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Current trends in liquid chromatography-mass spectrometry analysis of small interfering RNAs: A short review

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ABSTRACT

Small interfering RNA (siRNA) therapeutics represent one of the most rapidly advancing pharmaceutical modalities. Since the approval of the first siRNA-based drug in 2018, seven siRNA therapeutics have been approved to date. During manufacturing, "non-optimal duplexes" and "single-stranded impurities" can arise from synthetic byproducts such as shortmers and longmers, or from minor imbalances in strand quantification and mixing. Furthermore, phosphorothioate linkages introduce chirality, generating complex diastereomeric mixtures. The use of delivery systems, including lipid nanoparticles or *N*-acetylgalactosamine (GalNAc) conjugates, further increases the structural complexity and heterogeneity of the final product. Compared to single-stranded oligonucleotides, siRNA—being double-stranded—yields more complex chromatographic and mass spectral profiles, making analytical characterization particularly challenging. Therefore, the development of robust and reliable analytical methods is essential to ensure the quality of siRNA therapeutics. This review summarizes key analytical techniques for the quality evaluation of siRNA therapeutics. Liquid chromatography (LC) is one of the most widely used approaches, with separation modes such as ion-pair reversed-phase (IP-RP), anion exchange (AEX), size exclusion chromatography (SEC), and hydrophilic interaction chromatography (HILIC) being actively explored. Mass spectrometry (MS) is another powerful tool that provides molecular mass-based structural information not accessible *via* UV detection alone. The combination of LC and MS offers a highly effective platform for characterizing siRNA therapeutics, enabling both qualitative and quantitative assessment of impurities and isomers. Furthermore, tandem MS (MS/MS) can provide sequence-specific information for the identification of active pharmaceutical ingredients. In addition to quality assessment, the application of LC and MS techniques in the pharmacokinetic analysis of siRNA therapeutics is also briefly discussed.

1. Introduction

Small interfering RNA (siRNA) therapeutics are being actively studied as a promising class of oligonucleotide-based drugs, and their practical application is steadily progressing [1,2]. Since the approval of *patisiran* in 2018 as the first siRNA therapeutic, a total of seven drugs—*patisiran, givosiran, lumasiran, inclisiran, vutrisiran, nedosiran*, and *fitusiran*—have been approved to date (Table 1). siRNA is a short double-stranded oligonucleotide composed of two strands: the sense (or passenger) strand and the antisense (or guide) strand. Each strand is approximately 20 nucleotides in length and typically contains a two-base overhang at 3′-terminus, which remains unpaired and single-stranded. siRNA exerts its gene-silencing effect through the RNA interference (RNAi) mechanism [3]. It forms a complex with Argonaute 2 (Ago2) and some other proteins, known as the RNA-induced silencing complex (RISC), in which only the antisense strand is retained. This complex binds to complementary mRNA sequences and induces cleavage, thereby inhibiting protein translation.

As with other oligonucleotide therapeutics, unmodified siRNA is easily degraded in vivo and is not efficiently delivered to target tissues. Therefore, chemical modifications to the phosphate backbone or sugar moiety, as well as the use of delivery systems such as conjugates or lipid-based formulations, are essential for therapeutic application. One commonly used modification is the phosphorothioate (PS) linkage, in which a non-bridging oxygen atom of the phosphodiester (PO) linkage is replaced with a sulfur atom, thereby improving nuclease resistance [2,4,

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5]. Sugar modifications, such as substitution of the 2′-hydroxyl group with 2′-*O*-methyl (2′-OMe) or 2′-fluoro (2′-F), are also widely applied [2, 5]. In some cases, siRNA is encapsulated in lipid nanoparticles (LNPs) to protect it from nucleases and enhance cellular uptake [6]. Additionally, *N*-acetylgalactosamine (GalNAc) conjugation is employed to enable targeted delivery to the liver *via* the asialoglycoprotein receptor, which is abundantly expressed on hepatocytes [7].

In general, single-stranded oligonucleotides are chemically synthesized through the phosphoramidite method, which proceeds from the 3′ to the 5′ end [8,9]. The process begins with the removal of a protecting group from the initial hydroxyl functionality on the solid support, followed by coupling of a phosphoramidite monomer in the presence of an activator. The resulting phosphite triester is then subjected to either oxidation or sulfurization to form a phosphodiester or phosphorothioate linkage, respectively. After coupling, the protecting group of the newly added monomer is removed to prepare for the next cycle. This iterative process is repeated until the desired oligonucleotide sequence is synthesized. Various impurities can arise during this process. Since this method is carried out on solid supports, opportunities for intermediate purification are limited. Moreover, due to the relatively high molecular mass of the final product (several thousands to tens of thousands of daltons), chromatographic separation of structurally similar impurities becomes difficult. As a result, oligonucleotide therapeutics tend to contain more residual impurities compared to small molecule drugs. Common examples include shortmers and longmers (strand truncations or additions), and PS→PO impurities (where PS sulfur atoms are replaced by oxygen). These and other impurity types are summarized in Table 2 [10–12].

Even for single-stranded antisense oligonucleotides (ASOs), which have simpler structures than siRNA, the removal and accurate quantification of such impurities remain challenging. For siRNA, an additional annealing step is required to form the duplex, wherein the sense and antisense strands are mixed, heated, and gradually cooled to promote the formation of the thermodynamically stable duplex. As a result, siRNA therapeutics contain impurity profiles unique to double-stranded oligonucleotides. These include: (i) excess single strands resulting from slight mismatches in strand mixing ratios, (ii) non-optimal duplexes containing defective strands such as shortmers, PS→PO impurities, or 2′,3′-isomers, and (iii) side products generated during the annealing process, such as duplexes containing shortmers and 2′,3′-isomers. In addition to these impurities, other factors arising from the complexity of the active pharmaceutical ingredient (API) and formulation components can affect the quality of siRNA therapeutics. For example, PS modifications introduce a chiral phosphorus center, and the *R*p and *S*p diastereomers can differ in nuclease stability and RISC-loading efficiency [5,13,14]. While the analysis of diastereomeric distribution is important, the physicochemical similarities between these isomers make their separation extremely challenging. Similarly, LNPs require careful assessment of particle size distribution and siRNA loading efficiency, both of which influence drug efficacy. Quantification of LNP components—such as phospholipids, cholesterol, ionizable amino lipids, and PEG-lipids—as well as their degradation products, is also necessary for quality assurance. Furthermore, conjugated siRNA molecules, which often have higher molecular mass and distinct physicochemical properties, can complicate chromatographic analysis due to altered retention behavior.

Although liquid chromatography (LC) has been the most widely used technique for quality evaluation of oligonucleotide therapeutics, most studies have focused on ASOs, which are short single-stranded oligonucleotides [10,11,15]. Fewer analytical methods have been reported for siRNA. The duplex nature of siRNA makes its analysis more complex than that of single-stranded oligonucleotides. Some considerations must be taken into account when establishing analytical conditions for siRNAs, as follows. (i) Because siRNA is a polyanion possessing approximately 40 negative charges on the phosphate backbone, it shows little or no retention on conventional reversed-phase columns, which are widely used for various analyses. Therefore, the use of ion-pairing reagents is required to achieve retention. Other approaches, such as ion-exchange chromatography employing columns with positively charged functional groups, are also useful. (ii) siRNA forms a higher-order structure through non-covalent bonds, including Watson–Crick hydrogen bonding and base stacking. When maintaining the duplex form during LC analysis is required, conditions such as column temperature, mobile phase pH, and the ratio of organic solvents must be carefully optimized to prevent dissociation of base pairing and stacking interactions. (iii) siRNA is generally classified as a medium-sized molecule, with molecular masses typically ranging from several thousand to around 15,000, which are considerably larger than those of small-molecule drugs. Due to this intermediate molecular size and structural complexity, separation of closely related impurities is often more challenging than for small molecules. Additionally, because siRNA consists of two different strands, a broader range of peaks may appear than with single-stranded ASOs. (iv) Delivery tools, such as GalNAc and antibody, may be conjugated to siRNAs, and their physicochemical properties are completely different from those of the polyanionic siRNA itself. Depending on the characteristics of the delivery tool (e.g., hydrophobicity and molecular size), the retention behavior during chromatography may differ significantly from that of the naked siRNA molecule.

