Published in final edited form as:

Anal Biochem. 2014 March 15; 449: 106–108. doi:10.1016/j.ab.2013.12.022.

HPLC Purification of Chemically Modified RNA Aptamers

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Abstract

2'-Fluoro modified RNAs are useful as potential therapeutics and as special substrates for studying RNA function. 2'-Fluoro modified RNAs generally need to be purified after they are prepared either enzymatically or by solid-phase synthesis. Here we introduce a protocol by which 2'-fluoro modified RNAs with 57 and 58 nucleotides can be resolved and purified, using ion-pair, reverse-phase high performance liquid chromatography (HPLC). Because the size of our RNA samples is in the range of many known RNA aptamers of therapeutic values, our protocol should be generally useful.

Keywords

RNA purification and separation; HPLC; chemically modified RNA

Replacement of the 2'-hydroxyl (OH) group in the ribose of an RNA with different analogs is known to change the RNA properties for special uses [1–3]. Historically, the use of 2'-modified hammerhead ribozymes permitted the identification of the role of 2'-OH groups at specific nucleotide positions in their catalytic activities [1]. The incorporation of 2'-fluorouridine into the substrate facilitated the identification of a hydrogen bond between the hydroxyl group of the substrate and an adenosine in the catalytic core of the *Tetrahymena* ribozyme [4]. This study led to the suggestion that base-backbone tertiary contacts may be generally important for the organization of RNA tertiary structure. The use of the modified pre-mRNA revealed that 2'-OH at the 3' splice site is important for the second step in RNA splicing [5]. Furthermore, 2'-modified RNAs are more stable *in vivo* [6, 7]. Unmodified, RNA can be degraded in minutes when exposed to ribonucleases that are abundant in biological fluids [8]. In contrast, modified RNA by 2'-amino (NH₂) or 2'-fluoro (F) substitution on pyrimidines alone, for instance, exhibit half-life of over two days in serum due to strengthened resistance to ribonucleases [9]. To date, 2'-chemical modifications are routinely used in making antisense RNA, siRNA, miRNA and RNA therapeutics [8].

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Subject category: RNA Purification

Chemically modified RNA samples are generally impure regardless whether they are synthesized enzymatically or by solid-phase chemistry [10, 11]. Sample purification is therefore generally needed. Both polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC) are routinely used in RNA purification. Among other advantages, HPLC purification is automated with a short run time [12]. We previously reported that RNAs of 57–59 nucleotides (nt) in length can be resolved at a single nucleotide level or 305–345 Dalton (Da) by using an ion-pair, reverse-phase HPLC [11]. Here we ask whether HPLC can be used to resolve and purify chemically modified RNAs of similar lengths.

In this study, we used three 2'-fluoro modified RNAs. Fluorine substitution of the 2'-OH groups is a commonly used chemical modification to prepare ribonuclease-resistant RNA aptamers [6, 7]. The three 2'-fluoro modified RNAs are F-U 57-nt, F-U 58-nt and F-UC 58-nt (here F-U and F-UC stand for the replacement of the 2'-OH groups by 2'-F groups from all of the uridine (U) positions and the uridine/cytidine (C) positions in the RNA sequences, respectively). Because the fluorine replacement of a 2'-OH group only increases 2 Da in molecular weight, F-U 57-nt, F-U 58-nt and F-UC 58-nt RNAs are 26, 26, and 52 Da heavier than their unmodified counterparts (see their sequences in Supplementary Data). With these samples, we investigated whether 2'-fluoro modified aptamers that differ by one nucleotide can be resolved and whether a large scale purification is possible. It should be noted that the samples we used here are RNA aptamers that we previously isolated to inhibit the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, a subtype of the ionotropic glutamate receptor family [13]. Because the size of our samples (i.e., 57 and 58 nt) falls in the range of other RNAs or RNA aptamers of therapeutic values [14], the results from this study should be generally useful.

