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Synthesis of photolabile transcription initiators and preparation of photocleavable functional RNA by transcription

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ABSTRACT

Two new photolabile adenosine-containing transcription initiators with terminal thiol and amino functionalities are chemically synthesized. Transcription in the presence of the transcription initiators under the T7 phi2.5 promoter produces 5′ thiol- and amino-functionalized RNA conjugated by a photocleavable (PC) linker. Further RNA functionalization with biotin may be achieved through acyl transfer reactions from either biotinyl AMP to the RNA thiol group or biotin NHS to the RNA amino group. Photocleavage of the PC linker displays relatively fast kinetics with a half-life of 4–5 min. The availability of these transcription initiators makes new photolabile RNA accessible for affinity purification of RNA, in vitro selection of functional RNAs, and functional RNA caging.

RNAs with attached functional groups such as free amino and thiol groups may be used to conjugate diverse chemical and biochemical functionalities. Such functionalized RNAs have been commonly used in chemistry and biomedical research.[1–9] 5′ Thiol-labeled RNAs (5′ HS-RNA) have been used for RNA purification by affinity chromatography,[5,10] for RNA immobilization on gold surfaces,[11,12] and for construction of gold nanoparticle-siRNA (AuNP-siRNA) nanoplexes.[13,10] In addition, RNA conjugation with other macromolecules have been achieved with 5′ thiol-functionalized RNAs.[14–19]

In many RNA applications, it is highly desirable that conjugated chemical/biochemical functionalities be cleaved from RNA so that captured RNA is released from resins/surfaces[20] or that caged inactive RNA becomes active.[21–26] A cleavable chemical linker may be used to connect a chemical/biochemical group and RNA. To facilitate linker cleavage, the linker may be chosen such that it is sensitive to either reducing agents, acidic solutions, or photons. Among the different cleavage methods, photocleavage has several distinct advantages including clean cleavage at precise sites, avoidance of using cleavage chemicals, and easy realization of spatial and temporal cleavage control.[21–25,27,26]

Generation of functional ribozymes by in vitro selection[28–30] has been very successful. In vitro-isolated ribozymes can catalyze impressive diverse chemistries, including phosphodiester bond cleavage, phosphorylation, aminoacylation, RNA ligation, RNA polymerization, amide bond cleavage and formation, glycosidic bond formation, carboxyl activation, coenzyme incorporation and synthesis, thioester synthesis, carbon–carbon bond formation, Michael adduct formation, aldol reaction, alkylation, and 'high energy' phosphoanhydride bond formation. In principle, conventional in vitro selection methods can be designed to isolate desired self-modifying ribozymes that catalyze chemical transformations between a free substrate (often tagged for isolation, such as biotin-tagging) and an RNA-conjugated chemical functional group. However, there are multiple potential reaction sites on the RNA molecule, including the hydroxyl groups on the ribose, the nitrogen sites on the nucleobase, and the 5′ phosphate. Consequently, unintended side reactions within RNA internal sites can often overshadow the intended chemistry at a predefined site.[31–33]

The intrinsic limitation of conventional in vitro selection methods may be overcome by introducing a photocleavable linker (PC) between a pendant reactant group and RNA, which may be achieved by either photolabile guanosine derivative-initiated transcription[34–36] under the class III promoter (Φ6.5) or ligation with a oligoribonucleotide containing a reactant group and a PC.[37,38] Expanding our previously developed high efficiency RNA labeling methods[6,8,9,39,10] based on adenosine derivative-initiated transcription under the T7 Φ2.5 promoter, we describe the chemical synthesis of two new photolabile adenosine derivatives (**6** and **10**) that are efficient transcriptional initiators for the preparation of photocleavable RNAs. Compound **6** is a symmetrical adenosine derivative that contains two adenosines, two PC groups, and a disulfide bond. Photocleavage of **6** using a portable UV lamp

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(4 W) displays a relatively fast kinetics. Under standard transcription conditions, the disulfide bond is reduced to a free thiol group. As a result, the RNA transcript (**7**) initiated by **6** has a free thiol group at the 5′ end through a PC linker. Built on the half unit of **6**, **10** contains an adenosine and a free terminal amino group linked by a PC group. In vitro transcription in the presence of **10** results in amino-functionalized RNA transcript (**11**) which can be cleaved off by photon irradiation.

