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Nucleic Acid Crystallography via Direct Selenium Derivatization: RNAs Modified with Se-Nucleobases

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Abstract

Selenium-derivatized RNAs are powerful tools for structure and function studies of RNAs and their protein complexes. By taking the advantage of selenium modifications, researchers can determine novel RNA structures via convenient SAD and MAD phasing. As one of the naturally occurring tRNA modifications, 2-seleno-uridine, which presents almost exclusively at the wobble position of anticodon loop in various bacterial tRNAs (Ching et al., *Proc Natl Acad Sci U S A* 82:347, 1985; Dunin-Horkawicz et al., *Nucleic Acids Res* 34:D145–D149, 2006), becomes one of the most promising modifications for crystallographic studies. Our previous studies have demonstrated many unique properties of 2-seleno-uridine, including stability (Sun et al., *RNA* 19:1309–1314, 2013), minimal structural perturbation (Sun et al., *Nucleic Acids Res* 40:5171–5179, 2012), and enhanced base-pairing fidelity (Sun et al., *Nucleic Acids Res* 40:5171–5179, 2012). In this protocol, we present the efficient chemical synthesis of 2-seleno-uridine triphosphate (^{Se}UTP) and the facile transcription and purification of ^{Se}U-containing RNAs (^{Se}U-RNA).

Keywords

Selenium RNA; 2-Se-uridine triphosphate; SAD and MAD phasing; In vitro transcription

1 Introduction

RNA plays central roles in multiple cellular processes, including genetic information storage, transcription, translation [1], regulation [2] as well as catalysis [3, 4]. The multiple functions of RNAs require highly diversified 3D structures. Thus, elucidating the 3D structures of RNAs will help understanding the RNA functions and the biological processes. X-ray crystallography is one of the most widely used methods for high-resolution structure determination of RNAs and their protein complexes. In addition to crystallization, however, phase problem remains the major challenge in crystallographic studies. Scientists have discovered that large atoms, such as selenium (Se), can serve as anomalous scattering centers for MAD (Multiwavelength anomalous dispersion) and/or SAD (Single-wavelength anomalous dispersion) phasing. Seleno-Met derivatization has been successfully applied for structure determination of novel proteins [5] and nucleic acid–protein complexes [6]. Due to its chemical and biophysical similarity to oxygen and sulfur, selenium atom can be covalently introduced into proteins (yielding Se-Met protein) as well as nucleic acids

(yielding Se-NA) by replacing sulfur or oxygen atoms. Huang laboratory has pioneered and developed nucleic acid derivatization and X-ray crystallography with selenium modifications of nucleic acids [7–10]. Among all Se-derivatized nucleic acids, 2-Se-uridine is the only naturally occurring modification. Extensive experimental data show that the 2-seleno-modification has high stability [11] as well as virtually identical structure to the native in RNA structure [12]. One economical and efficient method to incorporate multiple ^{Se}Us into a RNA molecule is through in vitro transcription, since this strategy is well established and favored by most biology laboratories. Here, we present a facile and high-yield synthetic method for 2-selenouridine-triphosphate (^{Se}UTP) and in vitro transcription of ^{Se}U-containing RNAs (Scheme 1).

2 Materials

2.1 ^{Se}UTP Synthesis

1. Round bottom flasks (10 mL).
2. Stir bars.
3. Syringes (1 mL).
4. Needles (23G × 1½ in).
5. Magnetic stir plate.
6. Balloons.
7. Nitrogen gas.
8. Vacuum pump.
9. Rubber septa for 10 mL round bottom flasks.
10. Parafilm.
11. Ice bath.
12. Thin layer chromatography (250 µm).
13. UV lamp.
14. Digital balance.
15. Support stand with extension clamp.
16. Laboratory freezers (−80 °C).
17. High-speed centrifuge tubes (50 mL).
18. Laboratory bench-top centrifuge.
19. High-performance liquid chromatography (HPLC) system.
20. Reverse phase C-18 column (*see* Note 1).

¹We use Ultimate XB-C18 250 mm × 4.6 mm, 5 µm reverse phase columns.

