Non-Chromatographic Purification of Synthetic RNA Using Bio-Orthogonal Chemistry

Muhan He,[1] Xunshen Wu,[1] Song Mao,[1] Phensinee Haruehanroengra,[1] Irfan Khan,[1] Jia Sheng,[1] and Maksim Royzen[1,2]

[1]Department of Chemistry, University at Albany, SUNY, Albany, New York
[2]Corresponding author: *mroyzen@albany.edu*

Solid-phase synthesis of RNA oligonucleotides over 100 nt in length remains challenging due to the complexity of purification of the target strands from the failure sequences. This article describes a non-chromatographic procedure that will enable routine solid-phase synthesis and purification of long RNA strands. The optimized five-step process is based on bio-orthogonal inverse electron demand Diels-Alder chemistry between *trans*-cyclooctene (TCO) and tetrazine (Tz), and entails solid-phase synthesis of RNA on a photo-labile support. The target oligonucleotide strands are selectively tagged with Tz while on-support. After photocleavage from the solid support, the target oligonucleotide strands can be captured and purified from the failure sequences using immobilized TCO. The approach can be applied for purification of 76-nt long tRNA and 101-nt long sgRNA for CRISPR experiments. Purity of the isolated oligonucleotides should be evaluated using gel electrophoresis, while functional fidelity of the sgRNA should be confirmed using CRISPR-Cas9 experiments. © 2021 Wiley Periodicals LLC.

Basic Protocol: Five-step non-chromatographic purification of synthetic RNA oligonucleotides
Support Protocol 1: Synthesis of the components that are required for the non-chromatographic purification of long RNA oligonucleotides.
Support Protocol 2: Solid-phase RNA synthesis

Keywords: bio-orthogonal chemistry • oligonucleotide • purification • RNA • tetrazine • *trans*-cyclooctene

How to cite this article:
He, M., Wu, X., Mao, S., Haruehanroengra, P., Khan, I., Sheng, J., & Royzen, M. (2021). Non-chromatographic purification of synthetic RNA using bio-orthogonal chemistry. *Current Protocols*, *1,* e247. doi: 10.1002/cpz1.247

INTRODUCTION

Twenty-first century discoveries have created a strong need for robust solid-phase synthesis of RNA oligonucleotides that are over 100 nucleotides (nt) in length. One example is Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), perhaps the most effective approach for gene editing (Zheng, 2015) Solid-phase synthesis of 101-nt long single-guide RNA (sgRNA) allows sequence-specific incorporation of RNA modifications that can improve CRISPR efficiency and nuclease stability and reduce off-target activity (Yin et al., 2018).

CURRENT PROTOCOLS
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Current Protocols e247, Volume 1
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.247

Despite the strong need, solid-phase synthesis of RNAs that are 100-nt in length remains challenging and is seldom attempted. The limiting step of this otherwise highly optimized process is purification. The standard purification consists of either reversed-phase HPLC or preparative gel electrophoresis. Both are labor intensive, time consuming, and challenging procedures. The longer the RNA strand, the more challenging the purification procedure, because failure strands accumulate to some extent at every cycle of the solid-phase synthesis.

This article describes a non-chromatographic procedure that facilitates construction of RNA strands over 100 nt long, as schematically illustrated in Figure 1. The five-step purification procedure is based on bio-orthogonal inverse electron demand Diels-Alder (IEDDA) chemistry between *trans*-cyclooctene (TCO) and tetrazine (Tz) (Blackman, et al. 2008), allowing selective tagging and purification of structurally complex and increasingly long RNA strands to separate them from the failure strands that accrue during solid-phase synthesis. RNA synthesis is done on a CPG solid support modified with a photolabile linker. The linker does not impose any additional limitations on the standard solid-phase RNA synthesis. After the final synthetic cycle, our method takes advantage of the free 5′-OH group on the target strand, which provides an opportunity for selective bio-orthogonal tagging with Tz. Hydroxy groups of the failure strands are capped during each cycle of the solid-phase synthesis. Upon installing Tz on the target strand, oligonucleotides are cleaved from the solid support using UV irradiation. The

