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Review

Chromatographic methods for the determination of therapeutic oligonucleotides☆

A. Cary McGinnis[1], Buyun Chen[1], Michael G. Bartlett*

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA 30602-2352, USA

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ABSTRACT

Both DNA and RNA are being explored for their therapeutic potential against a wide range of diseases. As these new drugs emerge, new demands arise for the analysis and quantitation of these biomolecules. Pharmacokinetic and pharmacodynamic analysis requirements for drug approval place enormous challenges on the methods for analyzing these therapeutics. This review will focus on bioanalytical methods for DNA antisense and aptamers as well as small-interfering RNA (siRNA) therapeutics. Chromatography methods employing ultraviolet (UV), fluorescence and mass spectrometric (MS) detection along with matrix-assisted laser desorption/ionization (MALDI) will be covered. Sample preparation from biological matrices will be reviewed as well as metabolite analysis and identification. All of these techniques are important contributions toward oligonucleotide therapeutic development. They will also be important in microRNA (miRNA) biomarker discovery and RNomics in general, as more non-coding RNAs are inevitably discovered.

Contents

1. Introduction

Therapeutic oligonucleotides (oligos) have emerged as promising candidates for drug therapies for a wide range of diseases, including cancer, AIDS, Alzheimer's disease and cardiovascular disorders [1]. They have also become indispensible tools for genomic studies allowing for specific knockdown of proteins to study signaling pathways and identify therapeutic targets. Currently, there are two DNA therapeutics approved by the US FDA. Fomivirsen is an antisense oligonucleotide for the treatment of cytomegalovirus retinitis [2]. Pegaptanib is an aptamer for the treatment of neovascular age-related macular degeneration [3]. Table 1 shows the many DNA aptamer and antisense drugs, as well small-interfering RNA (siRNA) therapeutics that have been or are currently being used in human clinical trials [4].

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* Corresponding author. Tel.: +1 706 542 4410, fax: +1 706 542 5358.
E-mail address: Bartlett@rx.uga.edu (M.G. Bartlett).

[1] These authors contributed equally to this work.

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Table 1
Oligonucleotide therapeutics involved in clinical trials. Information includes the drug name, company sponsoring the clinical trial, the status, phase, and route of administration.

Drug	Company	Status	Phase	Administration	Disease
siRNA therapeutics					
TD101	Transderm, Inc.	Completed	I	Local injection	Pachyonychia Congenita
AGN211745	Allergan/Sirna	Completed	I/II	Local injection	Age-related macular degeneration; Choroidal neovascularization
AGN211745		Terminated	I	Intravitreal injection	Age-related macular degeneration; Choroidal neovascularization
CALAA-01	Calando Pharmaceuticals	Completed	II	IV injection	Cancer; Solid tumor
Atu027	Silence Therapeutics AG	Recruiting	I	IV injection	Advanced solid tumors
Bevasiranib	Opko Health, Inc.	Completed	II	Intravitreal injection	Diabetic macular edema
	Opko Health, Inc.	Completed	II	Intravitreal injection	Wet age-related macular degeneration
QPI-1007	Quark Pharmaceuticals	Recruiting	I	Local injection	Chronic optic nerve atrophy; Non-arteritic anterior ischemic optic neuropathy
PRO-040201	Tekmira	Terminated	I	IV injection	Hypercholesterolemia
siG12D	Silenseed	Not yet recruiting	I	Local Drug EluteR	Adenocarcinoma of the pancreas
I5NP	Quark Pharmaceuticals	Recruiting	III	IV injection	Delayed graft function in kidney transplantation
	Quark Pharmaceuticals	Active	I	IV injection	Kidney injury, Acute renal failure
SYL040012	Sylentis, S.A.	Recruiting	I/II	Opthalmic drops	Glaucoma, Ocular hypertension
Aptamer therapeutics					
ARC1905	Ophthotech Corporation	Active	I	Intravitreal injection	Dry age-related macular degeneration
E10030	Ophthotech Corporation	Recruiting	II	Intravitreal injection	Neovascular age-related macular degeneration
ARC1905	Ophthotech Corporation	Active	I	Intravitreal injection	Neovascular age-related macular degeneration
EYE001	Eyetech Pharmaceuticals	Recruiting	II/III	Intravitreal injection	Neovascular age-related macular degeneration
REG1	National Heart, Lung, and Blood Institute	Completed	I	IV injection	Anticoagulation system
Pegaptanib sodium (Macugen)	Eyetech Pharmaceuticals	Completed	IV	Intravitreal injection	Exudative age-related macular degeneration
AS1411	Antisoma Research	Recruiting	II	IV injection	Acute myeloid leukemia
NOX-E36	Noxxon Pharma AG	Completed	III	IV and Subcutaneous	Chronic inflammatory diseases, Type 2 diabetes mellitus, Systemic lupus erythematosus
NOX-A12	Noxxon Pharma AG	Recruiting	III	IV injection	Hematopoietic stem cell transplantation
ARC1779	Archemix Corp.	Completed	II	IV injection	Von Willebrand factor-related platelet function disorders
Bevacizumab	Medical University of Vienna	Recruiting	III	Intraocular injection	Diabetic retinopathy
ARC19499	Archemix Corp.	Not yet recruiting	I/II	Subcutaneous injection	Hemophilia
Antisense therapeutics					
EGFR Antisense DNA	University of Pittsburgh	Not yet recruiting	I/II	Intratumoral injection	Head and neck squamous cell carcinoma
	University of Pittsburgh	Active	I	Intratumoral injection	Head and neck squamous cell carcinoma
AEG35156	Aegera Therapeutics	Recruiting	I/II	IV injection	Advanced hepatocellular carcinoma
	Aegera Therapeutics	Recruiting	I/II	IV injection	Chronic lymphocytic leukemia
	Aegera Therapeutics	Terminated	I/II	IV injection	Advanced pancreatic cancer
	Aegera Therapeutics	Terminated	I/II	IV injection	Advanced breast cancer
	Aegera Therapeutics	Terminated	I/II	IV injection	Advanced Non-small cell lung cancer
	Aegera Therapeutics	Terminated	II	IV injection	Advanced cancer
	Aegera Therapeutics	Active	II	IV injection	Leukemia