All the 2'-fluoro modified RNAs used in this study were obtained from Trilink Biotechnologies (note that the samples were desalted after synthesis). As seen, they were impure (Fig. 1B). Using a linear gradient protocol and an XBridge column (see detail in the Fig. 1 legend and the Supplementary Data), we showed that the desired RNAs could be purified (Fig. 1A). This was based on the assessment of the quality of purification on a denatured PAGE (Fig. 1B) for samples taken before and after HPLC purification. It should be noted that the 2'-F modification of the 58-nt RNA prolonged its retention time, compared with the unmodified control. More chemical modifications on the same RNA extended the retention time even further. Specifically, the difference in retention time between the unmodified 58-nt RNA and the 2'-F-U counterpart was 2.2 min (Fig. 1C, the upper and middle chromatograms). However, when the 2'-OH group from both the 13 uridines and the 13 cytidines in the same sequence were replaced with 2'-F group, the resulting F-UC 58-nt RNA exhibited additional 2.9 min delay in de-absorption or elution from the column (lower HPLC trace as compared with the middle one in Fig. 1C).

2'-Fluoro modification either at U alone or at the U/C positions together in the same RNA sequence causes an insignificant increase of molecular weight, as compared with the unmodified RNA. For instance, 2'-fluoro replacement of 2'-OH groups in F-U 58-nt RNA increases the molecular weight by 26 Da, whereas the addition of one nucleotide from 57-nt to 58-nt RNA increases the molecular weight by 329 Da. Therefore, a significant delay in

elution (in this case by 2'-fluoro replacement as in Fig. 1C) most likely suggests a change of the property once an RNA is modified. From a crystallographic study, 2'-fluoro modification is thought not to affect significantly either the local or the overall helix geometry of a RNA [15]. This is probably expected given that the fluorine group is smaller in size than a hydroxyl group [16]. However, 2'-F substitution is thought to significantly reduce the hydration property of an RNA [15]. As we observed (Fig. 1C), a longer duration of the RNA absorbed onto a hydrophobic stationary phase or a longer retention time suggests a stronger hydrophobic interaction. Thus, an increase in hydrophobicity of a 2'-fluoro modified RNA should be the main contribution to the increase in retention time as compared with the unmodified RNA.

Next we tested whether we could resolve F-U 57-nt and F-U 58-nt RNAs from each other. F-U 57-nt and F-U 58-nt RNAs had identical 2'-fluoro replacements (i.e., 13 uridine positions were modified) but one nucleotide difference, which resulted in 329 Da molecular weight difference. As seen (Fig. 2A), the two RNAs could be resolved with a peak-to-peak difference of 1.4 min in retention time. However, despite a large molecular weight difference (as compared with that in Fig. 1C), F-U 57-nt and F-U 58-nt RNAs were not well separated. This result showed that the change of molecular weight in 2'-fluoro modified RNAs, due to a change of the number of nucleotides, affects little the retention time or the separation of 2'-fluoro modified RNAs. In contrast, the percentage by which a RNA sequence is modified affects significantly its retention, due to a major change of the hydrophobicity of the RNA (Fig. 1C).

In an attempt to better separate 2'-fluoro modified RNAs, we also tried to reduce the slope of a linear gradient. Gradient elution decreases the retention of later-eluting species so that they are eluted faster with narrower and taller peaks, as the concentration of an organic eluent increases [17]. However, as the gradient became shallower (which was in essence an increase of N, the theoretical plate number), the elution profile of F-UC 58-nt RNA, as an example, became worse (Fig. 2B). Specifically, in the second linear gradient, labeled as (b) to (c) in Fig. 2B, the methanol concentration rose from 24.6% to 28.65% in upper trace but only to 25.8% in the lower trace. Yet we observed a significant change of peak width and shape for F-UC 58-nt RNA. The half-height peak width from upper to the lower trace (Fig. 2B) was 0.8 and 2.2 min, whereas the half-height peak width for the unmodified 58-nt RNA as the control remained essentially unchanged, i.e., 0.24 min (upper) and 0.26 min (lower trace), respectively. In fact, the lower chromatogram displayed a broad, flat peak. This result demonstrated that even a slight decrease of the linear gradient slope causes a significant delay in elution and peak broadening of a 2'-fluoro modified RNA. Therefore, the use of a shallower gradient to achieve a *complete* separation of 58-nt from F-U 57-nt RNAs is not feasible with the 2'-fluoro modified RNA. This is mainly due to the fact that longer or larger RNAs continue to be challenging.

