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**ARTICLE** *in* ANALYTICAL CHEMISTRY · FEBRUARY 2005

Impact Factor: 5.64 · DOI: 10.1021/ac0487395 · Source: PubMed



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# Analysis of Biological and Synthetic Ribonucleic Acids by Liquid Chromatography−Mass Spectrometry Using Monolithic Capillary Columns

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**Ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) has been evaluated as a method for the fractionation and desalting of ribonucleic acids prior to their characterization by electrospray ionization mass spectrometry. Monolithic, poly(styrene−divinylbenzene)-based capillary columns allowed the rapid and highly efficient fractionation of both synthetic and biological ribonucleic acids. The common problem of gas-phase cation adduction that is particularly prevalent in the mass spectrometric analysis of ribonucleic acids was tackled through a combination of chromatographic purification and the addition of ethylenediaminetetraacetic acid to the sample at a concentration of 25 mmol/L shortly before on-line analysis. For RNA molecules ranging in size from 10 to 120 nucleotides, the mass accuracies were typically better than 0.02%, which allowed the characterization and identification of failure sequences and byproducts with high confidence. Following injection of a 500 nL sample onto a 60 × 0.2 mm column, the limit of detection for a 120-nucleotide ribosomal RNA transcript from *Escherichia coli* was in the 50−80 fmol range. The method was applied to the analysis of synthetic oligoribonucleotides, transfer RNAs, and ribosomal RNA. Finally, sequence information was derived for low picomole amounts of a 32-mer RNA upon chromatographic purification and tandem mass spectrometric investigation in an ion trap mass spectrometer. Complete series of fragment ions of the c- and y-types could be assigned in the tandem mass spectrum. In conclusion, IP-RP-HPLC using monolithic capillary columns represents a very useful tool for the structural investigation and quantitative determination of RNAs of synthetic and biological origin.**

RNAs play an essential role in a variety of biochemical processes, in which they serve as a temporary copy of genes that is used as a template for protein synthesis, function as adaptor molecules for translation of the genetic code, and catalyze the biochemical synthesis of proteins. In contrast to double-stranded, genomic DNA, RNA is a single-stranded polynucleotide that spontaneously folds into a variety of secondary and tertiary structures such as hairpins, bulges, pseudoknots, and internal loops.[1] These structural elements can either constitute binding sites for regulatory proteins or directly mediate a biological process.[2] In addition to these classical roles in cell biology, RNA is emerging as a key regulator of gene expression[3] and has been shown to be capable of catalyzing a range of chemical reactions both in vivo and in vitro.[4] Because of these multiple functions of RNA in many biochemical contexts, powerful analytical tools are required to determine their structure, sequence, purity, concentration, and spatial distribution. Moreover, the rapidly growing demand for chemically synthesized RNA necessitates the elaboration of suitable protocols for high-throughput quality assurance. Both electrospray ionization mass spectrometry (ESI-MS)[5] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[6] have been successfully applied as analytical methods to the characterization of in vivo RNA transcripts,[7] transfer RNA,[8,9] synthetic RNA,[10] ribosomal RNA subunits,[11,12] and RNA−RNA complexes.[13]

One characteristic of RNAs is their high affinity for metal ions, particularly bivalent cations that stabilize proper secondary and

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10.1021/ac0487395 CCC: $30.25 
Published on Web 12/17/2004

tertiary structures.[14] This represents a significant problem in ESI-MS of RNAs, as the substitution of some of the protons in the sugar−phosphate backbone by metal cations results in the formation of a multitude of adducted species. Sensitive and accurate mass measurements of RNAs are severely hampered by such cation adduction, because the numerous signals for the higher charge states of adducted species merge into one broad peak shifted to higher mass relative to that of the fully protonated species. Effective removal of cations is, therefore, essential to obtain mass spectra of high quality from which the correct molecular masses of RNAs can be deduced. As a consequence, several purification and desalting protocols such as multiple ethanol precipitation,[8] addition of organic bases, [8,15] chelating agents,[8] or cation-exchange beads,[13] solid-phase extraction,[16] affinity adsorption,[10] denaturing polyacrylamide gel electrophoresis,[10,13] and liquid chromatography,[17−19] as well as various combinations of the aforementioned methods, have been devised for the removal of cations and other impurities.

The prime prerequisites that have to be met for the applicability of purification protocols to routine analysis of RNAs are high desalting efficiency, low sample requirement, high analyte recovery, and potential for full automation. In this regard, we have employed miniaturized ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) using monolithic, poly(styrene−divinylbenzene) (PS−DVB)-based capillary columns for the purification of oligodeoxynucleotides and double-stranded DNA prior to investigation by ESI-MS.[20] Nevertheless, while IP-RP-HPLC has been shown to efficiently remove cationic adducts from DNA, the affinity especially of $Mg^{2+}$ ions for RNA is still strong enough to endure the chromatographic process. And although Taniguchi and Hayashi have shown that HPLC of RNAs in 150 × 0.3 mm columns packed with PS−DVB perfusion particles and a gradient of acetonitrile in 0.3 mmol/L tributylammonium acetate (pH 5.5) enabled the successful characterization of prepurified transfer RNAs,[17] we found that the desalting efficiency of this approach was not sufficient for ESI-MS analysis of unpurified transfer RNAs or raw products of RNA solid-phase synthesis. In this paper, we therefore report on an attempt to combine on-line liquid chromatographic purification under denaturing conditions[17,21] and addition of chelating agents[8] to efficiently desalt RNAs for ESI-MS analysis. This combination is shown to be very effective, permitting highly accurate mass measurements with small amounts of both synthetic and biological RNAs. Finally, IP-RP-HPLC in combination with tandem mass spectrometry is utilized to derive sequence information about the purified RNAs.