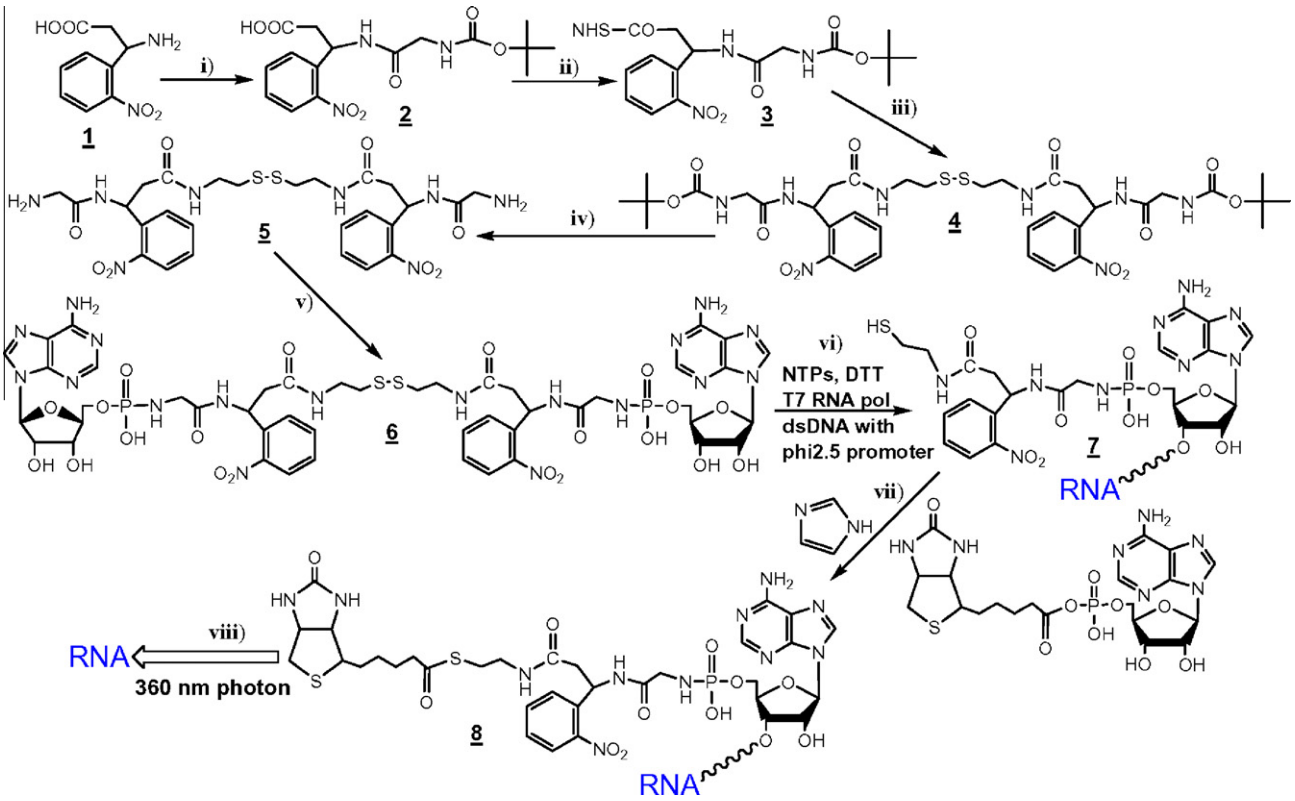
Following RNA preparation, both 5′ thiol and amino functionalized RNAs can be further modified by other chemical groups through acyl transfer reactions. Using biotin as an example, we have demonstrated that two forms of biotin-tagged RNA—5′ BiotinCO-S-PC-RNA (**8**) and 5′ BiotinCO-NH-PC-RNA (**12**), may be prepared through either imidazole-catalyzed thioesterization reaction[7] or biotinylation with an activated biotin ester. Finally, photocleavage kinetics of biotin-conjugated RNAs **8** and **12** are presented to show relatively fast PC linker cleavage upon photon irradiation.