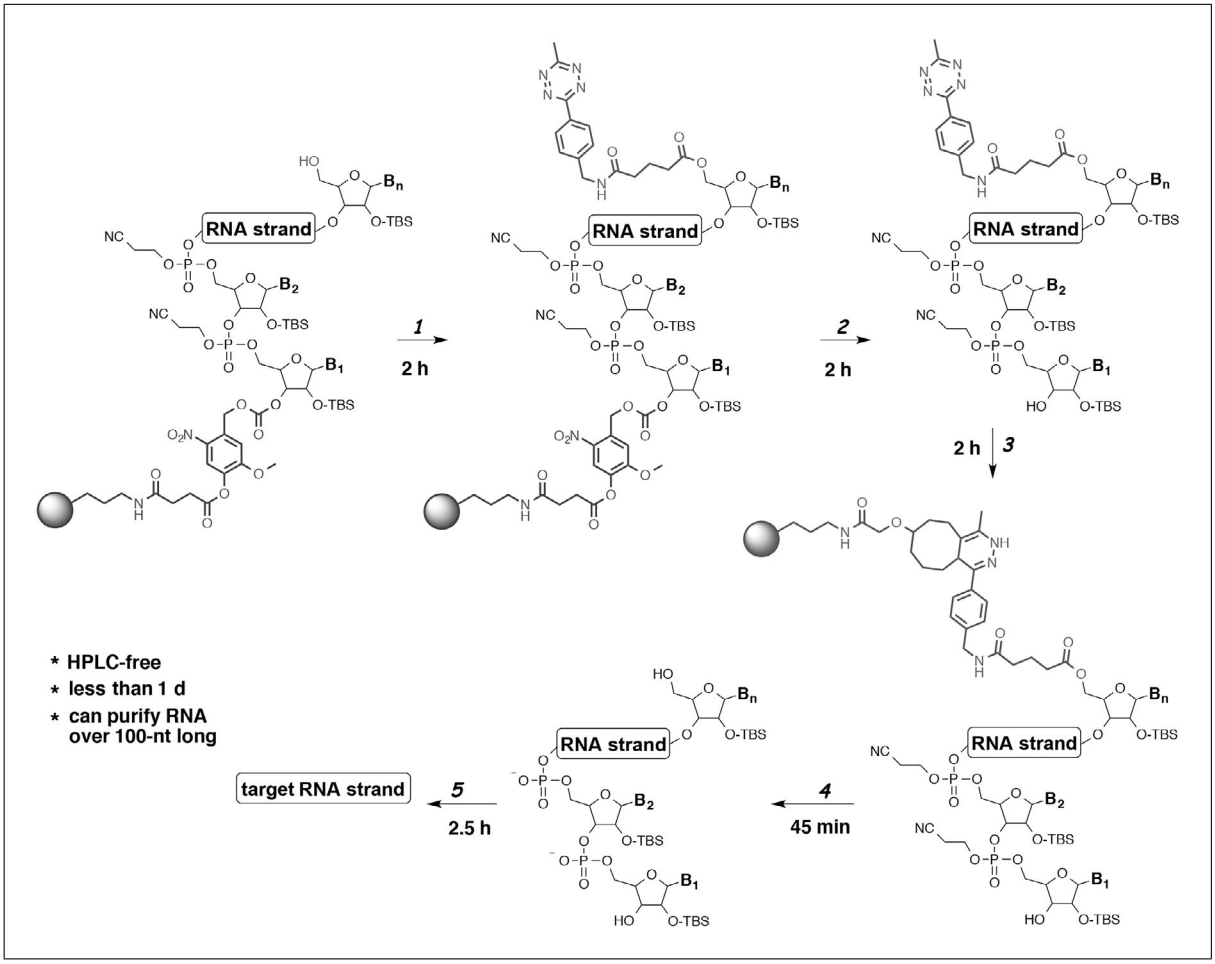

Figure 1 Non-chromatographic purification of synthetic RNA based on IEDDA chemistry. The outlined approach consists of five steps: (1) tagging of the target strand with Tz-anhydride; (2) photocleavage; (3) capture of the target strand with CPG-TCO; (4) deprotection; (5) desilylation and ethanol precipitation.

target strand is subsequently selectively immobilized using CPG modified with TCO, while all failure strands dissolved in the supernatant can be removed. Lastly, the target RNA strand is isolated using standard cleavage, deprotection, desilylation, and ethanol precipitation steps. This article describes the detailed protocol for the five-step process, which yields pure RNA strands that do not require any further HPLC purification. Support Protocol 1 describes chemical synthesis of the critical components necessary for the non-chromatographic RNA purification. Support Protocol 2 provides details of the solid-phase synthesis of RNA.

FIVE-STEP NON-CHROMATOGRAPHIC PURIFICATION OF SYNTHETIC RNA OLIGONUCLEOTIDES

BASIC PROTOCOL

This protocol describes a five-step procedure that allows purification of RNA oligonucleotides obtained from solid-phase RNA synthesis. The solid-phase synthesis has to be carried out using CPG support modified with a photolabile linker, which facilitates photolytic cleavage of the target RNA strand upon completion of the solid-phase synthesis. The purification procedure is based on bio-orthogonal chemistry between TCO and Tz, and utilizes reagents whose synthesis is described in Support Protocol 1.

Materials

RNA oligonucleotide obtained from standard solid-phase synthesis (*DMT-off*) using photolabile CPG support (see Support Protocols 1 and 2)
Freshly synthesized Tz-anhydride (see Support Protocol 1)
Diisopropylethylamine (DIPEA, anhydrous, 99.5%; Acros Organics)
4-Dimethylaminopyridine (DMAP, Reagent Plus, 99%; Sigma-Aldrich)
Methylene chloride (CH_2Cl_2, anhydrous, 99.9%; Acros Organics)
Acetonitrile (CH_3CN, anhydrous, 99.9%; Acros Organics)
TCO-CPG beads (Support Protocol 1)
AMA solution: 1:1 solution of aqueous methylamine (40% solution in water; Sigma-Aldrich) and aqueous ammonium hydroxide (28%-30% solution in water, Sigma-Aldrich)
Dimethylsulfoxide (DMSO, molecular biology grade; Sigma-Aldrich)
Triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$, 98%; Sigma-Aldrich)
Sodium acetate solution, 3 M, pH 5.5 (Invitrogen; ThermoFisher)
Ethanol (200 Proof; Spectrum)
MilliQ water

15-ml Falcon tubes (Corning, cat. no. 352196)
ThermoMixer C (Eppendorf)
Centrifuge 5418 R (Eppendorf)
SpeedVac vacuum concentrator and high-vacuum oil pump
Quartz cuvette with septum seal screw cap [Starna Cells, cat. no. GL14-S (*cuvette*); cat. no. GL14-S (*septum seal screw cap*)]
Micro stir bar
Nitrogen gas (tank with regulator)
Long and short syringe needles
Balloon to hold N_2 gas
Rayonet photochemical reactor (Rayonet, RPR-200), equipped with five UV lamps (Rayonet, RPR-3500A)
1.5-ml microcentrifuge tubes (Eppendorf)
NanoDrop microspecrophotometer

Additional reagents and equipment for HPLC (see Current Protocols article: Sinha & Jun, 2015) and PAGE of nucleic acids

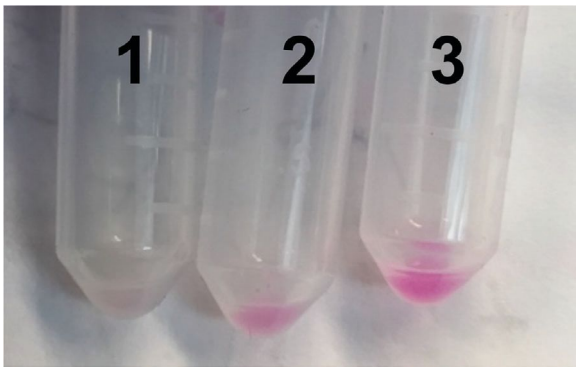

Figure 2 CPG beads after the tagging step: (1) unsuccessful tagging; (2) low-yielding tagging; (3) high-yielding tagging. The solid support becomes pink after conjugation to Tz.