Mass spectrometry (MS) is frequently employed to confirm the sequence of the API and to infer impurity structures based on molecular mass. However, siRNA generates more complex mass spectra than ASOs, making interpretation more difficult. Despite these analytical

Table 1
siRNA therapeutics approved by FDA.

Name (market name)	FDA Approval	Indication	Sugar modification	Phosphate modification	Delivery system
Patisiran (Onpattro)	2018	Polyneuropathy of hereditary transthyretin-mediated amyloidosis	2′-OMe	-	LNP
Givosiran (Givlaari)	2019	Acute hepatic porphyria	2′-OMe, 2′-F	PS	GalNAc conjugate
Lumasiran (Oxlumo)	2020	Primary hyperoxaluria type 1	2′-OMe, 2′-F	PS	GalNAc conjugate
Inclisiran (Leqvio)	2021	Heterozygous familial hypercholesterolemia Atherosclerotic cardiovascular disease	2′-OMe, 2′-F	PS	GalNAc conjugate
Vutrisiran (Amvuttra)	2022	Polyneuropathy of hereditary transthyretin-mediated amyloidosis	2′-OMe, 2′-F	PS	GalNAc conjugate
Nedosiran (Rivfloza)	2023	Primary hyperoxaluria type 1	2′-OMe, 2′-F	PS	GalNAc conjugate
Fitusiran (Qfitlia)	2025	Hemophilia A or B	2′-OMe, 2′-F	PS	GalNAc conjugate

"2′-OMe" indicates 2′-*O*-methyl, "2′-F" indicates 2′-fluoro, "PS" indicates phosphorothioate, "LNP" indicates lipid nanoparticle, and "GalNAc" indicates *N*-acetylgalactosamine.

Table 2
Impurities contained in siRNA therapeutics.

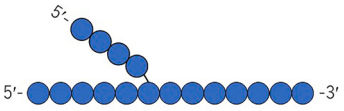
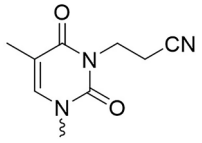
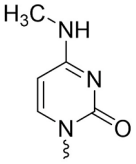
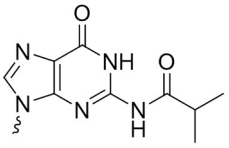
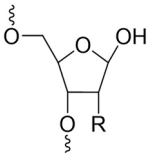
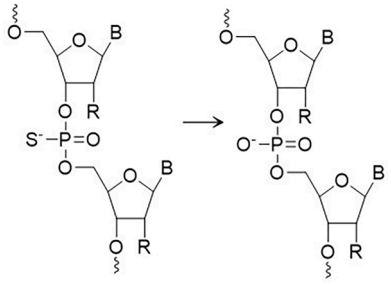
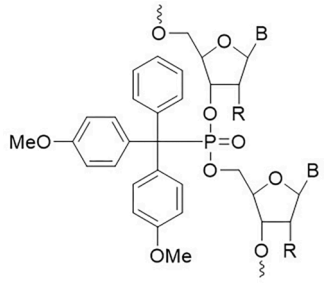
Type of impurity		Structure
Impurities related to phosphoramidite coupling	Shortmer	
	Longmer	
	Branchmer	
Impurities related to nucleobases	Cyanoethyl adduct	
	N4-methylcytidine	
	Isobutyryl adduct	
	Depurination	
Impurities related to phosphodiester backbone	Conversion of PS to PO	
	DMTr-phosphonate	

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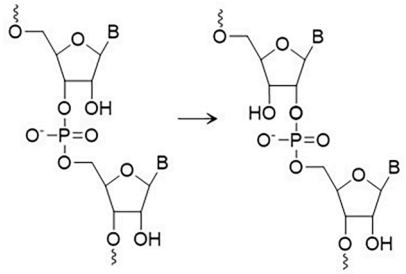
Table 2 (*continued*)

Type of impurity	Structure
2′,3′-isomer	

"PS" indicates phosphorothioate, "PO" indicates phosphodiester, and "DMTr" indicates 4,4′-dimethoxytrityl.

challenges, interest in siRNA analysis is growing as clinical implementation advances.

In this review, we summarize recent studies on siRNA analysis using LC and MS techniques, with a particular focus on their applications in impurity profiling and quality control. Applications in pharmacokinetic analysis are also briefly discussed. The chromatographic separation modes used in each study are compiled in Table 3, and the key characteristics of the major separation modes for siRNA analysis are summarized in Table 4.

2. Classification of chromatographic modes for siRNA and its impurities: denaturing vs. non-denaturing conditions

HPLC-based analytical methods for siRNA can generally be classified into two categories: denaturing chromatography and non-denaturing chromatography.

In *denaturing chromatography*, double-stranded siRNA is denatured into two single strands within the column, which then elute separately based on their retention characteristics. Denaturation is induced by analytical conditions such as elevated column temperatures, alkaline mobile phases, organic solvents, or ion-pairing reagents. When preservation of the duplex structure is not necessary, a broader range of analytical conditions can be applied. Conditions that promote sharper

Table 3
Separation modes utilized for siRNA analysis and their applications.

Application			Modification	Separation mode	Reference
Impurity analysis	Excess single strand		Natural	Non-denaturing IP-RP	[17]
			PS, 2′-OMe	Non-denaturing IP-RP	[18]
			PS, 2′-OMe	SEC	[18]
			Natural	Non-denaturing AEX	[19,28]
			Natural	SEC	[37]
			PS, Cyclic-peptide	SEC	[38]
			Natural	HILIC	[41]
			PS, 2′-OMe, 2′-F	Non-denaturing IP-RP	[42]
			PS, 2′-OMe, 2′-F	SEC	[42]
	Non-optimal duplex	Shortmer	Natural	Non-denaturing IP-RP	[17]
		Shortmer, longmer, 2′,3′-isomer	PS, 2′-OMe	Non-denaturing IP-RP	[18]
		Shortmer	Natural	Non-denaturing AEX	[19]
		2′,3′-Isomer	PS, 2′-OMe, 2′-F	Denaturing IP-RP	[42]
		2′,3′-Isomer	PS, 2′-OMe, 2′-F	Denaturing AEX	[42]
Diastereomer analysis			PS, 2′-OMe, 2′-F, GalNAc	Non-denaturing IP-RP	[24]
			PS, 2′-OMe	Denaturing IP-RP	[47]
			PS	Non-denaturing AEX	[48]
			PS, 2′-OMe, 2′-F	HILIC	[49]
Sequencing			di-siRNA	Denaturing IP-RP	[44]
			2′-OMe	Denaturing IP-RP	[52]
			2′-OMe	Non-denaturing IP-RP	[52]
LNP		Size distribution, molecular weight distribution, siRNA content	Natural	SEC	[54]
		siRNA content	-	SEC	[55]
		siRNA content	Natural	Denaturing IP-RP	[56]
		siRNA content	-	SEC	[57]
		LNP formulation, siRNA content	PS, 2′-OMe	IP-RP	[58]
GalNAc		Prepend denaturation, diastereomer separation	PS, 2′-OMe, 2′-F, GalNAc	Non-denaturing IP-RP	[24]
		Purification	PS, GalNAc	AEX	[59]
		Purification	PS, GalNAc	MMC	[59]
ARC		Purification	Antibody	SEC	[61]
Pharmacokinetics			PS, 2′-OMe, 2′-F	Non-denaturing IP-RP	[62]
			2′-OMe, 2′-F	Denaturing IP-RP	[63]
			PS, 2′-OMe, 2′-F, GalNAc	Denaturing IP-RP	[64]
			2′-OMe, 2′-F	Denaturing IP-RP	[65]
			Natural	Non-denaturing AEX	[66]

"2′-OMe" indicates 2′-*O*-methyl, "2′-F" indicates 2′-fluoro, "PS" indicates phosphorothioate, "LNP" indicates lipid nanoparticle, "GalNAc" indicates *N*-acetylgalactosamine and "ARC" indicates antibody–siRNA conjugates.

Table 4
Characteristics of the major separation modes for siRNA analysis and typical conditions.

