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Synthesis of symmetrical thiol-adenosine conjugate and 5′ thiol-RNA preparation by efficient one-step transcription

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ABSTRACT

A symmetrical transcription initiator containing two adenosines and conjugated thiol functionality (ThioAMP dimer) is chemically synthesized. Transcription in the presence of ThioAMP dimer under the T7 Φ2.5 promoter yields 5′ thiol-labeled RNA (5′ HS-RNA) with up to 90% labeling efficiency, depending on the concentration ratio of ThioAMP dimer to ATP. The resulting 5′ HS-RNA may be used directly or after thiopropyl Separose 6B affinity column purification. Biotinylation of 5′ HS-RNA and formation of gold nanoparticle-RNA nanoplexes are demonstrated.

Functionally derivatized RNAs have broad applications in chemistry and biomedical research.[1–6] 5′ Thiol-labeled RNA (5′ HS-RNA) may be used to immobilized RNA on thiopropyl Separose 6B affinity resins[1] and on gold surfaces.[7,8] Biotin-labeled RNA may be prepared from 5′ HS-RNA.[1,4] Recently, 5′ HS-RNAs have been used to construct gold nanoparticle-siRNA (AuNP-siRNA) nanoplexes.[9] In addition, 5′ thiol-functionalized RNA may be used to conjugate with other macromolecules.[10–15]

5′ HS-RNA may be prepared by either chemical synthesis via phosphoramidite chemistry,[8,10,11,9,12–15] in vitro transcription,[7,1–3,16–21] or polynucleotide-catalyzed thiophosphorylation.[8] For small RNA sizes, 5′ thiol-labeling of RNA by chemical synthesis is simple and commercially available. However, RNA synthesis and 5′ thiol-labeling by phosphoramidite chemistry become impractical when RNA sizes exceed a certain limit, typically around 60 nucleotides (nt), due to low yields of full RNA sequences and high costs of chemical synthesis. On the contrary, 5′ thiol-labeling of RNA by in vitro transcription is fast, relatively inexpensive for small quantities, and unrestricted from RNA size limit. 5′ Thiol-labeling of RNA may be achieved by transcription under either the T7 class II promoter (Φ2.5) or the class III promoter (Φ6.5). The former uses a thiol-conjugated transcription initiator containing an adenosine,[1–3] while the latter utilizes thiol-containing guanosine derivatives.[7,16–21]

We have previously demonstrated that transcription under the T7 Φ2.5 produces RNA transcripts that are superior to those prepared under the T7 Φ6.5.[22] Taking the advantage of the T7 Φ2.5 promoter, we have synthesized a number of adenosine-containing transcription initiators that have been successfully used to label the 5′ end of RNA with high efficiencies.[3,6,5,23] Herein we report the synthesis of a symmetrical transcription initiator (**4**) that contains two adenosines and a disulfide bond. Under transcription conditions, the disulfide bond is reduced to a free thiol group. Therefore, the resulting RNA transcript (**5**) is conjugated by a free thiol group at the 5′ end. Depending on the concentration ratio of the transcription initiator and ATP, 5′ thiol-labeling of RNA can reach 90% labeling efficiency. The resulting thiol-labeled RNA may be used directly in applications after a simple purification step by membrane filtration. Additionally, thiol-labeled RNA may be enriched to near 100% purity by thiopropyl column purification.[1] Following thiol-RNA preparation, we show that the RNA can be further modified by biotin through the imidazole-catalyzed thioesterization reaction.[4] Finally, we demonstrate the utility of our thiol-labeled RNA for the preparation AuNP-RNA nanoplexes.