Step 1: Tagging

The following steps will tag the 5´-end of a synthetic RNA with a Tz group.

1. Place the CPG beads from the 1-μmol solid-phase RNA synthesis (see Support Protocols 1 and 2) into a 15-ml Falcon tube. Add 7 ml of freshly prepared 10 mM solution of Tz-anhydride in CH_2Cl_2 to the Falcon tube. Add 50 μl of DIPEA and 10 mg of DMAP. Agitate at 1000 rpm at 25°C for 2 hr in a ThermoMixer.
2. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 2 ml anhydrous CH_2Cl_2. Repeat the process five more times and decant the last supernatant solution. Resuspend the beads in 2 ml of photocleavage solution (9:1 CH_3CN:H_2O). Repeat the process two more times and decant the last supernatant solution. Dry the CPG beads using SpeedVac.

   Upon successful tagging, the CPG beads should have a pink color, as illustrated in Figure 2.

Step 2: Photocleavage

The following steps will cleave the Tz-tagged RNA from the CPG solid support using UV light.

3. Make a suspension of CPG beads tagged with Tz in 3 ml of the photocleavage solution. Transfer the suspension into a 3.5-ml quartz cuvette. Add a micro stir bar to the quartz cuvette and seal using a cap with a silicon rubber septum.
4. Connect an N_2 balloon with the quartz cuvette through a long syringe needle and make sure there are no bubbles. Penetrate the septum with another short syringe needle. Position the short needle carefully so that the mixture is sparged with N_2, with medium bubbling for 15 min.
5. Place the quartz cell inside of Rayonet photochemical reactor equipped with a stirring plate and five RPR-3500A lamps. Ensure that the CPG beads are constantly stirred and the volume above the photocleavage solution is continuously sparged with N_2 during photolysis. Irradiate with 350 nm UV light for 2 hr.
6. Carefully decant the supernatant solution containing cleaved RNA oligonucleotides and concentrate the solution to 1 ml using SpeedVac.

Step 3: Capture of Tz-tagged RNA oligonucleotides

The following steps will selectively capture and immobilize the Tz-tagged RNA oligonucleotide using bioorthogonal IEDDA chemistry.

7. Add the concentrated supernatant solution from the photocleavage step to a microcentrifuge tube containing 100 mg TCO-CPG beads. Agitate at 1000 rpm, at 37°C for 2 hr.
8. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 200 μl of CH_3CN. Repeat the process two more times and combine all the supernatant solutions.
9. Dry the combined supernatant solution using SpeedVac.
10. Place the CPG beads in a 1.5-ml microcentrifuge tube and dry using SpeedVac.

Steps 4 and 5: Release and deprotection of full-length sequence

The following steps will cleave the captured RNA oligonucleotides from the CPG solid support and remove all nucleobase and 2´-OH protecting groups. The target RNA oligonucleotides are isolated by precipitation.

11. Add 800 μl AMA solution and agitate at 1000 rpm at 65°C for 45 min.

    This treatment will cleave the target RNA oligonucleotides from the CPG beads and remove the nucleobase protecting groups.

12. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Carefully remove the supernatant and evaporate the solvents using SpeedVac.
13. Dissolve the dry pellet in 100 μl of DMSO, add 125 μl of $Et_3N{\cdot}3HF$, and heat at 65°C for 2.5 hr.
14. Cool down the solution to room temperature. Precipitate the RNA with 25 μl of 3 M sodium acetate and 1 ml of cold ethanol at −20°C for 2 hr.

    Precipitation could also be done for 18 hr (overnight).

15. Centrifuge the microcentrifuge tube 15 min at 11,000 × *g*, 4°C. Carefully decant the supernatant and wash the pellet with 500 μl of 75% ethanol. Centrifuge 10 min at 11,000 × *g*, 4°C. Carefully remove the supernatant and dry the pellet using SpeedVac.
16. Resuspend the RNA pellet in 300 μl of MilliQ water. Check concentration using NanoDrop.
17. Confirm purity by PAGE and analytical HPLC (see Current Protocols article: Sinha & Jun, 2015).

SYNTHESIS OF THE COMPONENTS THAT ARE REQUIRED FOR THE NON-CHROMATOGRAPHIC PURIFICATION OF LONG RNA OLIGONUCLEOTIDES

SUPPORT PROTOCOL 1

This protocol describes the synthesis of: (**A**) CPG beads modified with a photolabile linker, which are used for the solid-phase synthesis of RNA oligonucleotides; (**B**) Tz-anhydride to be used as a tagging reagent; (**C**) TCO-CPG used to selectively capture Tz-tagged target RNA strands; and (**D**) Tz-DMT used to determine the loading of TCO-CPG beads (Fig. 3). These syntheses have previously been reported (He et al., 2021).