21. Lyophilizer.
22. 1-(5'-*O*-4,4'-dimethoxytrityl- β -D-ribofuranosyl)-2-selenouridine.
23. Trifluoroacetic acid.
24. Dichloromethane.
25. Methanol.
26. Trimethyl phosphate.
27. Proton-sponge (Sigma-Aldrich, Saint Louis, MI, USA).
28. Phosphorus oxychloride (POCl₃).
29. Tributylammonium pyrophosphate.
30. Tributylamine (TBA).
31. Dimethylformamide (DMF).
32. Triethylammonium bicarbonate (1 M).
33. 3 M NaCl.
34. Ethanol, 99.8 %.
35. Buffer A: 10 mM triethylamine-acetic acid (TEAAc) buffer solution in deionized, pH 7.4.
36. Buffer B: 10 mM TEAAc buffer solution in 50 % acetonitrile and deionized water (v/v), pH 7.4.

2.2 ^{Se}U-RNA Transcription

1. T7 RNA polymerase (*see* Note 2).
2. 10× Reaction buffer for transcription: 400 mM Tris-HCl (pH 7.5), 60 mM MgCl₂, 100 mM NaCl, 20 mM spermidine.
3. 100 mM ATP, CTP, GTP, and UTP solutions. UTP is substituted with ^{Se}UTP in ^{Se}U-RNA transcription experiment.
4. 100 mM DTT.
5. 100 mM MgCl₂.
6. RNase-free water.
7. Centrifuge tubes, 0.5 mL, sterile.
8. Pipettes (adjustable-volume).
9. Sterile pipette tips.

²We use commercially available AmpliScribe™ T7-Flash™ enzyme solution and this enzyme is included in the AmpliScribe™ T7-Flash™ Transcription Kit (Epicentre, Madison, WI, USA).

10. Transcription template. Linearized plasmid templates are applied in self-cleaving mutant and wild-type hammerhead-ribozyme transcription (50 ng/μL) and a double-stranded DNA template (55 nucleotides) is applied in non-self-cleaving hammerhead-ribozyme transcription (1 μM).
11. A heating block set at 37 °C.

2.3 Polyacrylamide Gel Electrophoresis for Se-RNA Purification

1. Acrylamide/bis-acrylamide (19:1), 40 % (w/v) solution. This solution is commercially available at many major scientific supply companies.
2. 5× TBE (Tris-Borate-EDTA) solution. This solution is commercially available at many major scientific supply companies.
3. 10 % (w/v) ammonium persulfate solution (APS) in water. Store at 4 °C.
4. *N,N,N,N*-tetramethyl-ethylenediamine (TEMED). Store at 4 °C.
5. Necessary polyacrylamide gel electrophoresis (PAGE) apparatus.
6. Gel loading-dye (pH 8.0): 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 0.2 M EDTA, 50 % glycerol, water.
7. Preparative high-speed centrifuge tubes (50 mL) sterile and preparative centrifuge.
8. UV lamp.
9. 3 M sodium chloride (NaCl) solution.
10. Ethanol, 99.8 %.
11. Laboratory freezers (−80 °C) and refrigerator (4 °C).

3 Methods

3.1 Selenouridine Nucleoside Synthesis and Purification

The synthesis of 2-selenouridine nucleoside was performed under argon. All organic solvents in this experiment should be redistilled, and all solid reagents should be dried under reduced pressure prior to use.

1. Weigh out 1-(5'-*O*-4,4'-dimethoxytrityl-β-D-ribofuranosyl)-2-selenouridine (compound **1**, Scheme 1, 0.4 mmol) and place in a 10 mL round bottom flask together with a stir bar.
2. Seal the round bottle flask with septa and seal the septa with parafilm.
3. Attach the flask to a high vacuum to dry the sample through a clean needle for 1 h.
4. Attach a balloon to a syringe and then inflate the balloon with argon.
5. Remove the round bottom flask from the high vacuum and quickly insert the balloon to it through a clean needle to fill the whole flask with argon.