Table 1 (*Continued*)

Drug	Company	Status	Phase	Administration	Disease
XIAP	Aegera Therapeutics	Completed	I/II	IV injection	Acute myelomonocytic leukemia
OGX-427	OncoGenex Technologies	Recruiting	I	Injection	Prostate, Ovarian, NSCL, Breast or bladder cancer
	Vancouver Coastal Health	Recruiting	I	Intravesical instillation	Superficial bladder cancer
LErafAON-ETU	Neopharm	Active	I	IV injection	Advanced cancer
	Neopharm	Completed	I	IV injection	Advanced solid tumors
	Neopharm	Completed	I	IV injection	Advanced malignancies
AVI-4658	Imperial College of London	Completed	I/II	Intramuscular injection	Duchenne muscular dystrophy
TGFB2-Antisense-GMCSF	Mary Crowley Medical Research Center	Recruiting	I	Injection	Advanced cancer
G3139	University of Chicago	Active	I/II	IV injection	Recurrent small cell lung cancer
Genasense (Oblimersen)	Genta Incorporated	Completed	I/II	IV injection	Chronic lymphocytic leukemia
	Genta Incorporated	Active	III	IV injection	Advanced melanoma
	Genta Incorporated	Active	I	IV injection	Solid tumors
	M.D. Anderson Cancer Center	Completed	II	IV injection	Recurrent non-Hodgkin's lymphoma
	Genta Incorporated	Active	I/II	IV injection	Chronic lymphocytic leukemia
	Genta Incorporated	Active	III	IV injection	Melanoma
	Genta Incorporated	Active	III	IV injection	Relapsed or refractory multiple myeloma
	California Cancer Consortium	Completed	II	IV injection	Metastatic renal cell cancer
	Genta Incorporated	Active	III	IV injection	Chronic lymphocytic leukemia
	University of Wisconsin, Madison	Completed	I	IV injection	Advanced solid tumors
	University of Chicago	Completed	I	IV injection	Extensive-stage small cell lung cancer
	Genta Incorporated	Active	II	IV injection	Acute myeloid leukemia
	British Columbia Cancer Agency	Completed	I	IV injection	Diffuse large B-cell lymphoma
	M.D. Anderson Cancer Center	Completed	I/II	IV injection	Metastatic or locally advanced breast cancer
	Jonsson Comprehensive Cancer Center	Active	I	IV injection	Advanced malignant melanoma
	Genta Incorporated	Active	II/III	IV injection	Lung cancer
	Genta Incorporated	Active	II/III	IV injection	Advanced melanoma
	Children's Oncology Group	Completed	I	IV injection	Relapsed or refractory solid tumors
	European Organization for Research and Treatment of Cancer	Active	II	IV injection	Hormone-refractory adenocarcinoma (cancer) of the prostate
	Memorial Sloan-Kettering Cancer Center	Completed	I/II	IV injection	Solid tumors
	San Antonio Cancer Institute	Completed	I/II	IV injection	Metastatic or recurrent colorectal cancer
	Cancer and Leukemia Group B	Completed	III	IV injection	Acute myeloid leukemia