Materials

Compound **1** (Venkatesan & Greenberg, 1996)
N,N-Dimethylformamide (DMF, anhydrous, 99.8%; Acros Organics)
Native amino lcaa CPG 1000Å (ChemGenes)

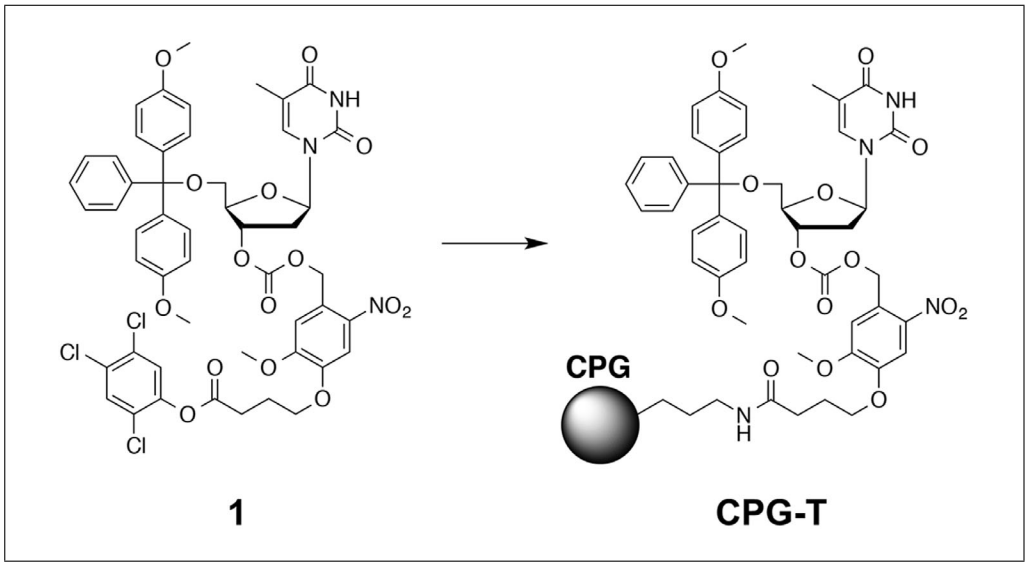

Figure 3 Synthesis of CPG-T.

1-Hydroxybenzotriazole hydrate (HOBt, 98%, containing 14% H_2O; Sigma-Aldrich)
Acetic anhydride (ACS reagent, 98%; Sigma-Aldrich)
4-Dimethylaminopyridine (DMAP, Reagent Plus, 99%, Sigma-Aldrich)
Pyridine (anhydrous, 99%; Acros Organics)
Ethyl acetate (EtOAc, anhydrous, 99.8%; Sigma-Aldrich)
Trichloroacetic acid (99%; Sigma-Aldrich)
Methylene chloride (CH_2Cl_2, anhydrous, 99.9%; Acros Organics)
Tetrazine (**2**) (Yang, Karver, Li, Sahu, & Devaraj, 2012)
Tetrahydrofuran (THF, anhydrous, 99.9%, Acros Organics)
Glutaric anhydride (95%; Sigma-Aldrich)
Diisopropylethylamine (DIPEA, anhydrous, 99.5%; Acros Organics)
Citric acid (anhydrous, 99%; Fisher Scientific)
Nitrogen gas (tank with regulator)
Sodium sulfate (Na_2SO_4, anhydrous, 99%; Fisher Scientific)
Silica gel [SiO_2, size 40-63 μm (230-400 mesh); Silicycle]
Methanol (MeOH; 99.9%, Sigma-Aldrich, cat. no. 646377)
N,*N*′-Dicyclohexylcarbodiimide (DCC; 99%, Sigma-Aldrich, cat. no. D80002)
trans-Cyclooctene (**5**) (Royzen, Yap, & Fox, 2008)
2,4,5-Trichlorophenol (95%; Sigma-Aldrich)
Heptane (liquid chromatography grade; Sigma-Aldrich, cat. no. 1043902500)
Dimethoxytrityl chloride (DMT-Cl; 95%, Sigma-Aldrich, cat. no. 100013)

15-ml Falcon tubes (Corning, cat. no. 352196)
ThermoMixer C (Eppendorf)
50-ml Falcon tubes (Corning, VWR cat. no. 89039-656)
SpeedVac vacuum concentrator and high vacuum oil pump
Rotavap (Buchi, Model R-124)
Centrifuge 5418 R (Eppendorf)
Cary 60 spectrophotometer (Agilent Instruments)
100- and 25-ml one-necked round-bottom flasks
Magnetic stir bars
Separatory funnel
Aluminum foil

Preparative silica TLC plates, 1000 μm thickness (Miles Scientific, cat. no. P02013)

Biotage Isolera One chromatography instrument

Additional reagents and equipment for flash chromatography (see Current Protocols article: Meyers, 2001), thin-layer chromatography (Meyers & Meyers, 2008), NMR, and HRMS

Synthesis of photolabile linker-T connected to CPG

1. Synthesize compound **1** using previously described procedure (Venkatesan & Greenberg, 1996). Confirm the correct structure using ^{1}H NMR.

   ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.58 (t, J = 2.4 Hz, 1H), 7.41 – 7.28 (m, 12H), 7.09 (s, 1H), 6.89 – 6.84 (m, 4H), 6.51 (dd, J = 9.3, 5.3 Hz, 1H), 5.61 (s, 2H), 5.43 (d, J = 5.7 Hz, 1H), 4.25 – 4.11 (m, 3H), 4.02 (s, 3H), 3.82 (s, 6H), 3.57 – 3.48 (m, 2H), 2.91 (t, J = 7.2 Hz, 2H), 2.54 – 2.30 (m, 4H), 1.40 (t, J = 6.1 Hz, 3H).

2. Add 6 ml anhydrous DMF to a 15-ml Falcon tube. Sequentially add 25 mg compound **1** (0.024 mmol), 800 mg native amino lcaa CPG 1000Å, and 3.2 mg HOBt (0.024 mmol).

3. Agitate at 1000 rpm for 18 hr at 25°C.

4. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 10 ml anhydrous DMF. Repeat the process two more times and decant the last supernatant solution.

5. Cap the unreacted amine groups of CPG with acetic anhydride. In a 50-ml Falcon tube, combine 5 ml acetic anhydride, 500 mg DMAP, and 40 ml pyridine. Agitate at 500 rpm for 1 hr at 25°C.

6. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 50 ml EtOAC. Repeat the process two more times and decant the last supernatant solution. Dry the CPG beads using Speedvac.

7. To determine loading, weigh out 5 mg of the modified CPG beads and add 10 ml of 3% trichloroacetic acid (w/w) in CH_2Cl_2. After 2 min, measure absorbance at 504 nm. Calculate the concentration of the released DMT group based on its extinction coefficient (e = 76 ml cm^{-1} μmol^{-1}).