## MATERIALS AND METHODS

**Chemicals and RNA Samples**. Acetonitrile (HPLC gradient grade) and disodium ethylendiaminetetraacetate dihydrate (EDTA; analytical reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO), and butyldimethylamine (analytical reagent grade) was purchased from Fluka (Buchs, Switzerland). A 0.50 M stock solution of butyldimethylammonium bicarbonate (BDMAB) was prepared by passing carbon dioxide gas (AGA, Vienna, Austria) through a 0.50 M aqueous solution of butyldimethylamine at 5 °C until pH 8.4 was reached. For preparation of all aqueous solutions, high-purity water (Epure, Barnstead Co., Newton, MA) was used.

5S ribosomal RNA from a 50S ribosomal subunit from *Escherichia coli* strain MRE600 was obtained from Sigma-Aldrich. Synthetic RNAs (32-mer, GGC GUU UUC GCC UUC GGG CGA UUU UUA UCG CU, $M_r$ = 10124.9; 55-mer, AGC GCC GAU GGU AGU GUG GGG UCU CCC CAU GCG AGA GUA GGG AAC UGC CAG GCA U, $M_r$ = 17837.9) were prepared by solid-phase synthesis[22] and used either without purification or with purification by means of anion-exchange HPLC (13-mer, 32-mer, 55-mer) or reversed-phase HPLC (21-mer).

The chemical synthesis and purification of aminoacylated tRNAs (protected *E. coli* Leu-$tRNA^{Gly}$-1), prepared by chemical ligation, GGG AGA AUA GCU CAG UUG GUA GAG CAC-(2′-*O*-NPEOM)-GAC CUU CUA AAG GUC GGG GUC GCG AGU UCG AGU CUC GUU UC-(2′-*O*-NPEOM)-U CCC UCC A-{(3′-*O*-NPEOM)-(2′-*O*-[(*N*-NPEOC)-Leu])}, $M_r$ = 25276.6, and the protected *E. coli* Leu-$tRNA^{Gly}$-2, prepared by enzymatic ligation, pGGG AGA AUA GCU CAG UUG GUA GAG CAC GAC CUU CUA AAG GUC GGG GUC GCG AGU UCG AGU CUC GUU UCU CCC UCC A-{(3′-*O*-NPEOM)-(2′-*O*-[(*N*-NPEOC)-Leu])}, $M_r$ = 24997.2; NPEOM = [1-(2-nitrophenyl)ethoxy]methyl; NPEOC = [1-(2-nitrophenyl)ethoxy]carbonyl) have been described in detail in refs 23−25.

**High-Performance Liquid Chromatography Interfaced with Electrospray Ionization Mass Spectrometry**. Monolithic capillary columns (60 × 0.20 mm i.d.) were prepared according to the published protocol.[21] The HPLC system consisted of a low-pressure gradient micropump (model Rheos 2000, Flux Instruments, Basel, Switzerland) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3 mm o.d. copper tubing which was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), and a microinjector (model C4-1004, Valco Instruments Co. Inc., Houston, TX) with a 500 nL internal sample loop.

ESI-MS was performed with a quadrupole ion trap mass spectrometer (LCQ, Thermo Finnigan, San Jose, CA) equipped with a modified nanospray ion source. The spray capillary (fused silica, 90 μm o.d., 20 μm i.d.; Optronis, Kehl, Germany) was directly connected to the capillary column by means of a T-piece (Upchurch Scientific, Oak Harbor, WA). A syringe pump equipped with a 250 μL glass syringe (Unimetrics, Shorewood, IL) and the T-piece were used for adding a makeup flow of 3.0 μL/min of acetonitrile. For analysis with pneumatically assisted ESI, an electrospray voltage of 5.0 kV and a nitrogen sheath gas flow of


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100 arbitrary units were employed. The temperature of the heated capillary was set to 250 °C. Total ion chromatograms and mass spectra were recorded on a personal computer with the LCQ Navigator software version 1.2 (Thermo Finnigan) with the biomass deconvolution module. Mass calibration and coarse tuning were performed in the positive ion mode by direct infusion of a solution of caffeine (Sigma, St. Louis, MO), methionylarginylphenylalanylalanine (Finnigan), and Ultramark 1621 (Finnigan). Fine-tuning for ESI-MS of oligonucleotides in the negative ion mode was performed by infusion of 3.0 μL/min of a 20 pmol/μL solution of $(dT)_{24}$ in 25 mmol/L aqueous BDMAB containing 10% acetonitrile (v/v).