	Advantage	Disadvantage	Typical conditions
IP-RP	Compatible with MS. High separation ability for impurities.	Duplex form may be denatured during the analysis.	Columns with hydrophobic functional groups such as C18 are used. Mobile phase contains ion pair reagents such as TEA and HA, and acetic acid or HFIP is commonly used as the counter anion.
AEX	High separation ability for impurities with selectivity different from IP-RP. Duplex form is maintained during analysis.	Incompatible with MS.	Columns with positively charged functional groups such as quaternary amines are used. Elution with a gradient of salts such as NaOH and $NaClO_4$.
SEC	Separation based on hydrodynamic radius. Duplex form is maintained during analysis.	Generally incompatible with MS (recent studies have shown compatibility using volatile buffers). Insufficient separation of impurities with similar molecular size.	Columns with sufficient pore size (e.g., 200 Å) is used. Buffers such as PBS are used as the mobile phase.
HILIC	Compatible with MS without the use of ion pairing reagents. Impurities can be separated based on hydrophilicity.	Duplex form may be denatured during the analysis (generally easier to maintain the duplex compared with typical IP-RP conditions). Insufficient separation of impurities.	Columns with hydrophilic functional groups such as amide and hydroxy groups are used. Mobile phase containing ammonium acetate is generally used, and elution is performed with a gradient of decreasing organic solvent content.

"TEA" indicates triethylamine, "HA" indicates hexylamine, and "HFIP" indicates hexafluoroisopropanol.

peaks and higher resolution—such as elevated column temperatures—can be utilized. Moreover, the molecular size of single-stranded siRNA is approximately half that of its duplex form, facilitating the separation of impurities.

In contrast, *non-denaturing chromatography* retains siRNA in its native double-stranded state during separation. To preserve the hydrogen bonds of Watson–Crick base pairs within the column, analytical conditions such as mobile phase composition and column temperature must be carefully controlled and are more limited. It has been demonstrated that high column temperatures lead to siRNA denaturation (Fig. 1), and that duplex stability varies depending on the mobile phase composition and separation mode [16]. Additionally, due to the larger molecular size of the duplex (~15 kDa), it becomes more difficult to resolve subtle structural differences between impurities and the API. Nevertheless, a key advantage of non-denaturing chromatography is that it allows assessment of purity in the intact form in which siRNA exerts its biological function. While non-denaturing methods are primarily used to separate unhybridized excess single strands, some studies have reported the separation of non-optimal duplexes, such as those containing shortmers, from the target duplex [17–19].

Gilar et al. [16] reported that, in addition to mobile phase composition and column temperature, the column's functional groups and separation mode also influence the denaturation behavior of siRNA. They compared the denaturation strength across several chromatographic modes, including size-exclusion chromatography (SEC), ion-pair reversed-phase (IP-RP), reversed-phase (RP), and hydrophilic interaction liquid chromatography (HILIC). The onset temperature of duplex denaturation differed significantly between RP and HILIC modes, even when using the same mobile phase containing ammonium acetate and acetonitrile (ACN). This suggests that duplex stability may be affected by the formation of aqueous or organic solvent layers on the surface of the stationary phase. Accumulating such knowledge is essential for developing effective strategies when optimizing analytical conditions for siRNA.

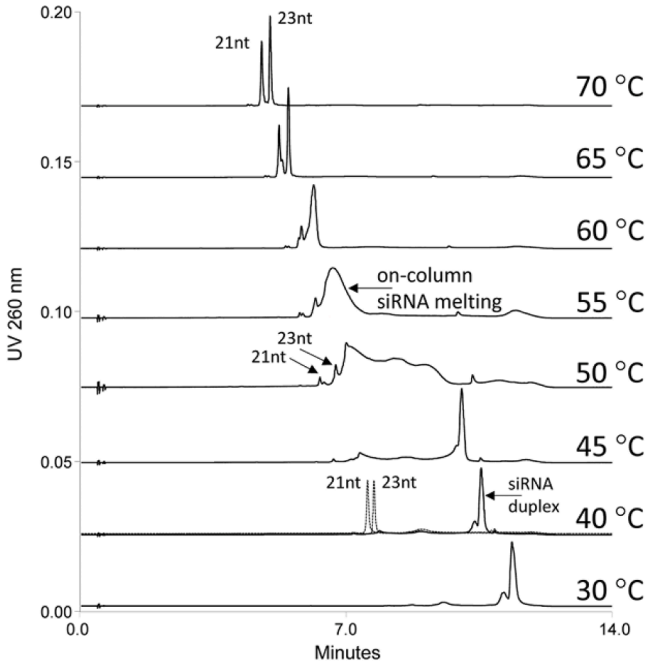


Fig. 1. IP-RP analysis of siRNA using 10/100 mM HA/HFIP ion-pairing system with methanol as eluent. siRNA duplex is well resolved from single stranded oligonucleotides (see 40 °C chromatogram). On column siRNA melting is apparent at 50 °C. Complete duplex melting was observed at > 65 °C. Reproduced with permission from [16].

2.1. *Ion-pair reversed-phase liquid chromatography (IP-RP)*

As with ASOs [15,20–22], which are single-stranded, IP-RP liquid chromatography is the most widely used analytical method for siRNAs. Cationic ion-pairing reagents such as triethylamine (TEA), hexylamine (HA), and tributylamine are commonly used, and two major mechanisms have been proposed to explain oligonucleotide retention. In the first model, the anionic phosphodiester linkages of the oligonucleotide form ion pairs with the cationic ion-pairing reagent, resulting in a neutral complex that interacts hydrophobically with stationary phase functional groups such as C18, C8, or phenyl. In the second model, the ion-pairing reagent first adsorbs onto the stationary phase, forming a positively charged surface, which then interacts electrostatically with the negatively charged oligonucleotide—resembling an ion-exchange mechanism. During gradient elution, increasing the concentration of hydrophobic organic solvents such as methanol (MeOH) or ACN promotes desorption of the ion-pairing reagent from the stationary phase, allowing the oligonucleotides to elute.

Additionally, nucleobases themselves contribute to hydrophobic interactions with the stationary phase, and the base composition affects retention time [23]. Generally, longer oligonucleotides exhibit stronger retention, making IP-RP effective for separating shortmers and longmers. This mode is also suitable for resolving impurities with hydrophobic protecting groups, such as cyanoethyl or dimethoxytrityl (DMTr). Although the use of organic solvents and ion-pairing reagents can destabilize the duplex form of siRNA in the column [16], the high

resolution and MS compatibility remain major advantages of IP-RP. Several studies have reported that siRNA denaturation can occur during IP-RP chromatography [16,17,24]. Nevertheless, considerable efforts have been made to develop non-denaturing IP-RP methods, and innovative strategies to preserve the double-stranded structure of siRNAs during analysis have also been proposed [24]. Analysis under non-denaturing conditions enables the assessment of purity and heterogeneity in the duplex state, which is more representative of the therapeutically active form. Generally, column temperature is set depending on whether analysis is performed under denaturing or non-denaturing conditions: low temperatures (typically $\leq$20 °C) are used to preserve the duplex structure, while high temperatures (typically $\geq$60 °C) are used to denature the duplex. On the other hand, systematic studies on other analytical parameters—such as the type and concentration of ion-pairing reagents (e.g., TEA, HA), the concentration of hexafluoroisopropanol (HFIP), and the choice of organic solvent—remain limited. Although method selection ultimately depends on the analytical objective, further comprehensive studies are needed to clarify how these variables affect chromatographic behavior and MS sensitivity. Such efforts will help elucidate the retention mechanisms of siRNAs and support the development of more robust and effective analytical methods.

One early report on siRNA analysis described non-denaturing IP-RP conditions using a mobile phase containing triethylammonium acetate (TEAA) at a column temperature of 20 °C [17]. Since the melting of DNA duplex was induced at higher column temperature and the melting point of siRNA was found to increase with higher guanine/cytosine content, a low-temperature setting was adopted. This method eluted the single strands faster than the double strands, allowing separation of excess single strands from siRNA. This behavior was attributed to the greater number of phosphates in duplexes interacting with ion-pair reagents than single strands. Moreover, non-optimal duplex impurities eluted little faster than the optimal duplex, enabling their separation. Additionally, a non-denaturing LC-MS method using hexylammonium acetate (HAA) as the ion-pairing buffer was developed, and MS analysis confirmed the presence of both sense and antisense strands within the duplex peak [17].