Table 1 (*Continued*)

Drug	Company	Status	Phase	Administration	Disease
	Genta Incorporated	Completed	I	IV injection	Solid tumors
	Southwest Oncology Group	Active	II	IV injection	Large B-cell non-Hodgkin's lymphoma
G4460	University of Pennsylvania	Active	II	Bone marrow transplantation	Chronic myelogenous leukemia
EZN-2968	National Cancer Institute	Recruiting	I	IV injection	Advanced solid tumors
	Enzon Pharmaceuticals, Inc.	Recruiting	I	IV injection	Advanced solid tumors or lymphoma
BP-100-1.01	Bio-Path Holdings, Inc.	Recruiting	I	IV injection	Leukemia
AP 12009	Antisense Pharma	Completed	II	Intratumoral injection	High-grade glioma
	Antisense Pharma	Recruiting	I	IV injection	Pancreatic neoplasms, Melanoma, Colorectal neoplasms
	Antisense Pharma	Recruiting	I	Intratumoral infusion	Anaplastic astrocytoma
LY900003	Eli Lilly and Company	Completed	II	IV injection	Non-small cell lung cancer
ISIS 113715	Isis Pharmaceuticals	Completed	I	Subcutaneous injection	Dermatologic effects
c-myb AS ODN	University of Pennsylvania	Recruiting	I	IV injection	Advanced hemotologic malignancies
SPC2996	Santaris Pharma A/S	Active	I/II	IV injection	Chronic lymphocytic leukemia
ISIS 104838	Isis Pharmaceuticals	Completed	II	Subcutaneous injection	Rheumatoid arthritis
Alicaforsen (ISIS 2302)	Isis Pharmaceuticals	Completed	III	IV injection	Crohn's disease
	Isis Pharmaceuticals	Active	III	IV injection	Crohn's disease
	Isis Pharmaceuticals	Completed	II	Enema	Mild to moderate active ulcerative colitis
	Isis Pharmaceuticals	Completed	II	Enema	Mild to moderate active ulcerative colitis
Lucanix™	NovaRx Corporation	Completed	II	Vaccine	Stages II–IV non-small cell lung cancer
Autologous Dendritic Cell Therapy	University of Pittsburgh	Recruiting	I	Vaccine	Type 1 diabetes
Mipomersen	Genzyme	Completed	I	Subcutaneous injection	Hypercholesterolemia
	Genzyme	Completed	I	IV injection	Cardiac repolarization
	Genzyme	Completed	I	IV injection	Assessment of blood clotting and thinning
GTX™ Drug Eluting Coronary Stent System	Cook	Terminated	I	Coronary Stent	Lesion in the coronary artery
VRX496-Modified Autologous T cells	VIRxSYS Corporation	Active	II	IV injection	HIV
Custirsen (OGX-011)	NCIC Clinical Trials Group	Completed	II	IV injection	Locally advanced or metastatic breast cancer
	NCIC Clinical Trials Group	Completed	I	IV injection	Metastatic or locally recurrent solid tumors
	NCIC Clinical Trials Group	Completed	I	IV injection	Prostate cancer
	University of British Columbia	Recruiting	II	IV injection	Localized prostate cancer
	OncoGenex Technologies	Recruiting	III	IV injection	Hormone-refractory prostate cancer
ISIS 2503	University of Alabama at Birmingham	Completed	II	IV injection	Colorectal cancer
	University of Alabama at Birmingham	Completed	II	IV injection	Pancreatic cancer
EGFRvIII peptide vaccine	Southwest Oncology Group	Active	I	Vaccine	Gastric, Prostate, or ovarian cancer
OGX-427	British Columbia Cancer Agency	Recruiting	II	IV injection	Hormone-refractory prostate cancer