## RESULTS AND DISCUSSION

**Effect of a Chelating Agent on Desalting Efficiency**. RNAs feature four potential sites for complexing metal ions, namely, the strongly acidic phosphodiester groups, the ribose hydroxyls, the nitrogens of the nucleobases, and the exocyclic keto groups of the nucleobases, the phosphate groups and the hydroxyl groups of which are the preferred binding sites.[26] As a consequence, RNAs have high affinity for cations both in aqueous solution and in the gas phase. Upon transfer of the RNA molecules from the liquid phase to the gas phase during electrospray ionization, cations may or may not remain associated with the RNAs. Different molecular species result with various degrees and types of cationization that differ in their molecular masses and show, depending on their charge states and the resolution of the employed mass analyzer, multiple or broadened signals in the mass spectrum. In the ion-pair reversed-phase chromatographic purification of RNAs using BDMAB as a mobile phase additive, the suppression of cationic adducts is based upon the exchange of metal cations with butyldimethylammonium ions from the mobile phase during the chromatographic process. In contrast to metal ions, butyldimethylammonium ions may dissociate into volatile butyldimethylamine and a proton that is left with the analyte molecules in the course of desolvation of analytes through the electrospray process, resulting in the reduction of metal cation-adducted species.

On the basis of the efficacy of desalting by means of IP-RP-HPLC, which enabled the mass spectrometric investigation of low femtomole amounts of double-stranded DNA restriction fragments up to a size of 500 base pairs,[21,27] we could expect that the method should perform equivalently in the analysis of RNAs. The analysis of 14 pmol of a synthetic 55-mer RNA by IP-RP-HPLC−ESI-MS is illustrated in Figure 1a. The sample was chromatographed at 70 °C with a gradient of 4−56% acetonitrile in 25 mmol/L BDMAB in 10 min. The poor signal-to-noise ratio in the raw mass spectrum (Figure 1a) as well as the multiple broad peaks in the deconvoluted mass spectrum (inset in Figure 1a) clearly proved that desalting was far from sufficient. Moreover, the lowest molecular mass of 17876 Da observed in the mass spectrum differed by 39 Da from the theoretical mass of 17837.9 Da of the 55-mer and indicated the prevalence of a potassium adduct. Two more intense signals were observed in the deconvoluted mass spectrum, which

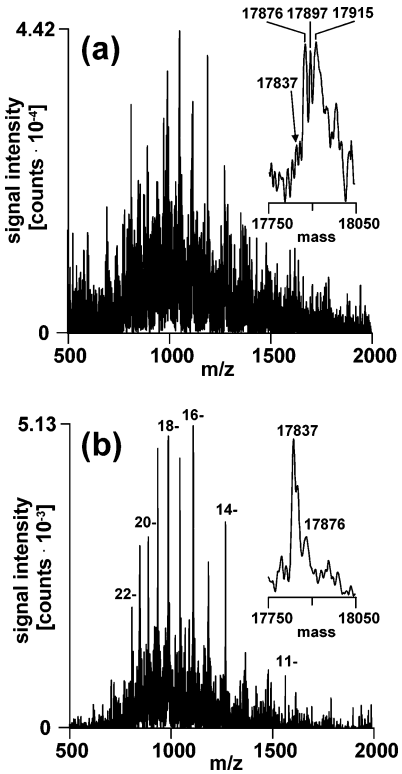


**Figure 1.** IP-RP-HPLC−ESI-MS of synthetic 55-mer RNA: column, PS-DVB monolith, 60 × 20 mm i.d.; mobile phase, (A) 25 mmol/L BDMAB, pH 8.40, (B) 25 mmol/L BDMAB, pH 8.40, 80% acetonitrile; linear gradient, 5−70% B in 10.0 min; flow rate, 2.0 μL/min; temperature, 70 °C; sheath liquid, 3.0 μL/min acetonitrile; sheath gas, $N_2$, 100 units; scan range, 500−2000 u; electrospray voltage, 5.0 kV; sample, (a) 14 pmol dissolved in water, (b) 14 pmol dissolved in 25 mmol/L EDTA solution.

were offset by 59 and 78 Da from the fully protonated molecule, probably mixed sodium/potassium and bipotassium adducts. The adduct-free species was visible in the mass spectrum only as a very small signal. Clearly, additional measures had to be taken to improve mass spectral quality.

The potential of removing cations from RNA by complexation with chelating agents such as EDTA, nitrilotriacetic acid, or *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid has been demonstrated by Limbach et al.[8] The agents were added at an approximately 3-fold molar excess to solutions of RNA, which had been prepurified by ethanol precipitation before their investigation by direct infusion ESI-MS. In this approach, however, the excess of complexing agent has to be kept as low as possible, because the agent is not separated from the analytes during direct infusion analysis, which may result in signal suppression due to competitive ionization. Chromatographic purification of RNAs may be advantageous in that complexed cations and excess chelating agents elute in the flow-through, while purified RNA is first retained on the column and subsequently eluted by an acetonitrile gradient.

Such an experiment is demonstrated in Figure 1b, in which the mass spectrum obtained by IP-RP-HPLC−ESI-MS analysis upon addition of EDTA to a final concentration of 25 mmol/L to a 28 μmol/L sample solution (900-fold molar excess) is shown. Multiply charged ions with charge states from 11− to 22− were clearly distinguishable in the mass spectrum and deconvoluted into an intact molecular mass of 17837 Da, which deviated only

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0.0050% from the theoretical molecular mass of the fully protonated species. Nevertheless, a monopotassium adduct at about 30% relative abundance was still observed in the deconvoluted mass spectrum. This result confirms earlier investigations, which showed that also monovalent cations, especially potassium, are very important in forming secondary and tertiary structures and can be found in tetraloops with a strong binding affinity.[28] Variation of the amount of EDTA added to the sample solution revealed that a minimum concentration of 1 mmol/L was necessary to induce effective cation complexation. Furthermore, increasing the concentration to 25 mmol/L had no untoward effect on chromatography, while desalting efficiency was slightly increased. Hence, we recommend the addition of 20−25 mmol/L EDTA for efficient cation removal, especially in the case of samples containing high salt loads.