The use of HFIP as a counter-anion, in place of acetic acid, has also been explored. Noll et al. demonstrated that a HA/HFIP buffer system is effective for non-denaturing IP-RP coupled with MS detection [18]. This system enabled the separation and MS identification of excess single strands and non-optimal duplexes (including shortmers, longmers, and 2′,3′-isomers) (Fig. 2), and showed significantly improved MS sensitivity compared to the HAA buffer. The study also examined how annealing conditions affect siRNA composition, showing that slow cooling after heating is important to reduce the peak area of excess single strands.

Another challenge in oligonucleotide analysis is the potential interaction of polyanionic oligonucleotides with metal surfaces in instrumentation, which can impair reproducibility and quantitative accuracy. To address this issue, both denaturing and non-denaturing IP-RP methods were implemented using C18 columns with high-performance surface (HPS) treatments [25]. For denaturing conditions, elevated temperatures (75 °C) and TEA/HFIP buffers were used to fully denature siRNA duplexes. For non-denaturing conditions, HA/HFIP buffer and a low column temperature (20 °C) were applied. The HPS column allowed robust analysis of siRNA without significant carryover. Notably, under non-denaturing conditions, peak broadening caused by PS diastereomers was observed, indicating that the presence of such stereoisomers could hinder the resolution of structurally similar impurities.

As previously discussed, one of the difficulties in siRNA analysis by IP-RP arises from the denaturation effects of organic solvents and ion-pairing reagents [16]. Enmark et al. demonstrated that the addition of salts to the mobile phase can enhance duplex stability during both UV and MS detection [24]. Specifically, the addition of phosphate-buffered saline (PBS) significantly reduced siRNA denaturation under IP-RP conditions. Subsequently, ammonium acetate—a more MS-compatible salt—was evaluated and yielded similar stabilizing effects. This study showed that salt-modified IP-RP conditions can support non-denaturing analysis while maintaining MS compatibility.

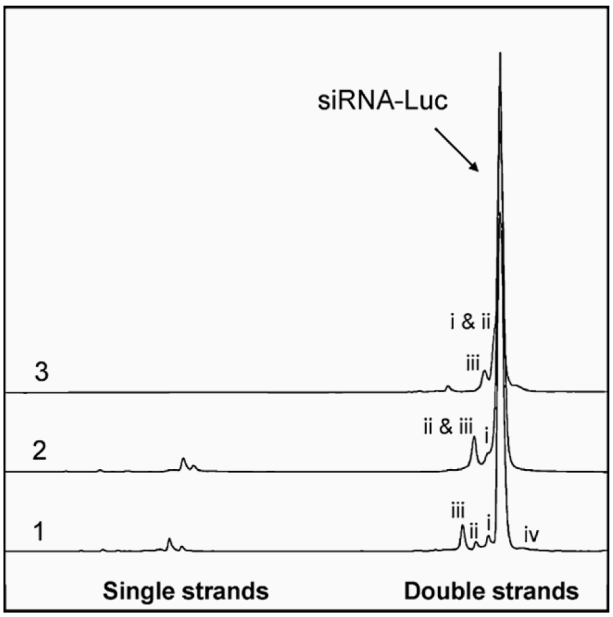


Fig. 2. Non-denaturing IP-RP analysis of siRNA. UV chromatograms are shown for buffer systems containing 25 mM HA and 100 mM HFIP (trace 1), 25 mM HAA (trace 2), or 16.5 mM TEA and 100 mM HFIP (trace 3). Non-hybridized single strands eluted earlier than the siRNA duplex. Peaks i, ii, iii, and iv correspond to non-optimal duplexes. Reproduced with permission from [18].

2.2. *Anion-exchange chromatography (AEX)*

AEX has also been widely used for the analysis of oligonucleotides. In AEX, stationary phases bearing quaternary or tertiary amine functional groups are employed, enabling electrostatic interactions with the negatively charged phosphate groups of oligonucleotides. Elution and separation are achieved by applying a salt gradient, in which the anions in the mobile phase compete with the oligonucleotides for binding to the stationary phase. Commonly used salts include sodium chloride, sodium perchlorate, and sodium bromide, with elution strength varying depending on the salt type. The mobile phase pH is typically adjusted to neutral or alkaline conditions. When analyzing single-stranded oligonucleotides with multiple PS modifications, strongly alkaline mobile phases (e.g., pH 12) are often employed to suppress diastereomeric separation and achieve sharp peak shapes. In addition, to minimize hydrophobic interactions with the polymer matrix of the column, organic solvents such as ACN are often added at approximately 20 % v/v.

In general, longer oligonucleotides exhibit stronger retention, allowing the effective separation of shortmers and longmers [26,27]. AEX is also capable of separating PS→PO impurities, which are often difficult to resolve by IP-RP chromatography [28]. Base composition additionally affects retention behavior [29]. Under alkaline mobile phase conditions, guanine, thymine, and uracil can become deprotonated, further increasing the negative charge of the oligonucleotide and thus enhancing retention *via* electrostatic interactions [28].

While the use of nonvolatile salts in the mobile phase limits compatibility with MS, AEX offers orthogonal selectivity to IP-RP and is a valuable complementary technique. Moreover, because organic solvents are not required for elution and because salt-based buffers help stabilize the secondary structures of polyanionic oligonucleotides, siRNAs can readily maintain their double-stranded form under AEX conditions. Consequently, AEX is a practical method for analyzing siRNAs

under non-denaturing conditions. On the other hand, by increasing the mobile phase pH or column temperature, hydrogen bonds within Watson–Crick base pairs can be disrupted, enabling AEX to also function under denaturing conditions. In one early study, Cook and Thayer demonstrated the utility of AEX for oligonucleotide analysis, showing that double-stranded siRNA could be effectively separated from its single-stranded components [28].

AEX is frequently employed in post-synthetic purification processes [30,31]. A notable example is the development of a simplified manufacturing and purification process for siRNA using non-denaturing AEX chromatography [32]. In this approach, crude single strands obtained directly after solid-phase synthesis were mixed and annealed prior to purification, and the purification was performed on the annealed duplex. A mobile phase composed of phosphate buffer (pH6.5) containing 10 %ACN was used, with sodium bromide as the eluting agent. The resulting product exhibited a higher proportion of optimal duplex compared to purification at the single-strand level, while overall purity levels were comparable between the two methods.

We have demonstrated that non-denaturing AEX enables efficient separation of siRNA impurities—such as excess single strands and non-optimal duplexes—and is well suited for siRNA purity analysis [19]. Under mildly alkaline conditions (mobile phase pH 9.0), excess single strands were clearly separated from duplexes (Fig. 3), and non-optimal duplexes containing shortmers (e.g., $n-1$ species) were eluted at distinct retention times relative to the optimal duplex (Fig. 4). In general, duplexes with greater numbers of deletions (e.g., from $n-1$ to $n-5$), with longer overhangs, exhibited stronger retention. This behavior was attributed to the presence of guanine and uracil residues at the overhangs, which become negatively charged under slightly alkaline conditions (the pKa values of the guanine N1-H and uracil N3-H groups are typically in the range of 9.2–10.5 [33,34]), thereby increasing interaction with the positively charged stationary phase.

2.3. Size-exclusion chromatography (SEC)

SEC is another practical separation mode for analyzing oligonucleotides [35,36]. It separates analytes based on their hydrodynamic radius, making it particularly suitable for quantifying excess single strands in siRNA. However, because non-optimal duplexes often have similar molecular sizes to the optimal duplex, SEC is generally ineffective for resolving these species. Like AEX, SEC utilizes salt-containing mobile phases without organic solvents, allowing siRNA to maintain its native double-stranded structure during analysis. Also, SEC has not been widely coupled with MS, similar to AEX, because its mobile phase typically contains salts. However, recent studies have demonstrated the feasibility of SEC-MS coupling using volatile buffers [37].

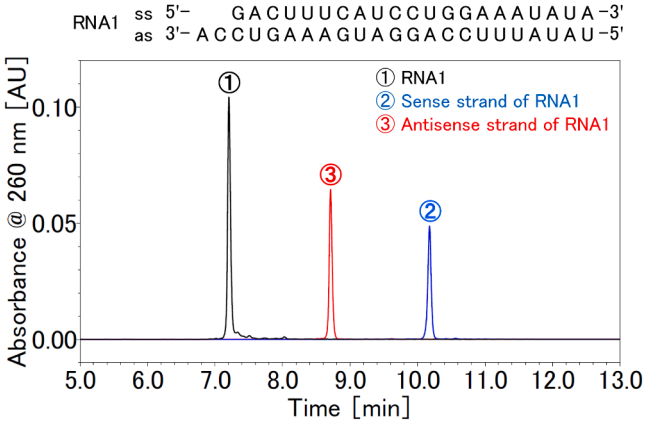


Fig. 3. Chromatographic separation of single- and double-stranded oligonucleotides. The siRNA duplex is shown in black, the sense strand in blue, and the antisense strand in red. AEX was performed using 20 mM Tris–HCl buffer at pH 9.0 (mobile phase A) and 1.25 M NaCl in mobile phase A (mobile phase B), with a DNAPac PA200 RS column (4 μm, 4.6 × 250 mm). Reproduced with permission from [19].