Although our protocol (Fig. 2A) is sufficient for detecting 2'-fluoro modified RNAs that differ by one nucleotide using HPLC, it is not practical for a large scale separation of 57-nt from 58-nt RNA. However, our protocol can be used with some conditions. First, a 2'-fluoro modified RNA sample prepared by solid-phase synthesis does not seem to contain a substantial amount of RNAs with the number of nucleotides closer to the desired one (see

Fig. 1). As such, a large scale purification of 2'-fluoro modified RNAs using HPLC can be achieved satisfactorily. Indeed, solid-phase synthesis is routinely used to prepare a large quantity of chemically modified, short RNAs (i.e., < 80 bases) [18]. Furthermore, dyes, spin labels, affinity labels and other functionalities can be incorporated to an RNA in a site-specific fashion by solid-phase synthesis [19]. Second, RNAs with extra nucleotides, such as the n+1 product, are frequently produced from transcription reactions [10]. However, the inclusion of the *glmS* ribozyme sequence in the transcription of an RNA can eliminate the production of the undesirable n+1 product, as we demonstrated previously in the enzymatic production of RNA aptamers of similar sizes [20]. For longer RNAs, enzymatic transcription has to be used, although enzymatic transcription reaction does not permit site-specific incorporation of chemical modifications at sugars, bases or phosphates. Therefore, for synthesis of longer, chemically modified RNA, using chemical synthesis and enzymatic RNA ligation in tandem can be a valuable approach [21].

In conclusion, using an ion-pair, reverse-phase HPLC, we show that 2'-fluoro modified RNAs with 57 and 58 nucleotides can be resolved and purified in a simple protocol. Our method extends the existing capability of using reverse-phase HPLC to purify chemically modified RNAs with < 30 nucleotides [22]. Because the size of our RNA samples is in the range of RNA aptamers of therapeutic values, our protocol should be generally useful. Our work also opens the possibility of separating RNAs of similar sizes but different chemical modifications, such as nucleobase methylations, uridine isomerization and hypermodified nucleotides in complex RNA maturation processes [19, 23]. The same method can be also explored with naturally occurring modifications in almost all classes of cellular RNAs [24].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from National Institutes of Health and Muscular Dystrophy Association (to L.N.).

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Fig. 1. Purification of 2'-fluoro modified RNAs by ion-pair, reverse-phase HPLC (here F-UC 58-nt RNA was used as an example). An XBridge C18 column mounted on a Waters Breeze HPLC system was used for RNA separation, monitored at 254 nm. All HPLC runs were at 50 °C. (A) The elution included two linear gradients. The first one started from (a) (15% of methanol) and ended at (b) (21.5% of methanol). The second one started from (b) and ended at (c) (29% of methanol). (B) RNA sample purity before and after HPLC purification was assessed on a denatured PAGE (10%, 8M urea). All samples (~100 ng each) contained 50% Bio-Rad Gel Loading Buffer II, and heated at 95 °C for 5 minutes before loading. The gel was run for 30 min at 25 V/cm, and stained with ethidium bromide. (C) HPLC chromatograms for 58-nt, F-U 58-nt and F-UC 58-nt RNAs. A faster protocol was used in running these samples through the HPLC column as compared with (A). In this protocol, a linear gradient of 15%–32.5% (20 min to 80 min) was used.

Fig. 2.

(A) Single-nucleotide resolution of F-U 57-nt and F-U 58-nt RNAs. The F-U 57-nt and F-U 58-nt RNAs were separated by 1.4 min in retention time. The two sections of linear gradient used were 15%–23% of methanol (20 min–50 min) and 23%–25% of methanol (50 min–140 min), respectively. (B) Effect of linear gradient on the peak shape and width of 2'-fluoro modified RNAs. The first linear gradient section started at 20 min, labeled as point (a), and ended at 50 min, labeled as point (b). The second linear gradient started at 50 min, labeled as point (b), and ended at 110 min, labeled as point (c). At point (c), the methanol concentration reached 28.65% in the upper trace and 25.8% in the bottom trace. The half-height peak width of 58-nt RNA in two traces was 0.24 min, and 0.26 min, respectively. In contrast, the half-height peak width of F-UC 58-nt RNA was 0.8 min (upper) and 2.2 min (lower traces), respectively.