The differences between RNA and DNA in the conditions required for efficient removal of cations probably rest within the divergence of the three-dimensional structures of the molecules. The high negative charge density of nucleic acids attracts a dense "cloud" of positive counterions which are usually delocalized and not associated with specific sites. These delocalized ions interact primarily through long-range Coulombic forces with the sugar−phosphate backbone and remain fully hydrated. The strength of interaction is determined by the charge of the ion and the magnitude of the electrostatic field. Double-helical DNA and regular single-stranded RNA are surrounded by such delocalized ions that can be rather easily replaced by other cations. This can change significantly in RNA with a globular structure containing pockets that are able to chelate the cations and displace some or all of its shell of tightly bound water molecules. This direct chelating of the ions makes it very difficult for mobile-phase additives to exchange the metal ions with protons.[29]

**Desalting of Ribosomal RNA**. To evaluate the effectiveness of desalting for larger RNAs forming quite stable secondary structures, we utilized the 120-nucleotide ribosomal 5S RNA (5S rRNA) subunit as analyte for IP-RP-HPLC−ESI-MS analysis. Figure 2 illustrates the analysis of 2 pmol (75 ng) of 5s rRNA strain MRE600 from *E. coli*. The components were separated at 70 °C with a gradient of 4−56% acetonitrile in 25 mmol/L BDMAB in 10 min without and with the addition of EDTA to the sample solution prior to injection (Figure 2a,d). In the absence of EDTA, the spectrum revealed charge states from 21− to 51− with very poor signal-to-noise ratios. The two molecular masses of 38 915 and 38 957 Da obtained from the deconvoluted mass spectrum were significantly higher with respect to the expected masses of the target compounds of 38 814.4 and 38 853.4 Da, which could be found only as a small peak and a shoulder in the mass spectrum (Figure 2c). This result demonstrates that cation adduction is also prevalent in biological RNA samples, necessitating additional desalting. Figure 2d depicts the chromatogram of the sample after addition of EDTA. Due to the elution of metal ion−EDTA complexes and excess EDTA, the intensity of the flow-through peak increased significantly, while that of the RNA peak remained practically constant. The effect of cation reduction can be clearly seen in the improved quality of the raw mass spectrum (Figure 2e), from which the molecular masses of 38 812 and 38 852 Da

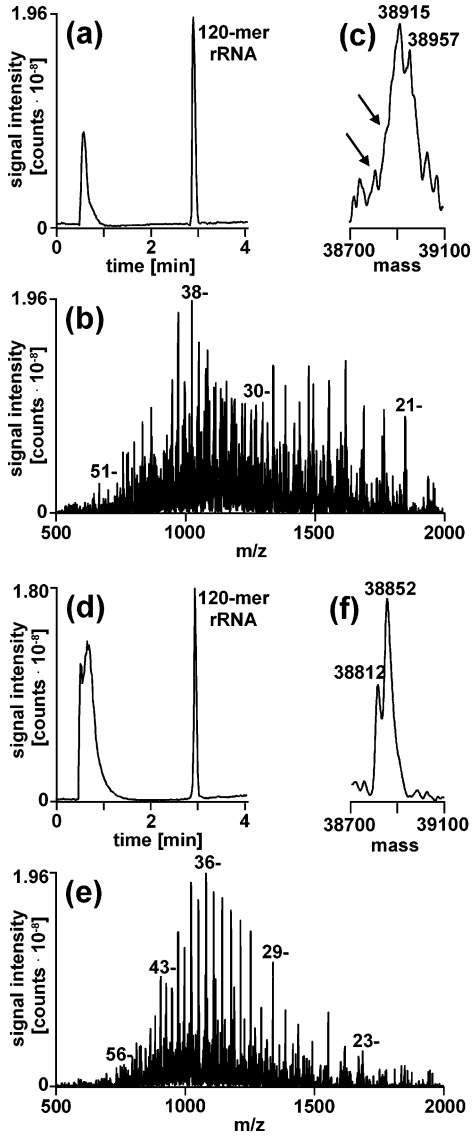


**Figure 2.** Analysis of 5S ribosomal RNA from *E. coli* strain MRE600: sample, (a) 2 pmol of 5S rRNA in water, (b) 2 pmol of 5S rRNA dissolved in 25 mmol/L EDTA solution. Other conditions as in Figure 1.

were deduced with no signs of cation adduction (Figure 2f). The two detected masses correspond to transcripts of the two 5S ribosomal RNA genes that occur in *E. coli* and correspond excellently to the theoretical molecular masses.[30]