Synthesis of the tagging reagent (Tz-anhydride; Fig. 4)

8. Synthesize the tetrazine **2** using previously described procedure (Yang et al., 2012).

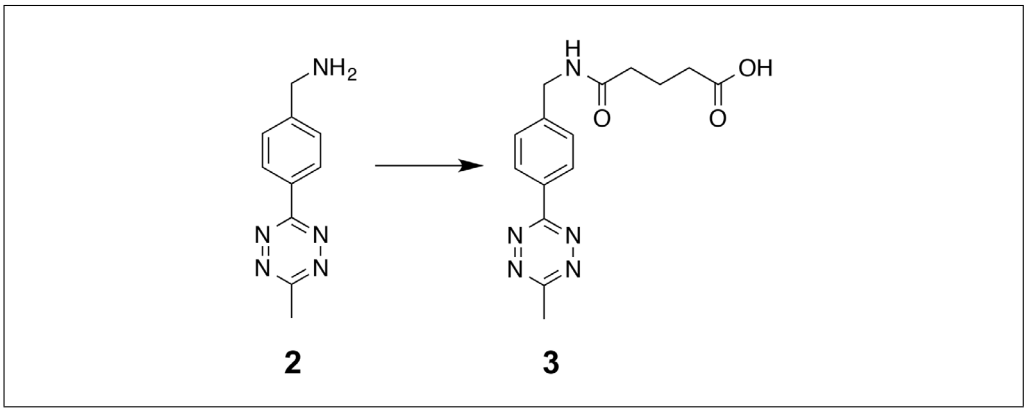

Figure 4 Synthesis of compound **3**.

9. In a 100-ml 1-necked round-bottom flask with a magnetic stirring bar, dissolve 300 mg compound **2** (1.5 mmol) in 10 ml THF. Add 205 mg glutaric anhydride (1.8 mmol). Dropwise add 775 mg DIPEA (6 mmol) over 5 min. Stir the reaction mixture at 25°C for 18 hr under N_2 atmosphere.

10. Dilute the reaction mixture with 100 ml CH_2Cl_2 and wash in a separatory funnel with 20 ml of 5% aqueous citric acid.

11. Extract the aqueous layer in the separatory funnel three times with 50 ml CH_2Cl_2. Combine the organic layers and dry with Na_2SO_4. Filter the supernatant by gravity.

12. Deposit the crude product on silica gel by adding 10 g of silica gel to the supernatant. Evaporate the solvents using Rotavap and reduced pressure.

13. Purify the product by silica gel flash chromatography (Isolera One; also see Current Protocols article: Meyers, 2001) using a gradient of MeOH in DCM (0%-20%).

    Use ACS-grade solvents.

    Yield = 0.330 g (70%).

14. Confirm that the correct product (**3**) was synthesized using NMR and HRMS:

    ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 – 8.38 (m, 2H), 7.59 – 7.49 (m, 2H), 4.39 (d, J = 6.0 Hz, 2H), 3.00 (s, 3H), 2.24 (dt, J = 9.4, 7.5 Hz, 4H), 1.83 – 1.70 (m, 2H).

    ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.65, 172.29, 167.54, 163.66, 145.01, 130.81, 128.51, 127.90, 34.86, 33.52, 21.30, 21.16.

    HRMS (ESI) Calc'd for $C_{15}H_{18}N_5O_3$ $[M+1]^+$ = 316.1404; found 316.1400.

15. Prepare fresh compound **4** before every oligonucleotide tagging experiment (Fig. 5) as described in the following steps.

16. In a 25-ml one-necked round-bottom flask with a magnetic stirring bar, dissolve 15 mg compound **3** (0.048 mmol), and 5 mg DCC (0.024 mmol) in 5 ml CH_2Cl_2. Stir the reaction mixture for 18 hr at 25°C under N_2 atmosphere.

17. Transfer reaction mixture into a 15-ml Falcon tube and centrifuge 1 min at 201 × *g*. Decant the supernatant and use it for the tagging step (Basic Protocol, step 1) without any further purification.

18. Confirm that the correct product (**4**) was synthesized using NMR. The compound is too unstable to be analyzed by HRMS.

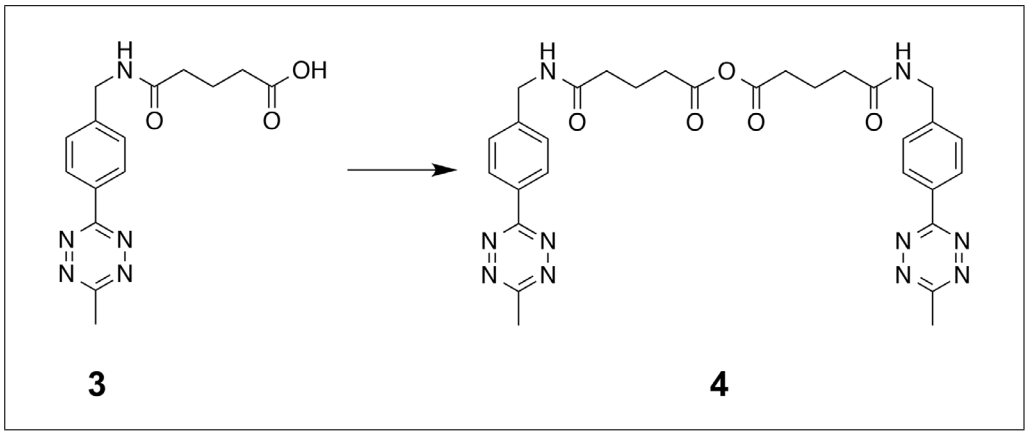

Figure 5 Synthesis of compound **4**.