*Synthesis of symmetrical thiol-containing transcription initiator—HS-PC-AMP dimer (**6**):* As shown in Scheme 1, the synthesis of **6** involved six chemical steps. To the starting 3-amino-3-(2-nitrophenyl)-propionic acid **1** (ANPPA, 110 mg, 0.52 mmol) dissolved in 1 mL of DMF, were added Boc-Gly-NHS (136 mg, 0.50 mmol) and triethylamine (0.075 mL, 0.54 mmol). The reaction was stirred overnight at room temperature to yield **2**. *N*-hydroxysuccinimide (69.0 mg, 0.60 mmol) and *N,N′*-dicyclohexylcarbodiimide (206 mg,1.0 mmol) were then added to the above solution. The mixture was stirred for 4 h at room temperature. The insoluble urea derivative from the reaction was removed by centrifugation and the resulting clear solution **3** was used directly for the next step reaction.

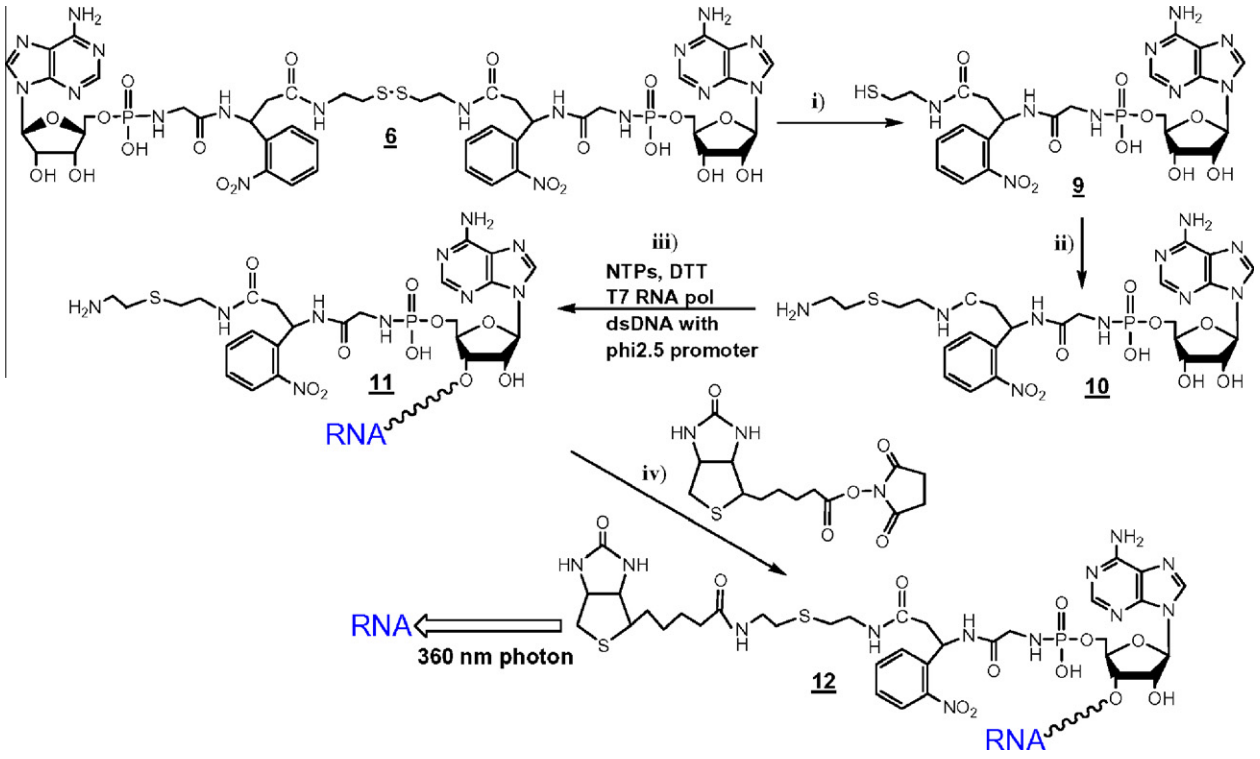
Cystamine (32 μL, 7 M, 0.22 mmol) was added to the solution **3** and stirred overnight at room temperature to yield disulfide-linked symmetrical **4**. Any unreacted NHS ester was hydrolyzed by adding a NaOH solution (1.2 mL, 0.3 M, stirring for 1 h). Water (3 mL) was then added to the solution to precipitate **4**, which was collected by centrifugation and washed by water (3 × 6 mL). The light yellow solid was dried under vacuum until reaching a constant weight (0.138 g, 74% yield). Both NMR and MS (Supplementary data) were consistent with the structure of **4**. The Boc protecting groups of **4** were removed by TFA treatment (1 mL of dichloromethane, 1 mL of TFA, and 0.1 mL of triisopropylsilane, 45 min at room temperature). The resulting **5** was precipitated by ether (30 mL) and collected by centrifugation, followed by washing with ether (3 × 20 mL). It was then dried under vacuum until constant weight (98 mg, 69% yield). The identity of **5** was confirmed by both NMR and MS (in Supplementary data).