Synthesis of a symmetrical transcription initiator—ThioAMP dimer (**4**). Starting with 6-aminohexanethiol (**1**, Dojindo Molecular Technologies), the transcription initiator ThioAMP dimer (**4**) was synthesized in two chemical steps, as shown in Scheme 1. In the first step, **1** was oxidized to form dithiobis(6-hexaneamine) (**2**) by 0.5 molar equivalent of hydrogen peroxide oxidation in water, pH 8. Extraction by dichloromethane followed by solvent removal led to a white powder **2** (90% recovered yield), whose structure was confirmed by NMR and MS: ^{1}H NMR (400 MHz, DMSO-d_6), δ = 1.248–1.647 (m, 20H, S–C–C_4H_8–C–NH_2), 2.499 (t, 4H, J = 7.2 Hz, S–CH_2), 2.691 (t, 4H, J = 7.2 Hz, N–CH_2), MS calcd for $C_{12}H_{28}N_2S_2$ $[M+H]^+$ 265.18, found 265.17 (ESI).

In the second step, **2** was incubated at room temperature with 4 molar equivalent of adenosine 5′-phosphorimidazolide (ImdAMP, **3**)[24] in DMF for 8 days to afford the symmetrical transcrip-

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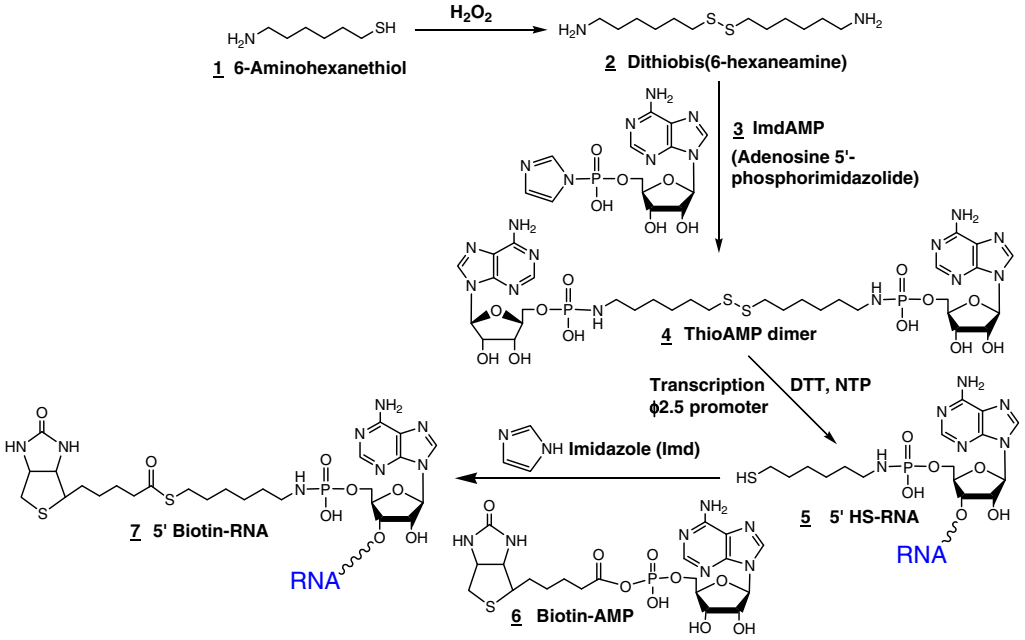

Scheme 1. Chemical synthesis of transcription initiator ThioAMP dimer (**4**), enzymatic preparation of 5′ thiol-labeled RNA (5′ HS-RNA) by transcription, and synthesis of biotin-labeled RNA (5′ Biotin-RNA) by imidazole-catalyzed biotinylation.

tion initiator **4**. After purification by HPLC (Fig. 1) and removal of solvent by lyophilization, a white powder (ThioAMP dimer **4**, 75% recovered yield) was obtained. The following NMR and MS data are consistent with the structure of **4**: ^{1}H NMR (400 MHz, D_2O), δ = 0.937–1.275 (m, 16H, S–C–C_4H_8), 2.307 (t, 4H, J = 7.2 Hz, S–CH_2), 2.618–2.691 (m, 4H, S–C_5–CH_2), 4.019–4.049 (m, 4H, P–O–CH_2), 4.367 (dd, 2H, J = 1.6, 4.0 Hz, H_{ribose}), 4.524 (t, 2H, J = 4.4 Hz, H_{ribose}), 4.782 (t, 2H, J = 5.2 Hz, H_{ribose}), 6.102 (d, 2H, J = 5.2 Hz, H_{ribose}), 8.163 (s, 2H, H_{Ar}), 8.519 (s, 2H, H_{Ar}). MS calcd for $C_{32}H_{52}N_{12}O_{12}P_2S_2$ $[M-H]^-$ 921.27, found 921.25 (ESI), calcd for $C_{32}H_{52}N_{12}O_{12}P_2S_2$ $[M-2H]^{2-}$ 460.13, found 460.17 (ESI).