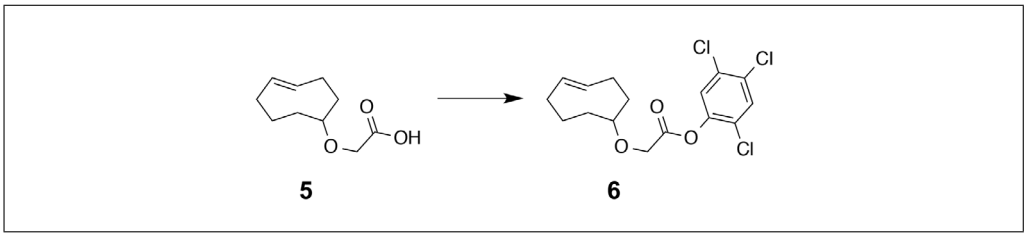

Figure 6 Synthesis of compound **6**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 8.47 – 8.40 (m, 2H), 7.59 – 7.45 (m, 2H), 4.39 (t, J = 5.2 Hz, 2H), 3.00 (d, J = 2.5 Hz, 3H), 2.40 – 2.14 (m, 3H), 1.70 – 1.56 (m, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 172.38, 172.00, 169.87, 167.54, 163.65, 144.92, 130.83,

128.53, 128.45, 127.90, 33.82, 25.80, 21.29.

Synthesis of CPG-TCO (Fig. 6)

19. Synthesize *trans*-cyclooctene **5** using previously described photoisomerization approach (Royzen et al., 2008).

20. In a 25-ml one-necked round-bottom flask with a magnetic stirring bar, dissolve 100 mg compound **5** (0.53 mmol) in 5 ml CH_2Cl_2. Add 156 mg 2,4,5-trichlorophenol (0.79 mmol) and 163 mg DCC (0.79 mmol). Stir the reaction mixture for 18 hr at 25°C under N_2 atmosphere.

21. Filter the precipitate using gravity and dilute the crude product with 50 ml CH_2Cl_2. Wash with 20 ml of water in a separatory funnel and dry the organic layer with Na_2SO_4.

22. Deposit the crude product on silica gel by adding 10 g of silica gel to the supernatant. Evaporate the solvents using Rotavap and reduced pressure.

23. Purify the product by flash chromatography using a gradient of EtOAc in heptane (see Current Protocols article: Meyers, 2001).

    Yield = 0.110 g (57%).

24. Confirm that the correct product (**6**) was synthesized using NMR and HRMS.

    ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.62 – 7.54 (m, 1H), 7.38 – 7.32 (m, 1H), 5.77 – 5.63 (m, 1H), 5.59 – 5.46 (m,

    1H), 4.42 – 4.32 (m, 2H), 3.78 (dd, J = 10.3, 4.8 Hz, 1H), 2.50 – 2.37 (m, 2H), 2.28 (ddd, J = 15.3, 9.2, 5.2 Hz, 2H), 2.00 – 1.73 (m, 4H), 1.62 – 1.55 (m, 2H).

    ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.26, 145.44, 135.93, 131.59, 131.15, 131.12, 125.27, 65.89,

    40.08, 34.45, 32.57, 29.72, 27.62.

    HRMS (ESI) Calc'd for $C_{16}H_{18}Cl_3O_3$ $[M+1]^+$ = 363.0316; found 363.0311.

25. Add 3 ml anhydrous DMF to 15-ml Falcon tube. Sequentially add 20 mg compound **6** (0.05 mmol), 400 mg native amino lcaa CPG 1000Å, and 5.1 mg HOBt (0.038 mmol). See Figure 7 for schematic.

26. Cover the Falcon tube with aluminum foil and agitate at 1000 rpm for 4 hr at 25°C.

27. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 10 ml anhydrous DMF. Repeat the process two more times and decant the last supernatant solution.

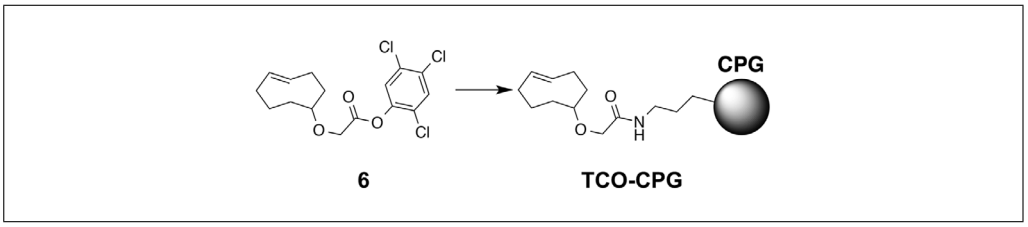

Figure 7 Synthesis of TCO-CPG.

28. Cap the unreacted amine groups of CPG with acetic anhydride. In a 50-ml Falcon tube combine 2.5 ml acetic anhydride, 250 mg DMAP, and 20 ml pyridine. Agitate at 500 rpm for 1 hr at 25°C.

29. Pellet the TPO-CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 50 ml EtOAC. Repeat the process two more times and decant the last supernatant solution. Dry the TPO-CPG beads using Speedvac.

30. To determine loading:

    a. Place 5 mg of TCO-CPG beads in 1.5-ml microcentrifuge tube. Add a solution of 5 mg of Tz-DMT (see steps 31-37, below) in 0.5 ml DMF. Agitate at 1000 rpm for 1 hr at 25°C.
    b. Pellet the CPG beads by centrifugation for 1 min at 201 × *g*. Decant the supernatant and resuspend the beads in 0.5 ml DMF. Repeat the process two more times and decant the last supernatant solution.
    c. Dry the CPG beads using Speedvac. Add 10 ml of the 3% trichloroacetic acid (w/w) in CH_2Cl_2.
    d. After 2 min, measure absorbance at 504 nm. Calculate the concentration of the released DMT group based on its extinction coefficient ($e = 76$ ml cm^{-1} μmol^{-1}).

31. Azeotrope compound **7** (0.63 mmol) with anhydrous pyridine to remove the residual moisture as follows. In a 25-ml 1-necked round-bottom flask with a magnetic stirring bar, dissolve 173 mg compound **5** (0.63 mmol) in 5 ml anhydrous pyridine. Rotavap under reduced pressure and leave under high vacuum for 18 hr. See schematic in Figure 8.