6. Securely place the round bottom flask above a magnetic stir plate, add 3 mL of dichloromethane into the reaction through a clean syringe and stir vigorously.
7. Inject trifluoroacetic acid dropwisely into the flask via a clean syringe (an immediate orange color should be observed once the acid is added) until the reaction pH reach 4 (Fig. 1).
8. Monitor the reaction for 1 h and check it with TLC plates using 10 % methanol in dichloromethane as eluent and use uridine sample as a reference to the 2-Se-uridine product (Fig. 2).
9. After the starting material is completely converted to 2-Seuridine product, slowly add methanol into the reaction drop by drop to quench the dimethoxytrityl group until the orange color entirely disappears (*see* Note 3).
10. The reaction solution is transferred into a high-speed centrifuge tubes (15 mL) and another 3 to 4 mL of dichloromethane is added into the same centrifuge tube, shake gently to dissolve impurity.
11. Centrifuge the suspension and the supernatant is removed.
12. Re-dissolve the pellet with a minimal amount of methanol and add another 5 mL of dichloromethane to re-precipitate the product and remove the by-products.
13. Repeat procedure 12 two times to obtain a pure 2-Se-uridine product.

3.2 Selenouridine Triphosphate Synthesis and Purification

The synthesis of 2-selenouridine triphosphate was performed under argon. All organic solvents in this experiment should be anhydrous, and all solid reagents should be dried under reduced pressure prior to use.

1. Weigh out 2-selenouridine nucleoside (compound 2, Scheme 1, 0.35 mmol), proton sponge (0.7 mmol) and tributylammonium pyrophosphate (0.53 mmol) and place each compound into separate round bottom flasks (10 mL).
2. Place a stir bar into each flask and cap with a dried septum. Use parafilm to wrap around the septa and make sure the flask is sealed properly.
3. Attach the flasks to high vacuum via a needle and dry the compounds for 2 h.
4. Remove the 2-selenouridine nucleoside flask (Flask 1) from the vacuum hose first and quickly insert argon balloon into the flask through a needle to fill the flask with argon gas.
5. Add 0.6 mL of trimethylphosphate into Flask 1 (Fig. 3) to dissolve the 2-selenouridine nucleoside (compound 2) and stir vigorously.
6. Prepare an ice bath and place it on the top of a magnetic stir plate.
7. Place Flask 1 in the ice bath securely (*see* Fig. 3 for the reaction setup) and make sure the septa area of the flask is above the ice.

³A white suspension is observed, indicating the formation of 2-Se-uridine product.

8. Remove the proton sponge flask (Flask 2) from the vacuum hose next and quickly insert argon balloon into it through a needle to fill the flask with argon gas.
9. Add 0.6 mL of trimethylphosphate into Flask 2 (Fig. 3) to completely dissolve the proton sponge.
10. Use a clean syringe to transfer the dissolved proton sponge from Flask 2 into Flask 1 and stir the mixture vigorously for 30 min on ice bath (**step 1**, Fig. 3).
11. Add phosphorus oxychloride (POCl_3 , 0.7 mmol, 0.065 mL) into Flask 1 reaction mixture dropwisely via a clean syringe and stir vigorously for 30 min in ice bath (**step 2**, Fig. 3).
12. After 30 min of stirring of the reaction mixture, check the formation of intermediate **3** with TLC plates by using 15 % methanol in dichloromethane as eluent. The TLC plate set up is shown in Fig. 4.
13. Remove the tributylammonium pyrophosphate flask (Flask 3) from the vacuum hose and quickly insert argon balloon into it through a needle to fill the flask with argon gas.
14. Dissolve the tributylammonium pyrophosphate in DMF (0.6 mL) completely and then add TBA (0.3 mL), mix well. Use a clean syringe to quickly inject the mixture from Flask 3 into the reaction (Flask 1) and stir vigorously in ice bath for 15 min (**step 3**, Fig. 3).
15. Add water (4 mL) to the reaction mixture (Flask 1) to hydrolyze compound **4** to final product 2-selenouridine triphosphate (compound **5**, **step 4**) and let the reaction continuously stir for another 3 h at room temperature.
16. Check the formation of final product 2-selenouridine triphosphate (compound **5**) by TLC plate, using isopropanol:ammonium hydroxide:water (5:3:2) as eluent and use uridine triphosphate (native UTP) sample as a comparison to the 2-Se-uridine triphosphate product. The TLC plate set up is shown in Fig. 5.
17. After the reaction, open the reaction flask (Flask 1) and transfer the reaction mixture into a clean centrifuge tube (50 mL).
18. Add 0.68 mL 3 M NaCl and 20 mL ethanol into the crude $^{\text{Se}}$ UTP, cap the centrifuge, use parafilm to seal and shake the tube to mix completely.
19. Place the tube into $-80\text{ }^{\circ}\text{C}$ freezer for half an hour and use centrifuge ($10,000 \times g$) to collect $^{\text{Se}}$ UTP sample (yellow pellet).
20. Remove the supernatant from the tube and let it air dry. Use water ($\sim 200\text{ }\mu\text{L}$) to re-dissolve the $^{\text{Se}}$ UTP yellow pellet.
21. The final concentration of $^{\text{Se}}$ UTP is measured and adjusted via UV-Vis to 100 mM for RNA transcription.
22. To analyze the purity of the synthesized $^{\text{Se}}$ UTP (compound **5**, Scheme 2), we use RP-HPLC and C18 column. The HPLC analysis was performed with a gradient