Preparation of 5′ ThioRNA (5′ HS-RNA, **5**). Following our standard transcription protocol,[1,2,22,23] 5′-thiol-labeled RNA (5′ HS-RNA, **5**) was prepared by in vitro transcription under the T7 Φ2.5 promoter and in the presence of ThioAMP dimer **4**. After transcription, RNA transcripts and thiol-labeled RNA transcripts of relatively small sizes (<100 nt) can be separated directly by polyacrylamide gel electrophoresis (PAGE). Figure 2 shows RNA thiol-labeling under different transcription conditions. The gel indicates that RNA thiol-labeling yields can vary, depending on the concentration ratio of ThioAMP dimer **4** and ATP. When the ratio is >8, RNA labeling yields can reach 90%.

Synthesis of 5′ Biotin-RNA (**7**)*:* Biotinylation of thiol-labeled RNA (5′ HS-RNA, **5**) may be achieved quantitatively in aqueous solutions by reaction with biotinyl adenylate (Biotin-AMP, **6**) in the presence of imidazole.[4] As can be seen from Figure 3, biotin-labeled RNA (5′ Biotin-RNA, **7**) can bind streptavidin to form stable biotin–RNA–streptavidin complexes, which can be separated easily from RNA by PAGE.[1,4] The biotinylation yield of thiol-labeled RNA transcripts (Fig. 3, lane 4) is the same as the RNA thiol-labeling yield (Fig. 2, lane 3). After 5′ HS-RNA was purified by thiolpropyl Sepharose 6B affinity column,[4] RNA biotinylation yield reached 96%.

Preparation of gold nanoparticle-RNA (AuNP-RNA) nanoplexes: To demonstrate the utility of 5′ thiol-labeled RNA (5′ HS-RNA) by the above described transcription method, we prepared a 5′ HS-RNA of 100 nt using a previous DNA template.[5,23] After transcription, the

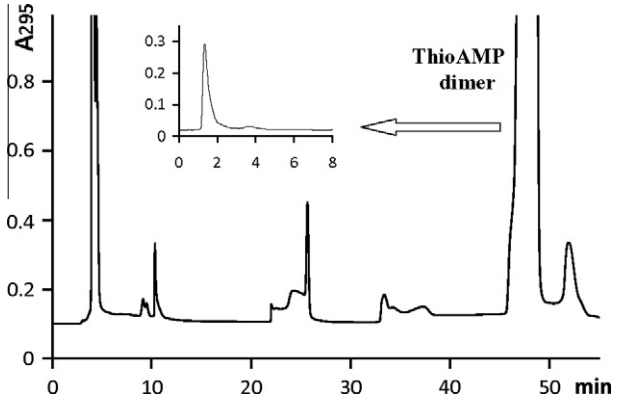

Figure 1. HPLC purification of ThioAMP dimer (**4**) and purify analysis of purified ThioAMP dimer (inset). HPLC conditions were: Econosphere C18 (Alltech) 10 × 250 mm, flow rate 4 mL/min. The column was equilibrated with 20 mM phosphate buffer (pH 7.0). Sample was loaded onto the column. At 18 min after sample injection, the solvent was changed to 100% water. At 28 min, 10% MeOH was introduced and the solvent was changed to 30% MeOH at 41 min. The major peak at 45.8–50 min (ThioAMP dimer) was collected. Its purity was confirmed (inset) by analytical HPLC (Econosphere C18 (Alltech) 4.6 × 50 mm, flow rate 1 mL/min, 40:60 MeOH:20 mM phosphate buffer, pH 7.0).