The final step to **6** was achieved by conjugation of **5** with adenosine 5′-phosphorimidazolide (ImdAMP).[40,10] Compound **5** (80 mg, 0.12 mmol) was dissolved in 280 μL DMF. ImdAMP (800 μL, 1 M in DMF) and triethylamine (120 μL) were added and the mixture was stirred for 4 days at room temperature to afford the photocleavable symmetrical thiol-containing transcription initiator **6**, which was then purified by HPLC to yield the pure product (52 mg, 40 μmol, 33% yield) (NMR, MS, in Fig. 1S in Supplementary data).

*Synthesis of photocleavable amino-containing transcription initiator—NH_2-PC-AMP (**10**)*: Based on **6**, a photocleavable amino derivative **10** was prepared in 2 simple steps, as shown in Scheme 2. In the first step, **6** (20 mg, 15 μmol) was dissolved in 160 μL DMF. To the solution, were added mercaptoethanol (60 μL) and triethylamine (30 μL). After overnight reaction, compound **9** was precipitated by ether and recovered by centrifugation. After multiple washing with ether, followed by centrifugation and finally drying, **9** was recovered in high yield (20 mg). The second step involved conjugation of an aminoethyl group with **9** through a thioether bond. Compound **9** (20 mg) was first dissolved in water (100 μL), following by adding 2-bromoethylamine (24 μL, 5 M) and NaOH



Scheme 1. Chemical synthesis of symmetrical HS-PC-AMP conjugate (**6**), enzymatic preparation of HS-PC-RNA (**7**) by one-step in vitro transcription, and posttranscriptional RNA biotinylation by imidazole-catalyzed thioesterification. (i) Boc-Gly-NHS, (ii) NHS, DCC, (iii) cystamine, (iv) TFA, (v) adenosine 5′-phosphorimidazolide (ImdAMP).



Scheme 2. Chemical synthesis of NH_2-PC-AMP conjugate (**10**), preparation of NH_2-PC-RNA (**11**) by in vitro transcription, and biotin-labeling of RNA (**12**) by chemical conjugation. (i) mercaptoethanol, (ii) 2-bromoethylamine.

(70 μL, 2 M). After 1 h reaction at room temperature, the solution pH was adjusted to 7.0 by using acetic acid. Compound **10** was isolated by HPLC to high purity (18 mg, 84% yield) (NMR, MS, in Fig. 2S in Supplementary data).