In a previous report, HPLC was not recommended as a suitable purification method for RNA, mainly because of a large sample requirement, low analyte recovery, and possible degradation of RNAs in the presence of heavy metal ions.[8] Nevertheless, using an HPLC pump with all wetted parts made of titanium or sapphire and a stainless steel injector which has been passivated with 1 mol/L nitric acid, we observed neither degradation products nor any signs of sample loss, as evidenced by blank injections that showed no memory signals. In the next step we evaluated the limits of detection of the IP-RP-HPLC−ESI-MS method for the analysis of large RNA molecules by analyzing a sample of 160 fmol of a 5S RNA sample containing 25 mmol/L EDTA. The

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quality of the raw mass spectrum was adequate to calculate the two masses with relative mass deviations of 0.0060% and 0.011%, respectively. On the basis of signal-to-noise ratios in the mass spectrum of approximately 10:1 to 6:1, the detection limit for the 5S RNA using this analytical system was estimated to be 50−80 fmol. With analysis times of less than 5 min, including sample preparation, this example clearly demonstrates the potential of on-line combined separation and analysis using IP-RP-HPLC−ESI-MS compared to other commonly applied techniques that require both more time and more sample material.

**Application to the Quality Control of Synthetic Oligoribonucleotides**. Synthetic oligoribonucleotides are considered as potential therapeutic agents for artificial regulation of gene expression.[31] Recent experiments have shown that double-stranded RNAs of approximately 20−25 nucleotides are able to induce posttranscriptional gene silencing in animals and plants. It has been demonstrated that short-chain RNA duplexes specifically suppressed expression of endogenous and heterologous genes in different mammalian cell lines.[32] The most prominent byproducts of chemical oligoribonucleotide synthesis are failure sequences resulting from incomplete chain elongation during solid-phase synthesis and partially protected sequences due to incomplete removal of protecting groups after complete assembly of the target sequence. As an example for the analysis of synthetic oligoribonucleotides, Figure 3a illustrates the reconstructed ion current chromatogram of a deprotected raw product of a synthetic 21-mer. Upon application of a shallow gradient of 2−12% acetonitrile in 25 mmol/L BDMAB in 10 min, the analytical system was capable of separating and identifying sequences from 7- to 20-mers together with the target product within 7 min. The results of the mass determinations and the identification of the different sequences, as well as the relative mass deviations, are summarized in Table 1. The mass spectrum extracted from the peak eluting at 3 min showed a predominant signal at $m/z$ 1035.4 representing the 2− charge state of the eluting oligoribonucleotide. A molecular mass of 2072.8 Da was calculated and correlated with the mass of the 7-mer, which constitutes the smallest detectable failure sequence. The following peaks were identified as the 8−21-mers, only the 12- and 13-mers, the 17- and 18-mers, and the 19-, 20-, and 21-mers of which coeluted at least partially.

The relatively high abundance of failure sequences, especially from 12- to 20-mers, indicates that the average coupling efficiencies during synthesis should be at least 98% to obtain the target in reasonable yields. Since the target sequence is generally the longest sequence generated by solid-phase synthesis, products eluting after the target sequence are most likely sequences from which removal of protecting groups was not exhaustive. A mass spectrum extracted from the peak eluting at 7 min yielded a molecular mass of 6707.2 Da, equivalent to a mass difference of 54.8 Da relative to the mass of the target sequence. This mass difference is compatible with an additional cyanoethyl protecting group attached to the 21-mer target sequence (theoretical mass difference 54.1 Da) and indicated that the removal of the cyanoethyl groups by elimination was incomplete.

The raw oligoribonucleotide obtained after solid-phase synthesis usually has to be purified by preparative gel electrophoresis or chromatography. In our example, the raw product was chromatographed at room temperature on a 250 × 4.6 mm column packed with an octadecyl stationary phase with a gradient of acetonitrile in 0.1 mol/L triethylammonium acetate. The peak of the main product was isolated and lyophilized. The success of preparative purification was checked by capillary IP-RP-HPLC−ESI-MS (Figure 3b). The chromatogram showed two dominant peaks in which four different oligoribonucleotides were identified on the basis of their molecular masses. Coeluting with the target

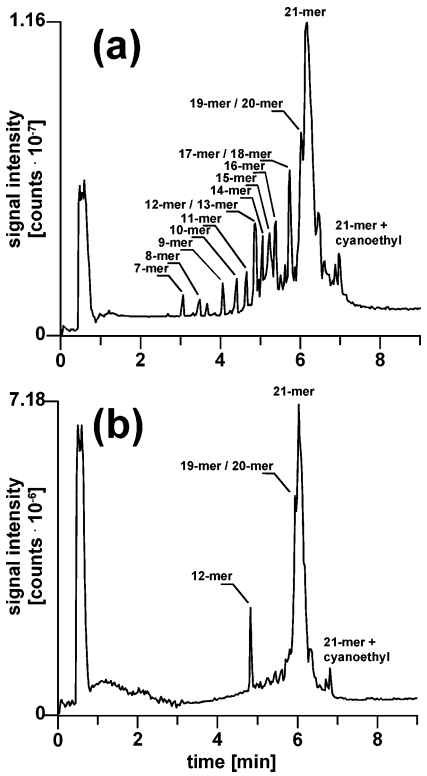


**Figure 3.** Quality control of a synthetic 21-mer oligoribonucleotide: mobile phase, (A) 25 mmol/L BDMAB, pH 8.40, (B) 25 mmol/L BDMAB, pH 8.40, 40% acetonitrile; linear gradient, 5−30% B in 10 min; sample, (a) 250 ng of raw product, (b) 250 ng of the product purified by reversed-phase chromatography at 25 °C dissolved in 25 mmol/L EDTA solution. Other conditions as in Figure 1.