Table 1 (*Continued*)

Drug	Company	Status	Phase	Administration	Disease
ISIS 3521/ISIS 5132	Eastern Cooperative Oncology Group	Completed	II	IV injection	Metastatic breast cancer
ISIS 3521	Isis Pharmaceuticals	Completed	III	IV injection	Non-small cell lung cancer
RevM10 gene	Systemix	Active	I/II	IV injection	HIV-related non-Hodgkin's lymphoma
Syngeneic Lymphocytes (CD4+) Cultured with OKT3 (Ortho) and Interleukin-2 (Chiro Miravirsen)	National Human Genome Research Institute (NHGRI)	Completed	I	IV injection	HIV infection
	Santaris Pharma A/S	Completed	I	IV injection	Hepatitis C
	Santaris Pharma A/S	Recruiting	II	Subcutaneous injection	Treatment-Naïve chronic Hepatitis C
	Santaris Pharma A/S	Active	I	IV injection	Hepatitis C
Aezea® (Cenersen)	Eleos, Inc.	Completed	II	IV injection	Acute myelogenous leukemia
	Eleos, Inc.	Not yet recruiting	II	IV injection	Acute myelogenous leukemia
ASM8	Topigen Pharmaceuticals	Completed	I/II	Inhalation	Asthma
GTI-2040	Lorus Therapeutics	Completed	I/II	IV injection	Renal cell carcinoma
RESTEN-MP	AVI BioPharma, Inc.	Terminated	I	IV injection	Coronary artery disease, Coronary stent restenosis
	AVI BioPharma, Inc.	Completed	I	IV injection	Coronary artery disease, Coronary stent restenosis

There are numerous challenges for analyzing DNA and RNA as therapeutics and as laboratory tools. Therapeutic oligonucleotides are generally 19–27 nucleotides (nt) long. They are characterized by their size (5–14 kDa) and high degree of negative charge. The IC_{50} is generally in the low nanomolar range (0.2–5 nM) [5]. Once inside a cell, they are powerful regulators of gene expression, but measuring these low levels requires sensitive, quantitative methods, especially for the determination of pharmacodynamic and pharmacokinetic parameters. Sensitive methods are required to determine metabolism, toxicological profiles and terminal phase elimination for therapeutic use. Laboratory use of oligonucleotides requires methods capable of measuring intracellular levels of these biomolecules. Since the mechanism of action of many oligonucleotides requires delivery of a sense-antisense duplex, the measurement of both the individual strands and the duplex are needed. Furthering the complexity and challenge of these biomolecules is sample preparation in plasma, tissues and cells. Exceptional techniques are required to extract oligos from biological matrices, separate them from interfering compounds, and then quantify, and characterize them.

Microbiological and imaging methods are available for analyzing RNA and DNA. Although some of these methods are quite sensitive, they often cannot distinguish between the parent molecule and their metabolites. Importantly, these methods also cannot differentiate unmodified from the modified oligos that are widely used in the lab and the clinic. Because they cannot distinguish between modified or unmodified small oligos, they cannot duplicate or directly measure whether these modifications remain intact as the molecules are metabolized. A thorough review of these methods, including immunoassays and qRT-PCR, has been previously reported and therefore will not be covered here [6]. This review will focus on bioanalytical methods to analyze RNA and DNA with an emphasis on therapeutic oligonucleotides. Specifically, sample extraction from biological matrices will be explored. Chromatographic separation methods with ultraviolet (UV) and fluorescence detection will be reviewed, as well as mass spectrometric and hyphenated methods involving combinations of these techniques. The mechanisms for DNA and RNA therapeutics will be discussed due to their relevance in selecting the most appropriate approach for analyzing particular oligonucleotides.