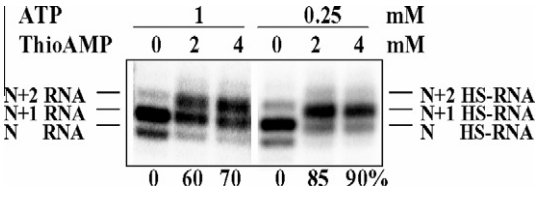

Figure 2. Polyacrylamide gel electrophoresis (PAGE, 8%) analysis of 5′ thiol-labeling of RNA by transcription under different transcription conditions. In addition to ATP and ThioAMP dimer **4**, a trace amount of [α-^{32}P] ATP was included in the transcription solution to label RNA internally by ^{32}P for the purpose of RNA analysis by PAGE and phosphorimaging. Depending on the concentration ratio of ThioAMP dimer and ATP, RNA 5′ thiol-labeling can reach 90% efficiency. The RNA in the experiment contained 35 nucleotides (nt).[22] This was a high resolution (single nucleotide resolution) gel, achieved by long gel-running time (1 h at 15 W for a 20 × 15 cm × 0.8 mm gel).

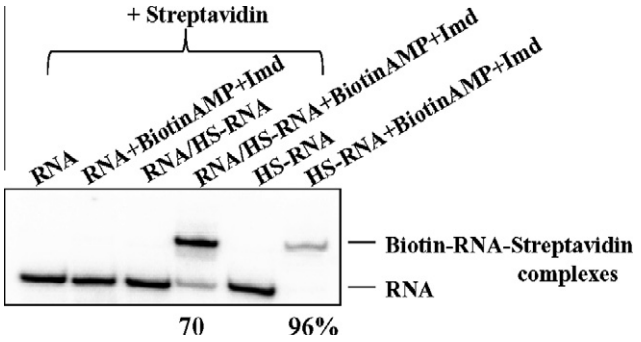

Figure 3. PAGE (8%) analysis of 5′ biotinylation of 5′ HS-RNA (**5**) by Bioitn-AMP (**6**) and imidazole. After reaction, unincorporated biotin was removed by EtOH precipitation and the recovered RNA was incubated with streptavidin, followed by PAGE. This was a low resolution (not capable of resolving RNA at single nucleotide resolution) gel, achieved by short gel-running time (10 min at 15 W for a 20 × 15 cm × 0.8 mm gel).[1,4] The RNA in this experiment contained 100 nucleotides (nt) that had been used in our previous studies.[5,23]

RNA sample was purified by Microcon (M30) filtration. The recovered 5′ HS-RNA was then used directly to prepare AuNP-RNA nanoplexes following the Mirkin procedure.[25] The final NaCl concentration reached 0.7 M. The formation of AuNP-RNA nanoplexes was monitored by measuring UV spectrum of AuNP solutions. As can be seen from Figure 4, when AuNPs (13 nm diameter)[26] were mixed with transcriptionally-prepared 5′ HS-RNA, the characteristic 520 nm plasma resonance peak of AuNPs red-shifted 9 nm. As a control, the same procedure was applied to AuNPs but without RNA. AuNPs quickly formed aggregation and precipitated out of solutions. The UV spectrum (Fig. 4) confirmed aggregation formation by NaCl. As another control, AuNPs were mixed with unlabeled RNA of the same sequence and size [prepared by transcription in the absence of ThioAMP dimer (**4**)]. NaCl was then gradually added to the sample to a final concentration of 0.7 M. As can be seen from Figure 4, although there were some interactions between AuNPs and unlabeled RNA, severe aggregation occurred and the dark red AuNP solution quickly turned to faint blue solution. With longer time, AuNPs precipitated out of solution.