**Table 1. Measured and Theoretical Masses of a Synthetic 19-mer and Its Failure and Partially Deprotected Sequences**

| oligonucleotide | retention time (min) | molecular mass (Da) measured | molecular mass (Da) theoretical | rel dev (%) |
|---|---|---|---|---|
| 7-mer | 3.09 | 2072.8 | 2074.3 | −0.075 |
| 8-mer | 3.51 | 2418.8 | 2419.5 | −0.032 |
| 9-mer | 4.09 | 2751.5 | 2748.7 | 0.100 |
| 10-mer | 4.44 | 3078. 5 | 3078.0 | 0.018 |
| 11-mer | 4.70 | 3407.3 | 3407.2 | 0.003 |
| 12-mer | 4.91 | 3736.5 | 3736.4 | 0.003 |
| 13-mer | 4.91 | 4041.3 | 4010.6 | −0.008 |
| 14-mer | 5.09 | 4347.4 | 4347.7 | −0.008 |
| 15-mer | 5.27 | 5654.1 | 4653.9 | 0.005 |
| 16-mer | 5.43 | 5000.3 | 4999.1 | 0.022 |
| 17-mer | 5.78 | 5329.1 | 5328.3 | 0.015 |
| 18-mer | 5.78 | 5673.0 | 5673.6 | −0.010 |
| 19-mer | 6.07 | 6003. 0 | 6002.8 | 0.004 |
| 20-mer | 6.07 | 6307.9 | 6309.0 | −0.001 |
| 21-mer | 6.22 | 6654.0 | 6653.2 | 0.013 |
| 21-mer + cyanoethyl | 7.03 | 6708.0 | 6707.2 | 0.011 |

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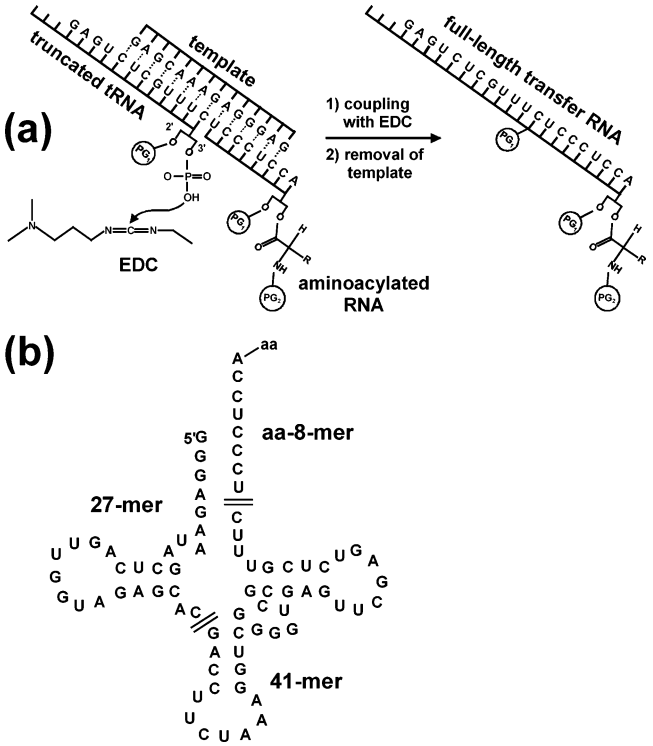


**Figure 4.** (a) Total synthesis of a 76-mer aminoacylated transfer RNA by ligation of a 27-mer, a 41-mer, and an aminoacylated 8-mer RNA. Only the coupling reaction between the 41-mer and the aminoacylated 8-mer is shown. PG = protecting group, and EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide. The template is a 2′-*O*-methylated RNA with a sequence complementary to that to be ligated. (b) Structure of the synthetic Leu-tRNA$^{Gly}$.

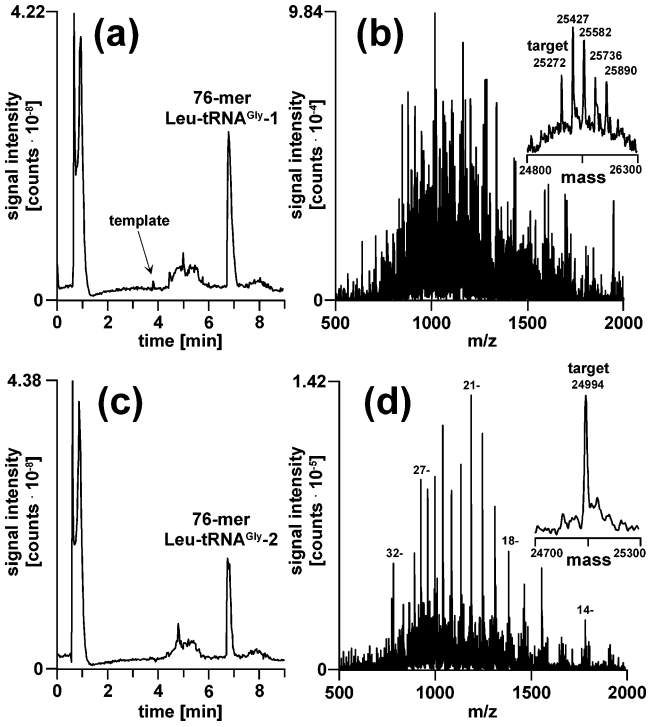


**Figure 5.** Analysis of synthetic *E. coli* Leu-tRNA$^{Gly}$ obtained by (a) chemical (Leu-tRNA$^{Gly}$-1) and (b) enzymatic (Leu-tRNA$^{Gly}$-2) ligation of 27-mer, 41-mer, and aminoacylated 8-mer RNA blocks: sample, 250 ng of product dissolved in 25 mmol/L EDTA solution. Other conditions as in Figure 1.