32. Add 257 mg DMT-Cl (0.76 mmol) and 20 ml pyridine. Stir the reaction mixture for 18 hr at 25°C under N_2 atmosphere.

33. Quench the reaction by adding 5 ml MeOH and stirring for 15 min.

34. Concentrate the reaction mixture by Rotavap under reduced pressure.

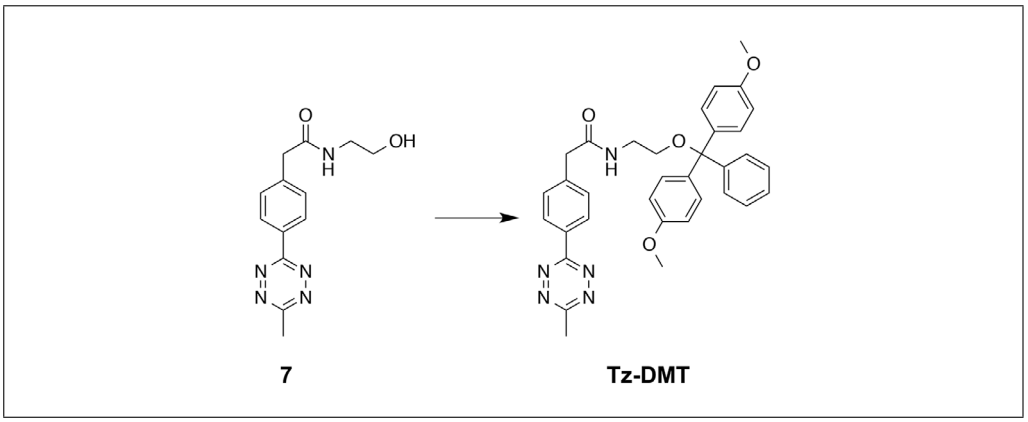

Figure 8 Synthesis of Tz-DMT.

35. Dissolve the crude product in 100 ml CH_2Cl_2. Wash in a separatory funnel with 20 ml of 5% (w/w) aqueous solution of citric acid, followed by 20 ml of water. Dry the organic layer with Na_2SO_4 and concentrate by Rotavap under reduced pressure.

36. Purify the product using preparative silica TLC (1000 μm thickness) using a 1:1 solution of EtOAc:heptane as a mobile phase.

    See Current Protocols article Meyers & Meyers (2008) for TLC protocols.

37. Confirm that the correct product (**Tz-DMT**) was synthesized using NMR and HRMS.

    ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.56 (t, J = 6.2 Hz, 2H), 7.53 – 7.45 (m, 2H), 7.34 – 7.13 (m, 9H), 6.88 – 6.73.

    (m, 4H), 4.13 (p, J = 7.0 Hz, 1H), 3.83 – 3.74 (m, 6H), 3.72 – 3.66 (m, 3H), 3.47 – 3.40 (m, 2H), 3.20 – 3.08 (m, 5H), 2.06 (s, 2H), 1.34 – 1.22 (m, 3H).

    ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.87, 167.27, 163.68, 158.45, 144.48, 139.71, 135.79, 130.97, 130.42, 129.80, 129.16, 128.51, 127.94, 127.85, 127.82, 127.80, 126.79, 113.11, 86.05, 77.32,

    77.07, 76.82, 61.67, 55.26, 55.21, 43.78, 39.70, 21.18.

    HRMS (ESI) Calc'd for $C_{34}H_{34}N_5O_4$ $[M+Na]^+$ = 598.2430; found 598.2411.

SOLID-PHASE RNA SYNTHESIS

SUPPORT PROTOCOL 2

This protocol describes the solid-phase synthesis of RNA oligonucleotides. The synthesis should be performed using commercial synthesizer equipment. The phosphoramidites used for the solid-phase synthesis should be TBDMS-protected at the 2′-OH group. Each synthetic cycle consists of activation, coupling, capping, and oxidation steps. The protocol describes the reagents and solvents used for each of these steps.

Materials

Control-pore glass (CPG-1000, Glen Research)
Phosphoramidites (TBDMS as the 2′-OH protecting group): rC is N-Ac protected and rG is N-iBu protected (ChemGenes)
Pac-A-CE phosphoramidite (Glen Research)
Activation reagent: 5-ethylthio-1H-tetrazole solution (0.25 M) in acetonitrile
Capping reagent: acetic anhydride/pyridine/THF 1/1/8 and 17.6% w/v *N*-methyl imidazole in acetonitrile
Oxidation step is done using I_2 (0.02 M) in THF/pyridine/H_2O solution
Detritylation step is done using 3% trichloroacetic acid in CH_2Cl_2
Oligo-800 synthesizer (Azco Biotech)

Solid-phase RNA synthesis

Perform all RNA oligonucleotide solid-phase syntheses on a 1.0-μmol scale using the Oligo-800 synthesizer using standard synthetic protocols. Perform solid-phase syntheses using control-pore glass (CPG-1000) modified with a photolabile linker, whose synthesis is described in Support Protocol 1. Carry out the detritylation steps using 3% trichloroacetic acid in CH_2Cl_2. Perform coupling of phosphoramidite monomers with 5-ethylthio-1H-tetrazole solution (0.25 M) in acetonitrile as the activator. Perform capping step with acetic anhydride. Carry out oxidation steps using I_2 (0.02 M) in THF/pyridine/H_2O solution.