2. Mechanisms of oligonucleotide therapeutics

The antisense DNA mechanism was first reported in 1978 by Zamecnik and Stephenson, who found that short complimentary DNA sequences could be used to suppress the expression of many disease-causing genes [7]. Antisense DNAs bind with the complimentary mRNA sequence following entry in to the cell and translocation to the nucleus (Fig. 1). The antisense DNA/mRNA duplex activates the nuclear enzyme RNase H, which then degrades the mRNA strand. The recognition site of RNase H is still not well understood, but DNA oligonucleotides as short as tetramers have been shown to activate RNase H [8]. Modifications to DNA, especially to the sugar/phosphate backbone can alter the mechanism of action, especially if they shift the structure of the DNA from B-form to a more RNA-like A-form configuration. These modified oligonucleotides typically no longer recognize RNase H but instead directly compete with mRNA substrates for interaction with many of the proteins involved in protein translation. A secondary mechanism for many modified DNA oligonucleotides is splicing inhibition, which involves interference with the production of mature mRNAs. One specialized application for oligonucleotides involves sequences containing unmethylated CpG motifs. These oligos have been shown to activate an immune response via stimulation of B cells and acceleration of monocyte maturation. This unexpected, highly sequence specific enhancement of the immune system was discovered during the development of antisense oligonucleotides. This mechanism leads to activation of natural killer (NK) cells, dendritic cells, macrophage and B cells and holds great promise for the treatment of many diseases and as vaccine adjuvants [9].

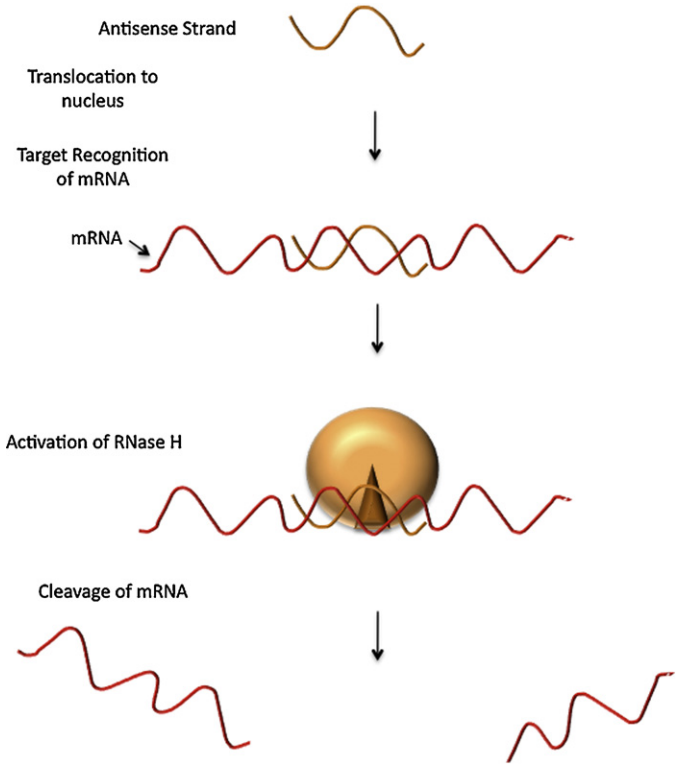

Fig. 1. Antisense DNAs enter the cell and are translocated to the nucleus. They bind with the complimentary mRNA sequence. The antisense DNA/mRNA duplex activates the nuclear enzyme RNase H, which then degrades the mRNA strand.