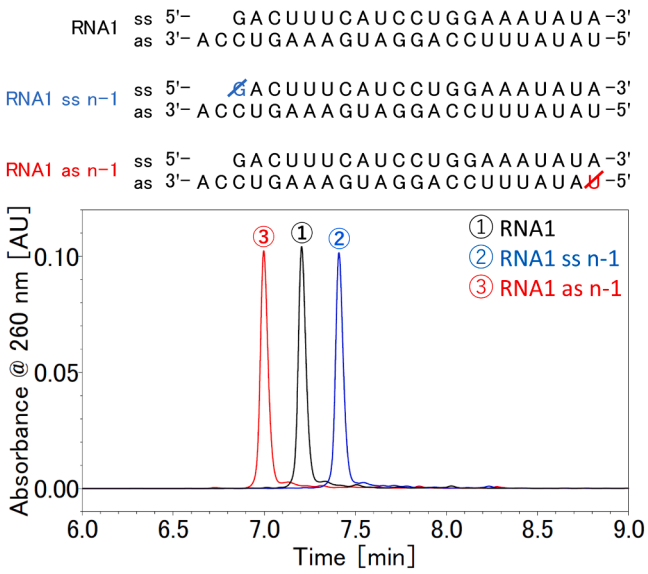


Fig. 4. Chromatographic separation of the optimal duplex from non-optimal duplexes containing an n-1 deletion. The optimal duplex is shown in black, while non-optimal duplexes are shown in blue and red. AEX was performed using 20 mM Tris–HCl buffer at pH 9.0 (mobile phase A) and 1.25 M NaCl in mobile phase A (mobile phase B), with a DNAPac PA200 RS column (4 μm, 4.6 × 250 mm). Reproduced with permission from [19].

The optimal separation mode for analyzing excess single strands in cyclic peptide-conjugated siRNA was investigated [38]. Under non-denaturing IP-RP conditions, both the sense and antisense strands eluted at the same retention time, making it difficult to identify which strand was in excess. Similarly, in non-denaturing AEX, the sense strand and the duplex exhibited similar retention times, making it challenging to confirm the presence of excess strands. These issues were likely due to interactions between the peptide conjugate and the stationary phase, which interfered with separation in both IP-RP and AEX modes. This may explain the difference from the study using AEX, which uses unconjugated siRNA [19]. In contrast, SEC successfully separated single strands from the duplex (Fig. 5). As expected based on the molecular mass, the antisense strand conjugated with a cyclic peptide—which had a larger hydrodynamic size—eluted earlier than the unconjugated sense strand. This result highlights the importance of selecting appropriate separation modes based on the structural characteristics of the analyte, such as the presence of conjugates.

As a recent advancement, it has been demonstrated that volatile buffers can be used to couple SEC with MS for siRNA analysis [37]. The use of 50 mM ammonium acetate as the mobile phase enabled direct MS detection, allowing separation of the duplex, sense, and antisense strands of siRNA by SEC, with their molecular masses subsequently confirmed by MS. Notably, the duplex structure was shown to remain at least partially intact during MS analysis.

2.4. Hydrophilic interaction liquid chromatography (HILIC)

In recent years, HILIC has been increasingly applied to the analysis of oligonucleotides, particularly single-stranded species [39,40]. Two primary mechanisms are proposed to explain retention in HILIC [39,40], and both seems to contribute in different proportions depending on the analyte and analytical conditions. The first mechanism involves hydrophilic partitioning between a highly organic mobile phase and a water-enriched layer immobilized on the surface of polar stationary phases (e.g., amide or diol groups). In this model, hydrophilic analytes

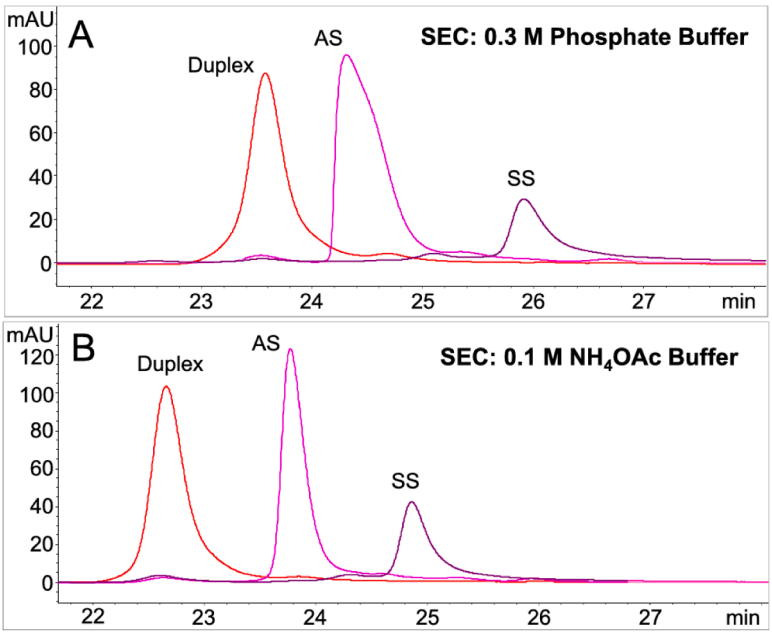


Fig. 5. SEC analysis of a cyclic peptide–siRNA duplex sample and its individual antisense (AS) and sense (SS) strands, using two Tosoh TSKgel SEC columns in series (UP-SW2000, 4.6 × 300 mm, 2 μm). (A) Mobile phase: 0.3 M NaCl, 50 mM phosphate buffer (pH 6.47, prepared using sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate); flow rate: 0.25 mL/min; column temperature: 25 °C; injection: 2 μL of 1 mg/mL. (B) Mobile phase: 0.1 M ammonium acetate (NH_4OAc); flow rate: 0.25 mL/min; column temperature: 25 °C; injection: 2 μL of 1 mg/mL. Reproduced with permission from [38].

are retained within the aqueous layer and gradually eluted as the proportion of organic solvent decreases during gradient elution. The second mechanism involves adsorptive interactions, such as hydrogen bonding and electrostatic interactions with polar functional groups on the stationary phase. It has been suggested that oligonucleotides are retained primarily through such interactions rather than partitioning [39,40]. Consequently, HILIC enables the separation of oligonucleotides based on differences in hydrophilicity, with longer strands generally exhibiting stronger retention. A key advantage of HILIC is its compatibility with MS, as it does not require ion-pairing reagents or HFIP, both of which may persist in instrumentation and interfere with MS analysis. Compared to IP-RP and AEX, HILIC currently faces limitations in impurity separation efficiency. Although partial denaturation can occur due to the use of organic solvents, HILIC generally maintains the duplex form more effectively than IP-RP under similar conditions.

Although applications to siRNA are still limited, a few studies have demonstrated its potential. For example, HILIC analysis using an amide column with larger pore size was reported under both non-denaturing and denaturing conditions, using the same mobile phase [41]. The mobile phase consisted of 50 mM ammonium acetate and ACN. The column, characterized by its large pore size and absence of apparent silanol activity, provided improved resolution of oligonucleotides. Sharp and reproducible peaks were observed across multiple column batches. Under denaturing conditions (i.e., high column temperature), the sense and antisense strands were successfully separated and identified based on their retention times. Under non-denaturing conditions (i.e., low column temperature), the duplex structure was preserved, allowing for detection of excess single strands.

2.5. *Combination of multiple analytical methods*

The siRNA manufacturing process gives rise to various impurities, making it challenging to accurately identify and quantify all impurity species using a single analytical method. Furthermore, analytical conditions—such as oligonucleotide concentration and buffer composition—often differ from those in the actual drug formulation, which may influence impurity profiles. To obtain a more comprehensive and accurate evaluation, it is useful to apply multiple separation modes and, when necessary, combine them with orthogonal techniques beyond chromatography and MS.

