA nanostructures. In vitro studies have demonstrated >70% uptake in primary neuronal cultures and zebrafish models within 2 hours of administration. Their design allows better cellular uptake while carrying more neurotransmitters through the blood-brain barrier, which provides an effective treatment for Parkinson's disease and other neurodegenerative conditions. Our recent study has confirmed that TDNs are effective carriers for delivering serotonin, epinephrine, and norepinephrine. The cellular absorption of TDNs proceeds through Clathrin-mediated endocytosis. The molecules are conjugated to TDNs by forming electrostatic bonds and hydrogen bonds. Neurotransmitters with positively charged amine groups in their protonated state are drawn toward DNA phosphates because they have negative charges. The DNA bases with oxygen atoms in their minor and major grooves form hydrogen bonds with neurotransmitter hydrogen atoms to create additional stability in the complex.

Materials

Biological materials: PC12 Cell line (ATCC No. - CRL-1721.1). Critical — In this protocol, a cell line has been used. It should be ensured that the handling of the cell line is done in a biosafety cabinet. The cells should be mycoplasma-free. Reagents: Caution — All the reagents should be handled after wearing personal protective equipment such as a lab coat and gloves. Caution — All the reagents should be used according to the manufacturer's protocol. Tetrahedron sequences (Table 1) were synthesized commercially by Sigma Aldrich. Table 1: Tetrahedron Sequence Sequence (5′ to 3′) M1: ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA M2: TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC M3: TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC M4: TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT M4Cy5: Cy5TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT Serotonin hydrochloride (Sigma Aldrich - H9523) Nuclease free water (SRL) F12K media (Gibco - 21127-022) Fetal Bovine Serum (FBS; Gibco - 10270-106) Horse Serum (Gibco - 16050122) Penstrap (Gibco - 15140122) MgCl2.6H2O (Emparta - DJ0D702365) 25 bp DNA ladder (Promega - G4511) 37% PFA 30% acrylamide-bisacrylamide (Himedia - ML037) Ammonia Persulphate (Loba Chem- 01321) N,N,N′,N′ -Tetramethylethylenediamine (TEMED) 4′,6-Diamidino-2-phenylindole (DAPI; Roche - 10236276001) Caution – DAPI is toxic. Wear appropriate personal protective equipment. Ethidium Bromide (EtBr; Himedia - MB071) Caution – EtBr is carcinogenic. Wear appropriate personal protective equipment. 6X gel loading dye (Promega - G190A) MPTP hydrochloride (Sigma Aldrich) Caution – MPTP hydrochloride is toxic. Wear appropriate personal protective equipment. Alpha-synuclein antibody (Cell Signaling Technology) Secondary antibody DCFDA Trypsin (Gibco) Tris Glacial Acetic Acid EDTA disodium salt Sodium chloride Potassium chloride Disodium hydrogen phosphate (Na2HPO4) Potassium dihydrogen phosphate (KH2PO4) Equipment: Thermal cycler (Biorad) Incubator (ThermoFischer Scientific) Microscope (Nikon) pH meter UV spectrometer Microplate reader (Biotek Cytation 3) Chemidoc imaging system (Biorad) DLS (Malvern Zetasizer) Confocal microscope (Leica Sp8) Vortex shaker Centrifuge Dessicator Vaccum pump Consumables: 200 µL, 1.5 mL and 2.0 mL tubes (Tarsons) 15 mL and 50 mL Falcon tubes (Tarsons) Cell culture flasks (Tarsons) Cell culture plates (Tarsons) 5 mL and 10 mL Serological pipettes (Tarsons) 12 mm Coverslips (Bluestar) Microscopic Slides (Bluestar) Quartz Cuvette Mica sheet Reagent Setup: 50X TAE Buffer: Add 2 M of tris, 1 M glacial acetic acid and 50 mM of EDTA disodium salt to prepare 50X TAE buffer. Make sure there are no precipitates. 1× TAE buffer. Mix 20 ml of 50× TAE buffer and 980 ml of Milli-Q water thoroughly. TAE buffer can be stored at room temperature until a precipitant form. 10% (wt/vol) APS: Dissolve 5 g of APS into 50 ml of Milli-Q water. Store APS buffer at 4 °C for ≤1 week in dark. EtBr Staining Solution: Dissolve 8 µL of 2 mg/mL EtBr in 50 mL of 1X TAE buffer. 1X Phosphate-buffered saline (PBS): Dissolve 8.0 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 in 800 mL deionized water, and adjust the final pH to 7.4 using HCl. Add deionized water to make the solution volume up to 1,000 mL. Filter-sterilize (0.22 µm), and store PBS solution at 4°C for no more than 6 months. 50 mM MgCl2.6H2O: Add 10.165 mg of MgCl2.6H2O in 1 mL of distilled water to make 50 mM MgCl2.6H2O F12K Complete Media: Add 1% PenStrap, 2.5% FBS and 15% Horse Serum to F12K serum free media to constitute F12K complete media. Store the complete media at 4°C.