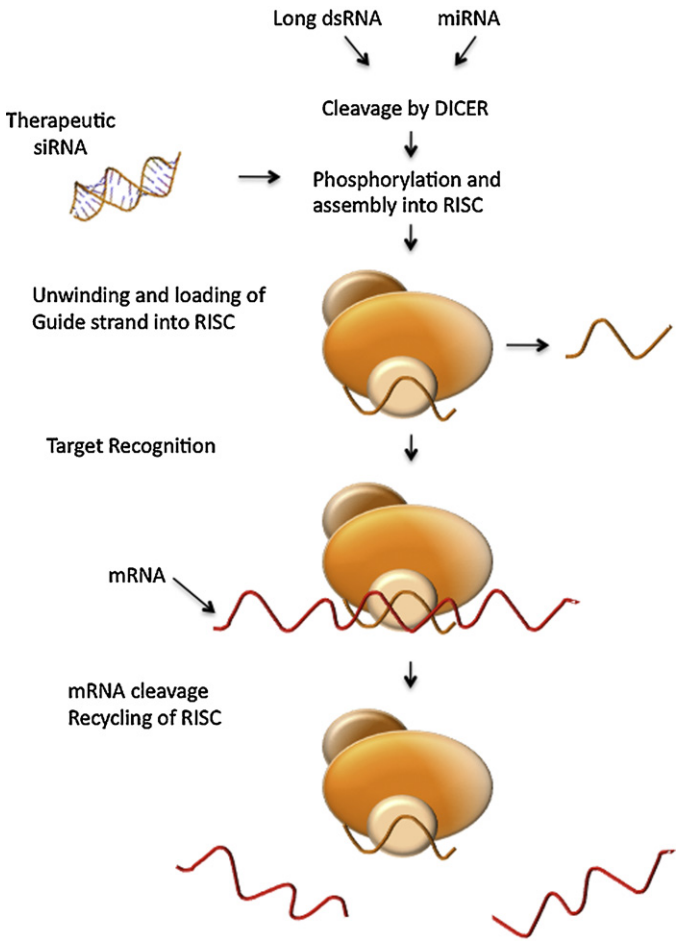

Fig. 2. RNA interference mechanism: The Dicer protein cleaves double stranded RNA in to 21–25 nt strands (siRNA). Dicer works with a double stranded RNA binding protein to guide siRNA in to the RNA induced silencing complex (RISC). Following phosphorylation of the 5′ ends, siRNA is incorporated in to RISC. The passenger strand is unwound, released and degraded leaving the guide strand bound to RISC. RISC recognizes mRNA through base pairing of a seed region that is 2–8 nt from the 5′ end. If the mRNA is perfectly complementary then it is cleaved. RISC is then recycled to perform further silencing.

RNA therapeutics include siRNA and microRNA (miRNA). Both use the RNA interference (RNAi) pathway, which is a natural defense mechanism against double stranded RNA (dsRNA) [10,11]. This silencing mechanism inhibits protein synthesis by destroying messenger RNA (mRNA) before it can be translated. This mechanism of inhibition is sequence specific and highly selective [11].

Fig. 2 shows the RNAi pathway [12,13]. RNA enters the pathway when the protein, Dicer [14], cleaves it in to 21–25 nt strands [15]. These small RNAs (siRNA or miRNA) are then incorporated into the RNA induced silencing complex (RISC) [16]. This is where most synthetic RNAi molecules enter the pathway. Synthetic siRNAs require phosphorylation of the 5′ end to be active. This occurs through the action of Clp1 kinase [17]. Following phosphorylation of the 5′ end, RISC binds to the 3′ end of the guide strand on the thermodynamically least stable end of the double strand [18,19]. The passenger strand is then unwound, cleaved and released leaving the guide strand bound to RISC [20–22]

Recognition of the target mRNA begins with a "seed" region that is 2–8 nt from the 5′ end of the guide strand [23]. This seed region pairs with the target mRNA. If the siRNA or miRNA is perfectly complementary, then the target mRNA will be cleaved between the 10th and 11th nt from the 5′ end [24]. If it is not perfectly complementary then translational repression will occur through competition with proteins involved in translational elongation or termination [19,25,26].

Knowledge of the RNAi mechanism allows us to make some general conclusions about the specificity for analytical methods that determine siRNA or miRNAs. These molecules are introduced as dsRNA, but once they are incorporated in the RISC complex, they exist as the single guide strand and possibly cleavage products of the passenger strand. Thus methods to determine siRNA levels should optimally measure both the single and double stranded forms. Measuring intracellular levels of siRNA will further challenge analytical methods because of the low levels of dsRNA and single stranded RNA (ssRNA).

3. Modifications

Therapeutic DNA and RNA are routinely modified to enhance their stability in plasma, tissues and cells [27]. The most common metabolic transformations of oligonucleotides occur from exonucleases and endonucleases, which initiate hydrolysis along the phosphate backbone. Therefore, it is not surprising that the majority of the modifications are used to decreases these reactions. The structures of the most common modifications are shown in Fig. 3. Besides decreasing degradation, modifications can also decrease toxicity and increase potency by increasing the volume of distribution and enhancing the binding specificity [23,27,28]. RNAs are routinely modified at the 2′ ribose position because it does not interfere with the A-form duplex structure that is necessary for activity [29]. Substitutions of this 2′ hydroxyl group protect against nucleases and increase affinity for the target mRNA. These include 2′-O-methyl (2′-O-Me), O-ethyl (2′-O-ethyl), O-methoxyethyl (2′-MOE), and fluorine (2′-F) substitutions as well as locked nucleic acids (LNA). LNAs have a bridge connecting the 2′ oxygen and the 4′ carbon. They enhance base stacking and are used on the