*Photocleavage kinetics of **6***: A sample of **6** was dissolved in D_2O and placed in a quartz NMR tube. After UV irradiation at 360 nm for different duration using a portable UV lamp (4 W), NMR spectra were taken. By quantitating four well-resolved NMR peaks (Fig. 3S in Supplementary data) that decreased over photo irradiation time, a photocleavage kinetic curve was obtained (Figure 4S in Supplementary Data). A half-life of 5–6 min under the photo irradiation conditions was obtained from the curve. The result in general agrees with those for other *o*-nitrobenzyl photocleavage.[41]

Preparation of 5′ HS-PC-RNA (**7**)**:** Using our standard transcription protocol,[5,42,43,39] 5′-thiol-PC labeled RNA (HS-PC-RNA, **7**) was prepared by in vitro transcription under the T7 Φ2.5 promoter and in the presence of HS-PC-AMP dimer **6** (Scheme 1). For relatively small RNA (<100 nt), the unlabeled RNA transcripts (pppRNA as a result of ATP initiation) and thiol-PC-labeled RNA transcripts can be separated directly by high resolution polyacrylamide gel electrophoresis (PAGE). Figure 1A shows RNA thiol-PC-labeling under different labeling conditions by a high resolution gel (single nucleotide resolution). The gel indicates that RNA thiol-PC-labeling yields can vary, depending on the concentration ratio of HS-PC-AMP dimer **6** and ATP. At 2:1 and 4:1 ratios, 5′-HS-PC-RNA yields can reach 70–80%.

Synthesis of 5′ BiotinCO-S-PC-RNA (**8**)**:** Biotinylation of thiol-PC-labeled RNA (HS-PC-RNA, **7**) may be achieved quantitatively in aqueous solutions by reaction with biotinyl adenylate (Biotin-AMP) to form a biotinyl thioester in the presence of imidazole.[7,10] As can be seen from Figure 1B (a low resolution gel), 5′ biotin-PC-labeled RNA (BiotinCO-S-PC-RNA, **8**) can bind streptavidin to form stable BiotinCO-S-PC-RNA-streptavidin complexes, which can be easily separated from unlabeled RNA by PAGE.[5,7,10] Under the biotinylation conditions, unlabeled RNA produced no RNA-streptavidin complexes (Fig. 1B, lane 2), whereas RNA/HS-PC-RNA mixture directly from transcription yielded 68% biotinylated RNA (Fig. 1B, lane 4). Following HS-PC-RNA purification from RNA/HS-PC-RNA mixture

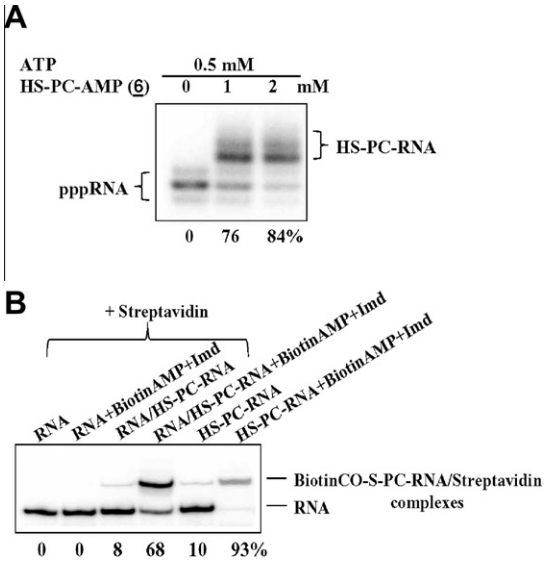


Figure 1. (A) High resolution polyacrylamide gel electrophoresis (PAGE, 8% with 7 M urea) analysis of 5′ thiol-PC-labeling of RNA in the presence of different concentrations of HS-PC-AMP conjugate (**6**). In addition to NTPs and **6**, a trace amount of [α-^{32}P] ATP was included in the transcription solution to label RNA internally by ^{32}P for the purpose of RNA analysis by PAGE and phosphorimaging. Bands of HS-PC-RNA were completely separated from those of unlabeled RNA (pppRNA). This high resolution (single nucleotide resolution) gel was achieved by long gel-running time (1 h at 15 W for a 20 cm × 15 cm × 0.4 mm gel). (B) Low resolution PAGE (8% with 7 M urea) analysis of streptavidin-Biotin–RNA complex formation under different conditions. Biotinylated RNA-streptavidin complex (top band) was easily fractionated from unlabeled RNA (bottom band). This low resolution gel resulted from short gel-running time (10 min at 15 W for a 20 cm × 15 cm × 0.4 mm gel). The RNA in the experiments contained 35 nucleotides (nt) as described previously.[43]