from 100 % buffer A (20 mM triethylammonium acetate in water) to 40 % buffer B (20 mM triethylammonium acetate in 50 % acetonitrile and 50 % water) in 15 min. The ^{Se}UTP HPLC analysis compared with native UTP is shown in Fig. 6.

3.3 2-Se-RNA Transcription

1. Add all the reaction components into a centrifuge tube (0.5 mL, sterile) and mix well, see Table 1. The total volume of the reaction is 100 μ L.
2. Place the reaction tube in the heating block (37 °C) and incubate for 3 h.
3. Heat inactivate T7 RNA polymerase at 70 °C for 10 min.
4. Transfer the transcribed ^{Se}U-RNA to a spin column to isolate transcribed RNA from unreacted NTPs.
5. Use a clean sterile tube to store the crude ^{Se}U-RNA.

3.4 Gel Purification of 2-Se-RNA

1. Mix the crude ^{Se}U-RNA with the gel loading-dye (1:1, v/v) and load the sample on a preparative gel.
2. Let the dyes run to proper positions and remove the gel from gel electrophoresis apparatus and place it on a flat surface.
3. Remove the glass plate from one side of the PAGE gel and place a layer of plastic wrap on top of it.
4. Place a preparative or analytical TLC plate on top of the plastic wrap (the silica-coated side faces the plastic wrap) and flip the entire glass plates over.
5. Carefully remove the remaining glass plate from the PAGE gel with a spatula.
6. Use a UV lamp to visualize the gel and use a clean knife to carefully cut off the RNA band.
7. Transfer the cutted gel to a clean tube and grind the gel into fine pieces.
8. Add RNase-free water into the tube (2 $\times$ gel volume) and soak the gel by rotating the tube for 4 h at room temperature. Collect the liquid and soak the gel again.
9. Centrifuge the collected and combined suspension at 1,000 $\times g$ for 10 min and transfer the supernatant to another tube.
10. Filter the supernatant with a 0.2 μ m nylon syringe filter to remove fine gel particles.
11. RNA was precipitated by NaCl/ethanol precipitation (following the procedure in Subheading 3.2, **steps 17–20**) to obtain a purified 2-Se-RNA. If the RNA volume is too big, lyophilize it (to reduce the volume) first before NaCl/ethanol precipitation.
12. Redissolve the ^{Se}U-RNA pellet with RNase-free water and quantify the RNA by UV–Vis absorption at 260 or 307 nm.

13. Store the ^{76}Se -RNA sample in $-80\text{ }^{\circ}\text{C}$ freezer. For longer-term storage, the ^{76}Se -RNA can be stored after lyophilization.