To further demonstrate AuNPs and 5′ thiol-labeled RNA interactions, we prepared 5′ thiol-labeled survivin siRNA[27] and purified HS-siRNA by thiopropyl Sepharose 6B affinity column according to our standard protocol.[1,4] The resulting HS-siRNA was then used

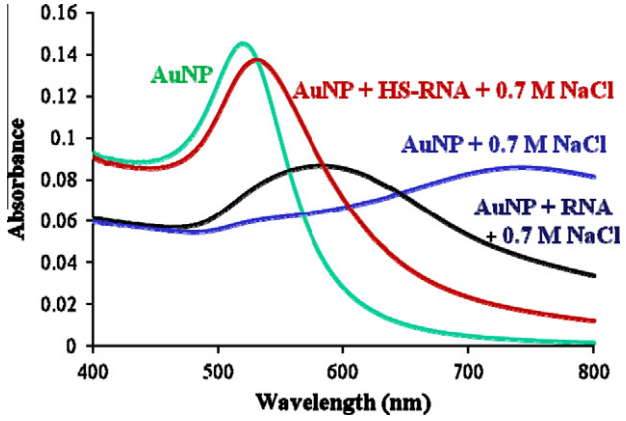

Figure 4. Formation of AuNP-RNA nanoplexes from AuNPs and 5′ HS-RNA (100 nt) prepared by transcription and purified by Microcon (M30). The resulting RNA preparation contained 30% unlabeled RNA (Figs. 2 and 3). AuNPs were first mixed with 5′ HS-RNA. NaCl was then gradually added to the sample over a course of 2 h at room temperature. The final NaCl reached 0.7 M. An aliquot of the sample was diluted for the measurement of UV spectrum. AuNPs (13 nm diameter) were prepared according to Mirkin.[26]

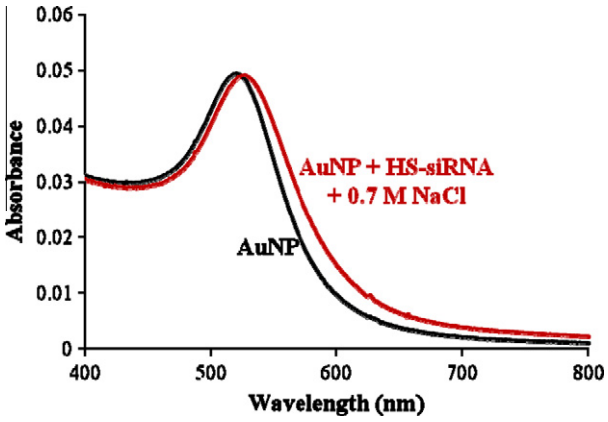

Figure 5. Formation of AuNP-siRNA nanoplexes from AuNPs and HS-siRNA prepared by transcription and purified by thiopropyl Sepharose 6B affinity column.[1,4] The resulting RNA preparation contained near pure RNA (Fig. 3, lane 6). The same procedure as in Figure 4 was used to prepare AuNP-siRNA nanoplexes.

to prepare AuNP-siRNA nanoplexes following the same procedure as described above. As indicated by UV spectra in Figure 5, our transcriptionally-prepared HS-siRNA was able to form AuNP-siRNA nanoplexes similar to those of AuNP-siRNA nanoplexes formed by chemically synthesized 5′ thiol-labeled siRNA.[26] The UV spectrum difference between the AuNP-RNA in Figure 4 and AuNP-siRNA in Figure 5 are mostly like due to different RNA sizes (100 nt vs 27 nt).

In summary, we have synthesized a new symmetrical thiol-containing transcription initiator (ThioAMP dimer **4**). Transcription under the T7 Φ2.5 promoter and in the presence of the ThioAMP dimer produces 5′ thiol-labeled RNA (5′ HS-RNA) with up to 90% labeling efficiency, depending on the concentration ratio of ThioAMP dimer and ATP. There is no sequence and/or RNA size limit of the described RNA 5′ thiol-labeling method. Although relatively small RNAs can be conveniently prepared by phosphoramidite chemistry, the described method is simple, highly efficient, and relatively inexpensive. It is particularly useful for 5′ thiol-labeling of relatively large RNA molecules. For small RNA sizes, the method is not only an option but also advantageous when the needed RNA quantity is small. Finally, when a large number of different thiol-labeled RNA sequences are needed, this method is particularly appealing.

Acknowledgments

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