by thiolpropyl Sepharose 6B affinity column,[7,10], high yield RNA biotinylation can be obtained (Fig. 1B, lane 6).

Preparation of 5′ NH_2-PC-RNA (**11**)**:** Following the same transcription protocol,[5,42,43,39] 5′-amino-PC labeled RNA (NH_2-PC-RNA, **11**) was synthesized by in vitro transcription in the presence of NH_2-PC-AMP (**10**) (Scheme 2). High resolution PAGE analysis indicated efficient RNA labeling by 5′-amino-PC (Fig. 2A, lanes 2, 3).

Synthesis of 5′ BiotinCO-NH-PC-RNA (**12**)**:** An alternative way to achieve RNA biotin-labeling is through Biotin-NHS reaction with a free amine on the RNA (Scheme 2, step iv). As can be seen in Figure 2B, Biotin-NHS reaction (5 mM in 50:50 DMSO:water, 30 min at room temperature) with RNA/NH_2-PC-RNA mixture directly from transcription resulted in high yield RNA biotinylation (Fig. 2B, lane 4), while unlabeled RNA produced very little biotinylated RNA under the same conditions (Fig. 2B, lane 2).

Photocleavage kinetics of **8** and **12:** After RNA biotinylation reactions (Scheme 1, step vii and Scheme 2, step iv), ^{32}P-labeled BiotinCO-S-PC-RNA (**8**) and BiotinCO-NH-PC-RNA (**12**) were purified by denaturing PAGE to remove unreacted Biotin-AMP and Biotin-NHS. Streptavidin was then mixed with BiotinCO-S-PC-RNA and BiotinCO-NH-PC-RNA separately. After UV irradiation of the streptavidin-RNA complexes for different duration, the samples were analyzed by PAGE. As shown in Figure 3, the PC linker in both BiotinCO-S-PC-RNA and BiotinCO-NH-PC-RNA was rapidly cleaved with similar kinetics to release free RNA, with a half-life of 4–5 min, similar to that of transcriptional initiator **6** photocleavage. From the kinetic curves, it appeared that a small fraction of Biotin-PC-RNA (~20%) had slower kinetics. This biphasic photocleavage kinetics (Fig. 3) of Biotin-PC-RNA (**8 or 12**) differs to some extent from transcriptional initiator **6** photocleavage kinetics (Figure 4S in Supplementary data). It is unclear what factor(s) from RNA contributed to such a difference.

In summary, we have synthesized two new photolabile adenosine-containing transcription initiators (HS-PC-AMP dimer **6** and NH_2-PC-AMP **10**) with terminal thiol and amino functionalities. High yield labeling of RNA (>80%, depending on the **6**:ATP or **10**:ATP ratio) by either thiol-PC or amino-PC may be achieved by transcription under the T7 Φ2.5 promoter and in the presence of **6** or **10**. Following RNA preparation, the RNA may be further derivatized post-transcriptionally with a broad range of acyl groups