Seiffert et al. investigated side reactions occurring during the siRNA annealing step using multiple chromatographic modes [42]. The impact of annealing temperature on unhybridized single strands in PBS or water was evaluated using SEC and non-denaturing IP-RP chromatography. In both analytical modes, the duplex content remained largely unaffected by the incubation temperature used during annealing. However, a decrease in full-length excess single strands with increasing temperature was observed exclusively in non-denaturing IP-RP analysis. This change was accompanied by an increase in earlier-eluting impurity peaks. MS analysis identified these impurities as shortmers and isomers of the sense strand. Denaturing IP-RP and AEX further revealed multiple impurity peaks eluting slightly before the main single-stranded components, which were attributed to 2′,3′-isomerization, strand scission, and fluorine elimination—side reactions likely induced during the annealing process. Importantly, the comprehensive use of multiple LC modes allowed for the detection and characterization of a wide range of side reactions that would have been difficult to capture using a single method. Overall, the study demonstrated that annealing parameters—such as RNA concentration, presence of cations, temperature, and heating duration—can significantly influence the extent of these side reactions.

The duplex-to-single-strand ratio is also affected by the measurement environment, including oligonucleotide concentration and buffer composition. Thus, hybridization or denaturation events occurring during sample preparation or analysis can bias the quantification of duplex content. To mitigate this, unbiased analytical techniques such as circular dichroism (CD) spectroscopy have been proposed for characterizing siRNA duplexes across various media prior to establishing non-denaturing chromatographic methods [43]. This approach enables optimization of both sample preparation and chromatographic conditions to minimize environmental influences on duplex structure, ensuring more reliable and reproducible analyses.

Beyond conventional siRNAs composed of a 1:1 duplex of sense and antisense strands, structurally complex variants such as divalent small

interfering RNAs (di-siRNAs) are also under development. Di-siRNAs form a 2:1 duplex structure, presenting additional analytical challenges. Analytical evaluation of di-siRNAs using both denaturing and non-denaturing chromatography has been reported [44]. Low-temperature IP-RP experiments indicated that di-siRNA sequences may undergo partial denaturation during analysis, while dilution of samples prior to SEC injection was found to underestimate di-siRNA aggregation states. Moreover, SEC data suggested that denatured di-siRNA samples could re-anneal under high-salt mobile phase conditions, as supported by melting temperature (T_m) studies. These findings highlight the importance of carefully selecting analytical conditions that preserve native conformations, particularly for structurally complex siRNA molecules.

In recent years, the application of two-dimensional liquid chromatography (2D-LC) to the analysis of oligonucleotides has also been actively pursued. A 2D-LC system was employed, in which SEC was used as the first dimension and denaturing IP-RP chromatography as the second [38]. This approach enabled the determination of the molecular masses of single-stranded components in non-optimal duplex that co-eluted with the optimal duplex in the first dimension. Such 2D-LC techniques broaden the analytical toolkit by enabling the coupling of MS-incompatible methods (e.g., AEX, SEC) with MS-compatible methods (e.g., IP-RP, HILIC) in the second dimension, thus facilitating both separation and structural identification by MS.

3. Separation of diastereomers of PS modification

PS modification is commonly introduced into siRNA therapeutics to enhance nuclease resistance [4,5], and six out of the seven approved siRNA drugs incorporate six PS linkages. The substitution of a non-bridging oxygen with sulfur at the phosphate center creates a chiral phosphorus atom, making each PS linkage a stereocenter. Consequently, an siRNA duplex with six PS linkages becomes a mixture of up to 64 (2^6) diastereomers. Recent studies have demonstrated that diastereomeric distribution influences key properties of siRNA, including RISC loading efficiency and nuclease resistance [5,13,14]. Therefore, the development of analytical methods capable of separating and characterizing these diastereomers is essential for ensuring the quality of PS-modified siRNA therapeutics. While most prior studies have focused on single-stranded oligonucleotides [45,46], several recent reports have successfully demonstrated diastereomer separation in PS-modified siRNA duplexes.

An IP-RP method was developed to achieve baseline separation of siRNA duplex diastereomers under denaturing conditions [47]. Once siRNA is denatured, it becomes two single strands. Since both the sense and antisense strands contain PS modifications, their simultaneous analysis produces more complex chromatographic patterns compared to the analysis of a single-stranded oligonucleotide. In this study, two diastereomers of a sense strand containing one PS bond and four diastereomers of an antisense strand with two PS bonds were successfully resolved. Using ACN as the organic solvent, mobile phases containing TEAA provided superior separation compared to other ion-pairing reagents. ACN was identified as a critical factor for efficient diastereomer separation, whereas other organic solvents yielded inferior results. Differential scanning calorimetry (DSC) further confirmed that ACN facilitates duplex denaturation, which appears to be essential for resolving diastereomers in this mode.

Enmark et al. reported that the addition of PBS to the mobile phase in non-denaturing IP-RP conditions not only enhanced duplex stability but also improved the resolution of PS diastereomers [24]. It is well established that salts reduce electrostatic repulsion between phosphate backbones, thereby stabilizing the duplex. Interestingly, their findings also suggest that the formation of higher-order structures enhances diastereomer separation. In contrast, replacing ACN with MeOH completely suppressed diastereomer separation, likely due to disruption of hydrogen bonding between the oligonucleotides and the stationary phase. These results differ from those obtained under denaturing conditions [47]. In both cases, ACN effectively promotes diastereomer separation; however, the ion-pairing reagents differ. At present, it remains unclear whether denaturing or non-denaturing IP-RP conditions are more favorable for diastereomeric separation. Further studies employing identical PS-modified siRNAs and ion-pair reagents will be required to elucidate the underlying separation mechanism.

We investigated the applicability of non-denaturing AEX for separating PS-modified siRNA diastereomers and found that diastereomer separation was more effective in the double-stranded form than in the single-stranded state [48], again indicating that higher-order structure facilitates separation. When the number of PS linkages was increased to four, the number of detected peaks matched the theoretical number of diastereomers ($2^4 = 16$) (Fig. 6). With six PS linkages, approximately 30 peaks were detected out of the theoretical 64 diastereomers ($2^6 = 64$) (Fig. 6). The observed separation was attributed to steric differences between inward-oriented *R*p and outward-oriented *S*p configurations of the sulfur atom, with *R*p isomers generally eluting earlier than *S*p isomers.

Similarly, the enhancement of diastereomer separation through higher-order structure formation was observed in HILIC-based analysis [49]. Improved resolution of PS-modified single strands was achieved under conditions of high ionic strength (100 mM ammonium acetate) and low column temperature (5 °C). These conditions helped preserve higher-order structures, even in mobile phases with high ACN content. Oligonucleotides with more rigid or limited conformations showed better diastereomeric selectivity. Since these conditions also stabilize the immobilized water layer on the amide column surface and suppress the ionic interactions, it was suggested that diastereomer separation in HILIC is primarily driven by hydrogen bonding. The method was further applied to PS-modified siRNA duplexes, where partial diastereomer separation was also confirmed (Fig. 7). Importantly, the siRNA duplex remained intact throughout both the chromatographic separation and subsequent MS analysis. Another study also reported that siRNA duplexes can be preserved and analyzed in their native form using HILIC-MS [50]. These findings suggest that HILIC mobile phases containing ammonium salts, such as ammonium acetate and ammonium formate, are particularly well-suited for detecting intact siRNA by MS.

4. Confirmation of the sequence *via* tandem mass spectrometry (MS/MS)

Fragmentation-based sequencing using tandem mass spectrometry (MS/MS) is a widely used approach for confirming oligonucleotide sequences and identifying siRNA molecules [11,51]. Due to its compatibility with MS, IP-RP chromatography is generally employed as the separation mode. A non-denaturing LC–MS/MS method was developed to confirm the sequence of siRNA using software for interpreting fragmentation data [52]. Under non-denaturing IP-RP conditions at low column temperature, complete sequencing of both co-eluted strands was achieved by employing a targeted MS/MS strategy, in which specific charge states corresponding to each strand were individually selected for fragmentation. In a parallel approach, denaturing IP-RP was performed at a column temperature of 60 °C, and data-independent acquisition (DIA) was applied using cone voltage-induced fragmentation without precursor ion selection. Under these conditions, the sense and antisense strands eluted separately, and complete sequencing was achieved solely by acquiring MS data.