Troubleshooting

Workflow

1 Primer Reconstitution

1.1 Spin down the vial of primer so that all the lyophilized oligonucleotides settle at the bottom of the tube.

1.2 Add an appropriate amount of nuclease free water (mentioned in the sheet received with the primer) to the vial to make a 100 µM stock.

1.3 Vortex properly, spin down and keep at 70°C for 1 hour 15 minutes at 450 rpm shaking on thermoshaker. It will help faster dissolution of the primers and denature any aggregates if present, ensuring that the primers are in a single stranded form.

1.4 Prepare 10 µM working stock using nuclease free water. Store the working stock at 4°C and main stock at -20°C.

2 DNA Tetrahedron Synthesis

2.1 Vortex and spin down the 10 µM working stock of the primers.

2.2 Prepare 4 tubes (Table 2) and add the primers in equi-molar ratio.

2.3 Add 2 mM MgCl2 from 50 mM stock.

2.4 Vortex and spin down the tube and proceed to thermalcycler.

2.5 Heat to 95°C for 30 minutes and anneal by decreasing the temperature by 5°C up to 4°C for 15 minutes at each step in a thermocycler.

2.6 Store at 4°C till further use.

3 Electrophoretic Mobility Shift Assay

3.1 To perform EMSA, prepare 10% Native-PAGE gel (Table 3) to confirm the formation of higher order structure.

3.2 Assemble the electrophoresis unit and make sure there is no leakage.

3.3 Prepare the loading sample containing 5 uL of TD, 3 uL of 1x TAE loading buffer and 1.5 uL of 6x loading dye.

3.4 Run the gel at 70 V for 90 minutes.

3.5 Stain the gel with EtBr staining solution and visualize it using Gel Documentation system.

4 Atomic Force Microscopy

4.1 Cut a small piece of mica sheet and place it on a clean slide using clear nail paint adhesive. Let it dry.

4.2 Prepare 1:10 dilution of TD in a PCR tube.

4.3 Cleve the top layer of mica sheet and drop cast the sample in a 1:10 dilution. Allow it to dry in a desiccator.

4.4 Image using AFM in tapping mode.

4.5 Process the image using JPK software.

5 TD:Ser Preparation

5.1 TD was combined with serotonin hydrochloride, epinephrine hydrochloride and norepinephrine hydrochloride in 1:50 ratio.

5.2 They were incubated at room temperature for 15 minutes in the dark. They were stored at 4°C until further use.

6 Fluorescence Quenching Study

6.1 The fluorescence quenching kinetic study is done to show the interaction between DNA and serotonin using Biotek multimode plate reader Cytation3.

6.2 96 well plate is taken and 100 µL of TD:Ser and Ser are added at different ratios. The excitation of 279 nm and emission of 320 nm is used.

6.3 The system is subjected to take reading every 20 minutes for 120 minutes and the data is further plotted using GraphPad Prism.