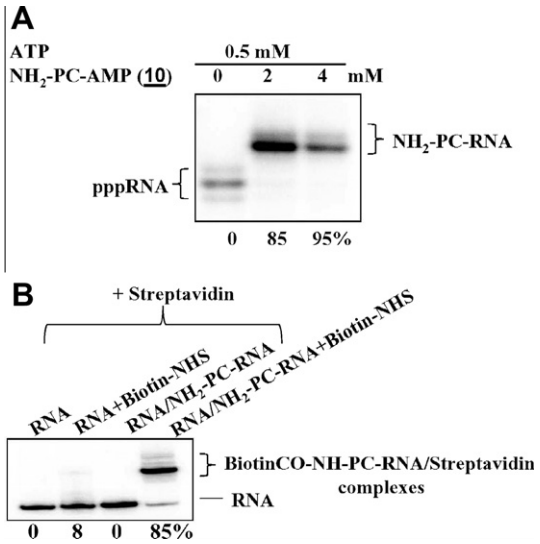


Figure 2. (A) High resolution PAGE (8% with 7 M urea) analysis of 5′ amino-PC-labeling of RNA in the presence of different concentrations of NH_2-PC-AMP conjugate (**10**). (B) Low resolution PAGE (8% with 7 M urea) analysis of streptavidin-Biotin–RNA complex formation. Transcription and PAGE conditions were the same as those in Figure 1.

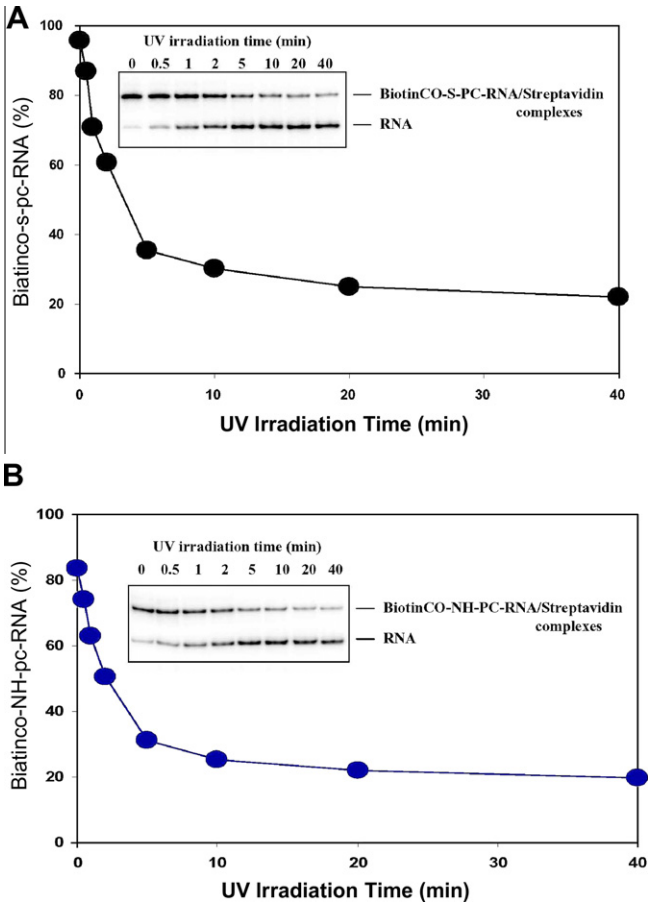


Figure 3. Kinetics of photocleavable linkage of Biotin-PC-RNA–streptavidin complexes under UV irradiation. The RNA-streptavidin samples (2 μL) were contained in 0.5 ml eppendorf tubes and placed on ice. A portable UV lamp (UVL-56, UVP, 4 W) was then placed in contact above the tubes. At the indicated time, the tubes were removed from UV irridiation. At the completion of UV-irradiation experiments, the samples were analyzed by low resolution PAGE (8% with 7 M urea) for photocleavage of Biotin-PC-RNA at different UV irradiation time. (A) BiotinCO-S-PC-RNA photocleavage, (B) BiotinCO-NH-PC-RNA photocleavage.

through the terminal thiol and amino groups by well-established acyl transfer reactions involving activated carboxylates (carboxylate-adenylates and carboxylate-NHS). In vitro cleavage of the photocleavable linker between RNA and the terminal functional group can be easily achieved by using simple portable long wavelength UV lamps (360 nm). The availability of these two adenosine-based transcription initiators makes new photolabile RNA accessible for affinity purification of RNA, in vitro selection of functional RNAs (aptamers and ribozymes), and functional RNA caging.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.028.

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