Denaturing IP-RP conditions were also applied to the sequencing of di-siRNA [44]. In this study, the covalently linked two 16-mer sense strands and the 21-mer antisense strand were successfully separated and subjected to MS/MS. Targeted fragmentation of both the antisense and sense strands yielded high-quality sequencing data. Full sequence coverage was obtained for the antisense strand, while the covalently linked sense strand—containing a tetraethylene glycol linker—achieved 93 % sequence coverage.

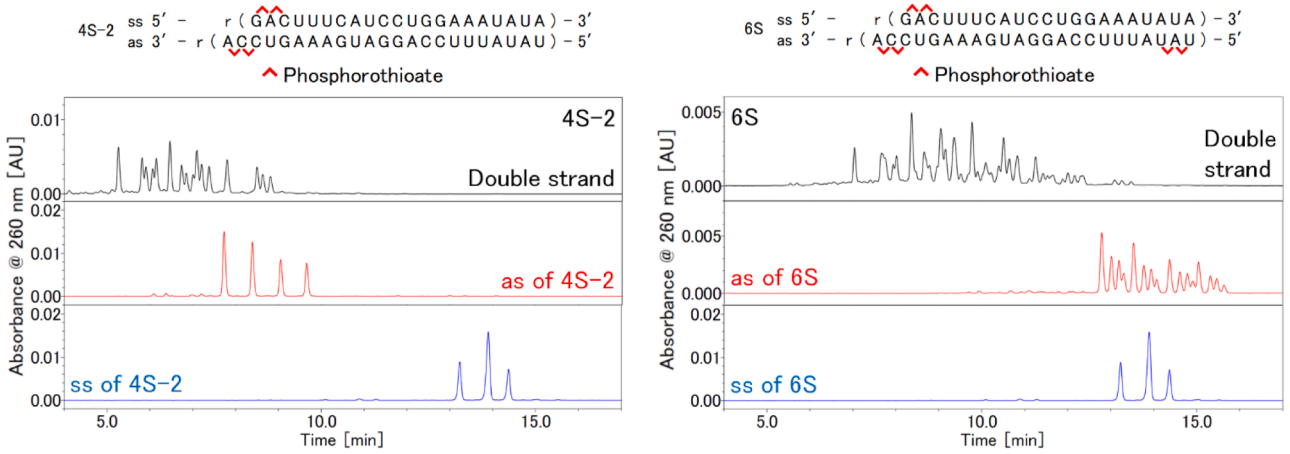


Fig. 6. Chromatographic separation of the diastereomers of double-stranded oligonucleotides containing four or six PS bonds, along with their corresponding single strands. "ss" indicates sense strand, and "as" indicates antisense strand. AEX was performed using 20 mM Tris–HCl buffer at pH 9.0 (mobile phase A) and 1.25 M NaCl in mobile phase A (mobile phase B), with a DNAPac PA200 RS column (4 μm, 4.6 × 250 mm). Reproduced with permission from [48].

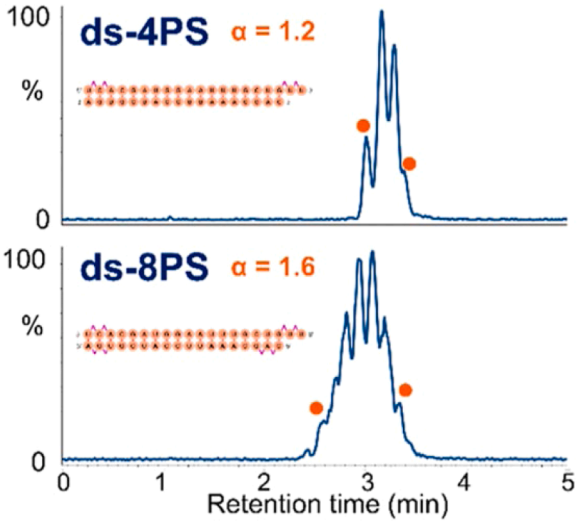


Fig. 7. HILIC analysis of intact double-stranded siRNAs. (A) Extracted ion chromatograms (EICs) of ds-4PS and ds-8PS using 100 mM ammonium acetate in the mobile phases and a column temperature of 5 °C. Selectivities were calculated between the first and last detectable peaks within the apparent cluster, as indicated by orange dots. Reproduced with permission from [49].

5. Analysis of LNP-based siRNA, GalNAc-conjugated siRNA and antibody-siRNA conjugates

The first approved siRNA drug, *patisiran*, is formulated using LNPs. LNPs are employed to overcome the inherent challenges of systemic siRNA delivery. Due to their large molecular size, susceptibility to nuclease degradation, and strong negative charge, siRNAs cannot efficiently cross cell membranes. Therefore, effective delivery systems such as LNPs are essential for achieving therapeutic efficacy. LNPs are also used in mRNA therapeutics, notably in COVID-19 vaccines, prompting active research to improve their design and performance.

Unlike small molecule drugs, which are typically highly pure and chemically uniform, LNP-based therapeutics exhibit heterogeneity in both particle size and lipid composition. Since LNP characteristics such as particle size and lipid impurities influence efficacy [53], analytical methods are required to assess these parameters and quantify individual lipid components.

Zhang et al. developed a SEC method to analyze the size distribution of LNP-based siRNA formulations [54]. To reduce nonspecific interactions, a column with cationically modified resin was employed. Well-shaped peaks corresponding to siRNA-containing LNPs were observed, and separation efficiency was confirmed. Elution time increased with decreasing particle size, indicating that separation occurred *via* a size-exclusion mechanism. By coupling SEC with multiple detection techniques—UV–Vis absorbance, multi-angle light scattering (MALS), and refractive index (RI) detection—the distributions of particle size, molecular mass, and siRNA encapsulation were comprehensively characterized. Despite similar manufacturing processes, LNP A exhibited a narrower particle size distribution and higher siRNA loading than LNP B, highlighting the importance of analytical control over LNP formulation consistency.

Also, facile and rapid analytical method for determining RNA content in LNPs has been established [55]. Because increasing LNP particle size causes light scattering that affects UV absorption, online correction was achieved by combining SEC with MALS and RI detection. Double-stranded RNA was encapsuled in four LNP formulations, and the results obtained using this online approach were consistent with those from offline quantitation by RP analysis, demonstrating its suitability for accurate RNA content measurement.

In addition, simple and user-friendly LC methods have been developed to quantify total siRNA content in LNP formulations [56]. Isocratic chromatographic conditions were optimized using reversed-phase or cation-exchange columns maintained at 30 °C, with mobile phases composed of 0.1 M ammonium bicarbonate in water containing 20–30 % v/v ACN. These alternatives to traditional SEC were selected to address its limitations—long run times due to low flow rates (to avoid high backpressure) and shorter column lifetimes when exposed to lipid-rich samples. In these methods, siRNA peaks were clearly separated from system peaks, and the approach was validated according to International Council for Harmonisation (ICH) guidelines. Among the columns tested, those with a 130 Å pore size provided better separation than those with 90 Å, owing to the higher molecular mass of siRNA relative to buffer components and excipients.

A fast and user-friendly method for quantifying the siRNA content in LNPs using IP-RP HPLC with UV detection has also been reported [57]. Addition of a non-ionic surfactant to the LNP samples before injection improved siRNA recovery, and accurate quantification was achieved using a mobile phase containing 100 mM TEAA and ACN with a column temperature of 80 °C which is above the T_m of the duplex.

LNPs typically consist of a cationic (or ionizable) lipid, a phospholipid, a PEGylated lipid, and cholesterol. Chemical analysis of these components is essential for ensuring drug quality. A denaturing IP-RP method was developed for simultaneous separation and detection of both siRNA and lipid species in LNP formulations [58]. siRNAs were

detected using a diode array detector, while lipids—lacking UV chromophores—were monitored by charged aerosol detection. The retention of siRNA correlated with the alkyl chain length of the ion-pairing reagent, whereas phospholipid retention was relatively unaffected by ion-pairing conditions. Optimal separation was achieved using a ethylene bridged hybrid (BEH) phenyl column and dibutylammonium acetate as the ion-pairing reagent. This method enabled selective detection of siRNA, lipid components, and potential degradation products within LNP formulations.