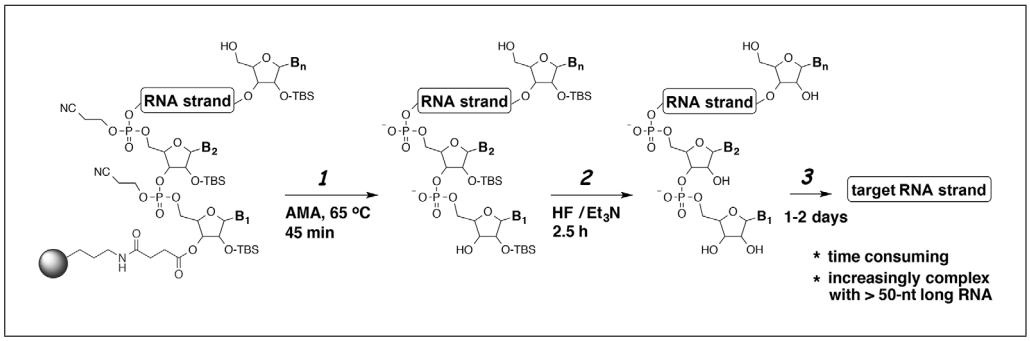

Figure 9 The three-step standard procedure for purification of synthetic RNA oligonucleotides: step 1, cleavage from CPG and deprotection; step 2, desilylation; step 3, HPLC purification.

COMMENTARY

Background Information

There is a strong need for a procedure that facilitates robust solid-phase synthesis and purification of RNA strands that are over 100 nt in length. Currently, solid-phase synthesis of RNAs that are >100 nt in length remains challenging and is rarely attempted (Taemaitree, Shivalingam, El-Sagheer, & Brown, 2019). The limiting step of the otherwise highly optimized process is purification, illustrated in Figure 3 (Grajkowski, Cieslak, & Beaucage, 2016). The standard purification process entails cleavage of oligonucleotides from the solid support and concomitant deprotection of the nucleobases using AMA solution (a 1:1 aqueous solution of methylamine and concentrated ammonium hydroxide). Subsequently, desilylation of the 2-hydroxy groups is done using a fluoride reagent, such as $Et_3N{\cdot}3HF$. After ethanol precipitation, the target RNA strands are purified using reversed-phase HPLC or preparative gel electrophoresis (see Fig. 9). The latter is the most labor-intensive, time-consuming, and challenging step. Longer RNA oligonucleotides become increasingly more challenging to purify from failure strands that accumulate to some extent at every step of the solid-phase synthesis.

Several non-chromatographic oligonucleotide purification approaches have been previously reported. The classical 'DMT-on' approach has been used for purification of oligonucleotides that are 20- to 30-nt long (Deshmukh, Cole, & Sanghvi, 2000). The large and hydrophobic DMT group serves as a handle for separation of DMT-protected (DMT-on) full-length oligonucleotides from the failure sequences. The Fang group has described an approach for capping the failure sequences with an acrylated phosphoramidite (Fang et al., 2011). The failure sequences are removed by subsequent polymerization. Bergstrom and co-workers described 5′-end biotinylation of synthetic RNAs (Fang et al., 2004). After cleavage and deprotection, the target strands can be isolated using NeutrAvidin-coated microspheres. Beaucage developed DNA strands that are modified with 5′-siloxyl ether linkers. Synthetic DNA can be captured through an oximation reaction with aminopropylated silica gel (Grajkowski et al., 2016). None of these approaches have been successfully applied to purify 100-nt long RNA strands that present a substantially higher degree of complexity than the ones described.

This article describes protocols that allow robust construction of RNA strands that are over 100-nt in length. The ultimate goal is to convince the RNA community that our approach can expand the scope of solid-phase RNA synthesis toward longer and more complex oligonucleotides. The described methodology is based on the bio-orthogonal reaction between *trans*-cyclooctene and tetrazine, which are highly selective for each other and have minimal cross-reactivity with other functional groups found in RNA. As a result, lEDDA chemistry does not interfere with the intricate structural elements of RNA.

The bio-orthogonal click chemistry is highly efficient, even at very low concentrations of TCO and Tz. Thus, it can still be effectively applied toward isolation of long synthetic RNA strands obtained in low overall yield.

Critical Parameters and Troubleshooting

The tagging reagent, Tz-anhydride, is highly unstable. It should be freshly synthesized ahead of the purification procedure and used without prolonged storage. While synthesizing CPG, loading of over 30 should be avoided, as that will result in lower purification

yields. Both CPG-T and CPG-TCO should be stored at −20°C in the dark.

Synthesis of compound **6** is low-yielding because of the inherent instability of TCO. To maximize the yield, TCO compounds should be protected from direct exposure to strong light during the reaction and purification. The TCO-containing compounds should be stored at −20°C.

Anticipated Results

The described protocols have been utilized for purification of 76-nt long lysine transfer RNA. The solid-phase synthesis was carried out on 1-µmol scale and the target 76-nt long RNA strand was isolated at 15.4% (77 nmol) with over 95% purity. The described protocol has also been utilized toward purification of 101-nt long sgRNA for CRISPR experiments. The solid-phase synthesis was again carried out on 1-µmol scale, and the target 101-nt long RNA strand was isolated at 10% (50 nmol) with over 95% purity. The isolated RNA would be sufficient for over 800 CRISPR experiments.

Time Considerations

The non-chromatographic RNA purification protocol consists of five steps. Their duration is:

(1) Tagging of the target strand with Tz - anhydride: 2 hr

(2) Photocleavage: 2 hr

(3) Capture of the target strand with CPG-TCO: 2 hr

(4) Deprotection: 45 min

(5) Desilylation and ethanol precipitation: 2.5 hr.

Acknowledgments

This work was supported by the NIH grant R21CA228997-01 to M.R., the NSF grant 1664577 to M.R., and the NSF MRI grant 1726724 to SUNY Albany. The authors would like to thank Prof. Greenberg (Johns Hopkins University) for his suggestions regarding synthesis of the photolabile linker.

Author Contributions

Muhan He: conceptualization, data curation, investigation, writing original draft; **Xunshen Wu**: data curation, investigation; **Song Mao**: validation; **Phensinee Haruehanroengra**: validation; **Irfan Khan**: formal analysis, validation; **Jia Sheng**: supervision, validation; **Maksim Royzen**: funding acquisition, supervision, validation, writing review and editing.

Conflict of Interest

There are no conflicts of interest to declare.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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