sequence were the 19- and 20-mer sequences, which could obviously not be sufficiently separated by preparative reversed-phase HPLC. All other failure sequences have been removed quite efficiently, except the 12-mer that eluted as a clear and abundant peak at 4.9 min. This surprising finding could be explained by a strong interaction between the 12-mer and the longer sequences, which resulted in a shift of elution of the 12-mer toward longer retention time in the preparative separation, which was performed under nondenaturing conditions. Moreover, the small peak of the mono(cyanoethyl)-protected 21-mer was still present. To prepare RNA sequences of good quality, HPLC purifications, even of relatively short sequences, need to be carried out under denaturating conditions (e.g., anion-exchange HPLC at pH 11.5 and 25 °C or at pH 7 and 80 °C),[31] thereby avoiding the formation of structures which result in peak-broadening and insufficient separation of failure sequences and partially protected products.

**Verification of the Total Chemical Synthesis of Aminoacyl-tRNA**. In the translation of the genetic code into an amino acid sequence, aminoacyl transfer RNAs serve as the carriers of the amino acids during the ribosomal biosynthesis of proteins. The total chemical synthesis of transfer RNAs facilitates the specific incorporation of alternative or modified amino acids, which constitutes a powerful tool for site-directed modification and mutagenesis. Automated solid-phase synthesis enables, in principle, the assembly of linear chains of polynucleotides up to chain lengths of 100 and more.[22,33] Nevertheless, because of low total yields even at high coupling efficiencies per individual cycle as well as contaminations with failure sequences that are difficult to remove from the target, the total synthesis of tRNA is very challenging. Hence, it is strongly advisable to assemble the biopolymers by the conjunction of smaller pieces of oligomers that are easier to synthesize and purify. The preparation of aminoacyl transfer RNA described in this example includes the synthesis and purification of two RNA blocks and an aminoacyl-RNA fragment and their subsequent ligation as outlined schematically in Figure 4.

Figure 5 illustrates the IP-RP-HPLC−ESI-MS analysis of 250 ng (~10 pmol) of the transfer RNA product after purification by polyacrylamide gel electrophoresis. The sample was separated at 70 °C with a gradient of 4−56% acetonitrile in 25 mmol/L BDMAB in 10 min. While both slab gel electrophoresis and chromatography using UV detection revealed only a single band or peak, respectively, mass spectrometry readily identified additional components in the sample mixture. The deconvoluted spectrum of the product peak that eluted at 6.85 min showed the target product with a molecular mass of 25472 Da as well as at least four additional masses with differences of 155, 310, 464, and 618 Da relative to the mass of the target product (Figure 5b). These mass differences are compatible with the addition of one, two, three, and four 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) molecules to the target aminoacyl transfer RNA (theoretical mass differences 155.2, 310.5, 465.8, and 621 Da). EDC has been used as a coupling reagent during synthesis of the transfer RNA.[25] A plausible side reaction to explain the addition of EDC is the reaction with amino groups of the nucleobases, resulting in guanidine derivatives. The relative peak intensities in the decon-

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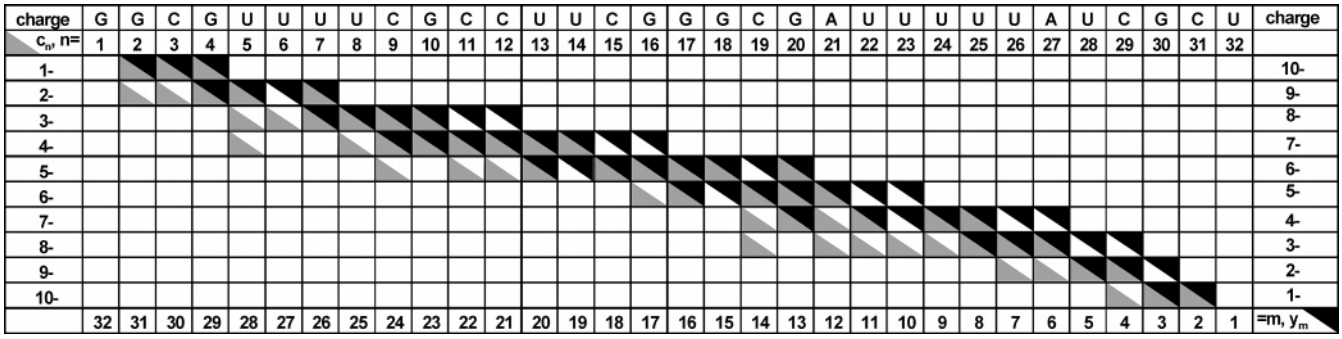