6.4 The concentration and timepoint giving a significant decrease in fluorescence intensity is selected, in this case 1:50. It is done using GraphPad Prism software.

7 DLS and Zeta Potential

7.1 The sample is prepared by diluting TD and TD:Ser 20-fold.

7.2 Then they are subjected to Malvern analytical Zetasizer Nano ZS instrument to measure the hydrodynamic size.

7.3 The same instrument is used to measure zeta potential as well.

7.4 The data is further analyzed using GraphPad Prism software.

8 UV-Visible Spectroscopy

8.1 TD, serotonin and TD:Ser were diluted at a 1:20 ratio and absorption is taken using UV-Vis spectroscopy.

8.2 The results are further plotted using GraphPad Prism.

9 Stability Assay

9.1 Stability of TD and TD:Ser are checked using serum stability assay.

9.2 TD and TD:Ser are incubated with 10% FBS at 37°C for different time points till 360 minutes.

9.3 10% native PAGE (Table 3) is run to check the band intensity.

9.4 Assemble the electrophoresis unit and make sure there is no leakage.

9.5 Prepare the loading sample containing 5 µL of TD or TD:Ser, 3 µL of 1× TAE loading buffer and 1.5 µL of 6× loading dye.

9.6 Run the gel at 70 V for 90 minutes.

9.7 Stain the gel with EtBr staining solution and visualize it using Gel Documentation system.

10 Cell Culture

10.1 PC12 cells are cultured in F12K media with 15% horse serum, 2.5% FBS and 1% PenStrap.

10.2 The cells should be maintained at 37°C in a humidified incubator and 5% CO2.

11 MPTP Treatment

11.1 The cells are treated with 500 µM of MPTP hydrochloride for 12 hours once they are 80% confluent.

11.2 Following that, TD or TD:Ser treatment is given for 6 hours at 200 nM concentration in the presence of MPTP hydrochloride.

12 Cellular Uptake

12.1 The cells are seeded in 24 24-well plate on coverslips and allowed to grow till 80% confluency, followed by the MPTP hydrochloride treatment (as mentioned in above section).

12.2 After the treatment, cells are incubated with 200 nM TD-Cy5 or TD-Cy5:Ser for 1 hour at 37°C.

12.3 The cells are washed with 1X PBS two times to remove any unbound or surface TD or TD:Ser.

12.4 The cells are then fixed with 4% PFA for 15 minutes at 37°C, washed three times with 1X PBS and later mounted with DAPI + Mowiol.

12.5 The slides are stored at 4°C until imaging.

13 a-synuclein Immunostaining

13.1 The cells are seeded in 24 24-well plate on coverslips and allowed to grow till 80% confluency, followed by the MPTP hydrochloride and TD or TD:Ser treatment.

13.2 They are fixed with 4% PFA for 15 minutes at 37°C and washed three times with 1x PBS.

13.3 The cells are permeabilized with 0.1% Triton-X 100 for 15 minutes at 37°C and then blocked with blocking buffer (10% FBS + 0.05% Triton-X 100) for 1 hour at 37°C.

13.4 The cells are subjected to a-synuclein primary antibody at 1:100 dilution for 2 hours at 37°C followed by washing two times with 1X PBS to remove any unbound antibody.

13.5 The cells are then treated with secondary antibody for 2 hours at 37°C.

13.6 The cells are then washed two times with 1X PBS and mounted with DAPI + Mowiol.

13.7 The slides are stored at 4°C until imaging.

14 Confocal Microscopy

14.1 The cells are visualized using 63X oil immersion objective lens in Leica Sp8 laser scanning confocal microscope.

14.2 The 405 nm laser is used for DAPI and 633 nm laser is used to analyze Cy5 labelled TD or TD:Ser or A647 secondary antibody.

14.3 4-5 areas are images per condition along with their Z-stacks.

14.4 The further analysis is done using Fiji ImageJ software.

14.5 At least 30 cells are quantified, and the data is further plotted using GraphPad Prism software.