In parallel with LNP development, GalNAc-conjugated siRNAs have emerged as a major delivery platform. While some studies have examined GalNAc-siRNA conjugates [24], limited information exists regarding the impact of the GalNAc moiety on chromatographic retention. Kazarian et al. investigated the influence of GalNAc conjugation on AEX analysis [59]. In AEX, the sense strand bearing a GalNAc conjugate eluted earlier than the unconjugated strand, and the yield and purity during purification were insufficient. This behavior was attributed to steric hindrance from the bulky conjugate, which appeared to disrupt the interaction between the phosphate backbone and the positively charged amine groups of the column. However, when analyzed using mixed-mode chromatography (MMC) incorporating both cationic and hydrophobic functional groups, the elution order was reversed: the GalNAc-conjugated strand exhibited stronger retention and was more efficiently separated. This enhanced retention was attributed to the hydrophobic nature of the GalNAc moiety. These results suggest that selection of an appropriate separation mode—taking into account the physicochemical properties of conjugated structures—is critical for effective purification and characterization of GalNAc-siRNA.

Also, ion-pairing HILIC (IP-HILIC) is attracting attention as a novel technique, and its application to impurity profiling of GalNAc-conjugated ASOs has been recently investigated [60]. In this study, ion-pairing reagents such as TEA were added to the HILIC mobile phase to mask the negative charges on the phosphate backbone, thereby enhancing selectivity based on nucleobase composition and the presence of GalNAc conjugation. This enabled the separation of various impurities, including deamidated products that are difficult to resolve by conventional methods. These findings suggest that IP-HILIC could be a promising method for siRNA analysis.

In recent years, to expand the therapeutic scope beyond liver-targeted diseases, conjugation of monoclonal antibodies to siRNA has been actively investigated as a viable delivery strategy. SEC conditions for purifying antibody-siRNA conjugates (ARC) have been reported [61]. In that study, SEC enabled the purification of ARC by exploiting the difference in molecular size between conjugated and unconjugated species. In addition, the identity and the degree of conjugation were confirmed by SEC-MS using 0.1 M ammonium acetate as the mobile phase.

6. Analysis for pharmacokinetics

Pharmacokinetic analysis of siRNA typically relies on LC-MS, owing to its high sensitivity and molecular specificity. Among various separation modes, IP-RP chromatography using HFIP as the counter anion and alkylamines as ion-pairing reagents is most commonly employed. A major advantage of LC-MS is its ability to infer metabolite structures based on accurate molecular mass measurements. LC-MS was used to investigate the siRNA metabolites that had transferred to urine and rabbit ocular vitreous humor [62]. The mobile phase consisted of HFIP/TEA and MeOH, and electrospray ionization (ESI) under these conditions preserved the duplex structure. This study demonstrated that siRNA metabolism is influenced by the position of chemical modifications, and that duplex structures are more resistant to nuclease degradation than single strands.

High-resolution mass spectrometry (HRMS) was also used to study siRNA metabolites after incubation in rat and human serum, as well as liver microsomes [63]. Following liquid–liquid extraction and solid-phase extraction, HRMS enabled distinction between metabolites differing by <1 Da. Distinct metabolic patterns were observed: in serum, the antisense strand underwent greater degradation, whereas in liver microsomes, the sense strand was more susceptible.

A validated plasma assay for GalNAc-conjugated siRNA and its metabolites was also developed using a high-resolution time-of-flight (TOF) mass spectrometer [64]. Despite the significantly larger molecular mass of siRNA compared to small-molecule drugs, the assay met bioanalytical method validation criteria as outlined in regulatory guidance for small-molecule pharmacokinetics. This method was successfully applied to pharmacokinetic studies in monkeys.

Hybridization LC-MS/MS, recently established as a highly sensitive and specific method for quantifying single-stranded oligonucleotides such as ASOs in biological matrices, has also been applied to siRNA analysis [65]. A major challenge in applying this technique to double-stranded oligonucleotides is strand competition with the capture probe during hybridization, which can lower extraction recovery. To overcome this issue, peptide nucleic acid (PNA) probes with higher affinity than DNA or RNA were employed, resulting in satisfactory recovery. The optimized method enabled quantification of siRNA in monkey plasma, cerebrospinal fluid, and tissue homogenates across the range 2.00–1000 ng/mL.

In addition to IP-RP, other separation modes have also been explored for siRNA pharmacokinetics. McGinnis et al. reported a non-denaturing AEX method with UV detection for quantifying siRNA and its metabolites [66]. A one-step sample preparation protocol using lysis buffer containing proteinase K was employed to extract siRNA from cells and media efficiently. This AEX method achieved a lower limit of quantitation of 6 ng/mL and was applied to separate and quantify siRNA and its chain-shortened metabolites in both formulated media and cell culture matrices. Metabolite identification was accomplished by comparing retention times with those of synthetically prepared standards.

7. Conclusions and perspectives

This review has summarized various LC separation modes and MS techniques employed in the analysis of siRNA therapeutics, highlighting the advantages and limitations of each method. siRNA represents one of the most rapidly advancing classes of therapeutics, with ongoing research extending into candidates featuring increasingly complex architectures, including multiple chiral centers and diverse conjugates. Compared to single-stranded oligonucleotides, siRNA—being a double-stranded—yields more complex chromatographic and mass spectral profiles, rendering analytical interpretation more challenging. In particular, non-denaturing methods require stringent control of parameters such as column temperature and mobile phase pH to preserve duplex integrity. The combined use of both denaturing and non-denaturing techniques offers a practical and comprehensive approach for siRNA characterization. As this modality continues to evolve, the development of robust and reliable analytical methods remains essential to ensure product quality.

IP-RP chromatography remains the most widely used separation mode due to its strong resolution and compatibility with MS. One limitation of IP-RP is the tendency of siRNA duplexes to denature in the presence of organic solvents and ion-pairing reagents; however, recent studies have shown that the addition of volatile salts can significantly improve duplex stability, thereby expanding the applicability of IP-RP. AEX is another widely adopted technique. Its high-salt mobile phase makes it suitable for maintaining duplex structure during analysis. Although AEX is generally incompatible with MS, the use of 2D-LC, where a second MS-compatible dimension follows AEX separation, offers a promising solution for structural elucidation of complex peaks. SEC separates components based on hydrodynamic radius, offering a simpler separation mechanism. It is particularly useful when IP-RP or AEX are unsuitable due to sample matrix or structural complexity. Given that many siRNA formulations include large delivery systems such as

LNPs and conjugates, SEC will continue to play an important role in their analysis. Recently, several reports have indicated that the mobile phase composition used in HILIC not only helps preserve the siRNA duplex during separation but also during the MS ionization process. Currently, few studies have systematically examined how HILIC parameters (e.g., stationary phase type, mobile phase composition, and ion-pairing additives) affect duplex integrity, ionization efficiency, and impurity separation in siRNA analysis. Further mechanistic and comparative investigations are needed to establish optimized, duplex-preserving HILIC-MS methods and to better inform the development of analytical methods for siRNA. MS remains a powerful analytical tool, capable of detecting components that cannot be separated chromatographically and facilitating structural prediction from molecular mass. Enhanced understanding of duplex stability during ionization will help expand the capabilities of MS in siRNA analysis. Moreover, the development of mobile phases that are both highly sensitive and free from ion-pairing reagents or HFIP (which risk contaminating instrumentation) is highly desirable. New separation techniques—such as 2D-LC, MMC, supercritical fluid chromatography (SFC), and ion mobility spectrometry (IMS)—have been applied to single-stranded oligonucleotides [67,68,26,69–71], and their application to siRNA may help overcome analytical challenges that current techniques cannot address.

While numerous analytical techniques are available, the choice of method should be carefully guided by the research objective, considering the balance between sensitivity, specificity, and practicality. Continued advancement in analytical technologies will undoubtedly support the development of high-quality siRNA therapeutics and facilitate their broader clinical application.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT (ChatGPT 4o and ChatGPT 5) to improve readability and language of the text. Following the use of this tool, the authors thoroughly reviewed and edited the content and take full responsibility for the final version of the manuscript.

CRediT authorship contribution statement

Hiroyuki Togawa: Writing – original draft, Visualization, Conceptualization. **Takao Yamaguchi:** Writing – review & editing, Writing – original draft, Conceptualization. **Junji Kawakami:** Writing – review & editing. **Satoshi Obika:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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