**Figure 6.** Sequence coverage diagram for the tandem mass spectrometric sequencing of a 32-mer oligoribonucleotide: mobile phase, (A) 25 mmol/L BDMAB, pH 8.40, (B) 25 mmol/L BDMAB, pH 8.40, 40% acetonitrile; linear gradient, 5−70% B in 10 min; isolated *m*/*z*, 1011.3; isolation width, 4 u; relative collision energy, 18%; sample, 12 pmol dissolved in 20 mmol/L EDTA solution. Other conditions as in Figure 1. Gray triangles represent fragments of the c ion series and black triangles those of the y ion series identified in the tandem mass spectrum.

voluted mass spectrum suggest that the products with one and two adducted EDCs were those of highest abundance in the reaction mixture. The small peak at 3.85 min showing a molecular mass of 4657.8 Da was identified as the short template RNA (theoretical mass 4659.1 Da).

On the basis of the results of the previous analysis, assembly of the transfer RNA was improved by means of enzymatic ligation of the building blocks using T4-DNA-ligase.[23] The ligated product was purified by means of polyacrylamide gel electrophoresis and subjected to characterization by IP-RP-HPLC−ESI-MS (Figure 5c,d). Both the chromatogram and mass spectrum showed that there is a relatively pure main product eluting at 6.8 min, which represents the target molecule having a molecular mass of 24997.2 Da. Please note that the molecular masses of the chemically and enzymatically ligated target products are different due to phosphorylation of the latter and differences in the protecting groups. At this point we want to emphasize that neither polyacrylamide electrophoresis nor ion-exchange or ion-pair reversed-phase chromatography was able to reveal the side reaction in the chemical ligation. Hence, chromatographic separation in combination with mass spectrometry represents a very powerful tool for both optimization and structure verification in the total synthesis of macromolecular RNA.

**Mass Spectrometric Sequencing of Synthetic RNA.** The past two decades have seen nucleic acid sequencing evolve from specialized, complicated, and labor-intensive procedures to fully automated, high-throughput, commonplace techniques. With the advent of soft ionization techniques for oligonucleotides[34,35] in combination with fragmentation by collision-induced dissociation, tandem MS has become an alternative method to conventional ladder sequencing. The fragmentation reactions of oligodeoxyribonucleotides[36−38] and oligoribonucleotides[11,19,39] upon collisional activation have been studied extensively. Nevertheless, sequencing by tandem MS has been usually performed by direct infusion of prepurified RNA samples, requiring relatively large amounts of sample material. As a consequence, we aimed at applying our miniaturized and fully automatable approach of on-line purification by capillary HPLC to the generation of sequence data by tandem MS.

In an attempt to obtain sequence information about a 32-mer oligoribonucleotide by IP-RP-HPLC−ESI-MS/MS, a 12 pmol sample was purified and the 10− charge state was selected for fragmentation with a relative collision energy of 18%. Assignment of the generated fragments in the mass spectra revealed that only a few a-B and w ions, which are the most common fragments in spectra of oligodeoxyribonucleotides, and mostly c and y ions were generated, which confirms published results obtained with shorter oligoribonucleotides.[19] The sequence coverage with c and y fragments is shown in Figure 6, which reveals that each position in the RNA sequence is covered by at least two, but in most cases even more, c and y fragment ions having different charge states. This example clearly demonstrates that ion trap tandem mass spectrometry is suitable for the generation of sequence information about longer RNAs. However, due to the limited mass accuracy and mass resolution of ion trap mass analyzers and the mass difference of only 1 Da between cytosine and uridine, it will be difficult to derive unequivocal de novo sequence information for oligoribonucleotides longer than approximately 15 nucleotides.

## CONCLUSIONS

IP-RP-HPLC using monolithic capillary columns is highly suitable for the on-line sample preparation and purification of femtomole amounts of both synthetic and biological ribonucleic acids for subsequent investigation by ESI-MS. The main advantages of this technology rest within the high robustness of the monolithic purification system, the minimal amount of sample required, the time saving because no other sample preparation step is required, and its applicability to the fully automated routine analysis of RNA molecules ranging in length from a few to more than 120 nucleotides. RNA differs significantly from DNA in its ability to form relatively strong complexes with cations, which significantly impairs ESI-MS investigations of RNA. As a consequence, strong competitive chelating agents have to be added to RNA samples to remove cationic adducts. Since the distinction of RNAs is based on differences in both chromatographic retention and molecular mass, IP-RP-HPLC−ESI-MS may be regarded as a

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two-dimensional analytical system with enhanced selectivity and specificity as compared to chromatographic or mass spectrometric methods alone. Finally, sequence information may be obtained for RNAs upon chromatographic purification, collisionally induced dissociation, and mass analysis of the obtained fragment ions. In contrast to DNA, which predominantly fragments into ions of the a-B and w types, c and y ions are identified as the most abundant fragments in tandem mass spectra of RNA. The mass spectrometric sequencing of RNA oligonucleotides longer than 15 nucleotides is more challenging compared to its DNA counterpart, because of difficulties in the discrimination of cytosine and uridine differing only by 1 Da.


## ACKNOWLEDGMENT

This work was supported by a grant from the Austrian Science Fund (P14133-PHY).

Received for review August 24, 2004. Accepted October 29, 2004.

AC0487395