References

1. Watson JD. Involvement of RNA in the synthesis of proteins. *Science*. 1963; 140:17–26. [PubMed: 13999211]
2. Serganov A, Patel DJ. Ribozymes, riboswitches and beyond: regulation of gene expression without proteins. *Nat Rev Genet*. 2007; 8:776–790. [PubMed: 17846637]
3. Schmeing TM, Ramakrishnan V. What recent ribosome structures have revealed about the mechanism of translation. *Nature*. 2009; 461:1234–1242. [PubMed: 19838167]
4. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell*. 2009; 136:629–641. [PubMed: 19239885]
5. Hendrickson WA, Horton JR, LeMaster DM. Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. *EMBO J*. 1990; 9:1665. [PubMed: 2184035]
6. Ferre-D'Amare AR, Zhou K, Doudna JA. Crystal structure of a hepatitis delta virus ribozyme. *Nature*. 1998; 395:567–574. [PubMed: 9783582]
7. Carrasco N, Ginsburg D, Du Q, Huang Z. Synthesis of selenium-derivatized nucleosides and oligonucleotides for X-ray crystallography. *Nucleosides Nucleotides Nucleic Acids*. 2001; 20:1723–1734. [PubMed: 11580197]
8. Caton-Williams J, Huang Z. Biochemistry of selenium-derivatized naturally occurring and unnatural nucleic acids. *Chem Biodivers*. 2008; 5:396–407. [PubMed: 18357549]
9. Sheng J, Huang Z. Selenium derivatization of nucleic acids for X-Ray crystal-structure and function studies. *Chem Biodivers*. 2010; 7:753–785. [PubMed: 20397215]
10. Lin L, Sheng J, Huang Z. Nucleic acid X-ray crystallography via direct selenium derivatization. *Chem Soc Rev*. 2011; 40:4591–4602. [PubMed: 21666919]
11. Sun H, Jiang S, Caton-Williams J, Liu H, Huang Z. 2-Selenouridine triphosphate synthesis and Se-RNA transcription. *RNA*. 2013; 19:1309–1314. [PubMed: 23887148]
12. Sun H, Sheng J, Hassan AEA, Jiang S, Gan J, Huang Z. Novel RNA base pair with higher specificity using single selenium atom. *Nucleic Acids Res*. 2012; 40:5171–5179. [PubMed: 22323523]

Fig. 1.
Illustration of apparatus for selenouridine nucleoside synthesis

Fig. 2.
Illustration of TLC analysis of selenouridine product, starting material and uridine (comparison)

Fig. 3.

Illustration of the synthesis of 2-selenouridine triphosphate, includes four steps, reagents and reaction time

Fig. 4.
Illustration of TLC analysis of intermediate phosphorodichloridate **3**, starting material and proton sponge

Fig. 5.
Illustration of TLC analysis of 2-selenouridine triphosphate and native UTP (comparison)

Fig. 6. HPLC profiles: native UTP monitored at 260 and 307 nm (retention time: 11.2 min); 2-^{Se}UTP monitored at 260 and 307 nm (retention time: 14.1 min); coinjection of both native UTP and ^{Se}UTP monitored at 260 and 307 nm (retention time: 11.2 min and 14.1 min)

Scheme 1.

Chemical synthesis of $^{76}\text{SeUTP}$ [1] and transcription of RNA containing ^{76}SeU [2]. **(a)** 4 % trifluoroacetic acid; **(b)** POCl_3 , Me_3PO ; (tri-*n*-butyl) amine, pyrophosphate, *N,N*-dimethylformamide; the H_2O hydrolysis; **(c)** RNA transcription

Scheme 2.

Chemical synthesis of ^{Se}UTP [1]. (1) Proton sponge, Me₃PO₄; (2) POCl₃, Me₃PO₄; (3) tributylammonium pyrophosphate, TBA, DMF; (4) H₂O hydrolysis

Table 1

Reaction components of the RNA transcription

10× Reaction buffer	10 µL
100 mM Dithiothreitol (DTT)	10 µL
100 mM ATP	5 µL
100 mM CTP	5 µL
100 mM GTP	5 µL
100 mM 2- ^{sc} UTP	10 µL
Inorganic pyrophosphatase (0.1 U/µL)	4 µL
100 mM MgCl ₂	5 µL
DNA template (200 ng/µL)	10 µL
T7 RNA polymerase	10 µL
Water (RNase-free)	26 µL