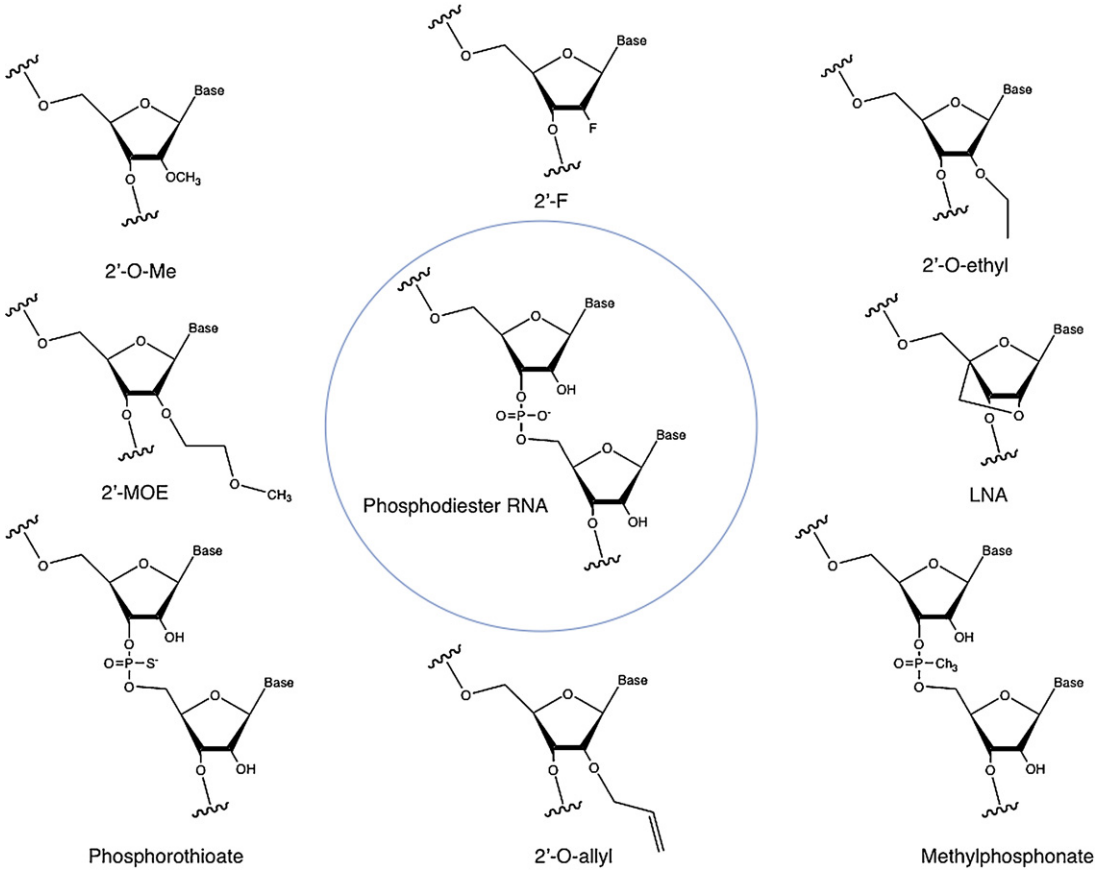

Fig. 3. Structures of RNA modifications. Unmodified RNA is shown in the center. DNA modifications are identical with the absence of the 3′ hydroxyl group on the ribose. Modifications of RNA and DNA include 2′-O-methyl (2′-O-Me), O-ethyl (2′-O-ethyl), O-methoxyethyl (2′-MOE), and fluorine (2′-F) substitutions as well as locked nucleic acids (LNA). Backbone modifications include phosphorothioate and methylphosphonate.

passenger strand of duplex RNAs to enhance the binding specificity of the guide strand in the RISC complex [30] The most frequently used 2′ modifications are the 2′-O-Me, and 2′-F substitutions [5,31,32]. The positions of 2′ modifications are often made after testing the oligonucleotide to locate the most labile linkages.

Besides enhancing binding selectivity of the guide strand, 2′-O-Me modifications also dramatically increase the elimination half-life of oligonucleotides up to 30 days in plasma and tissues from a wide-variety of species. 2′-F modifications reduce the hydrophobicity of the oligonucleotide which aids with entry into the cell [27]. Both modifications increase binding affinity and stability against nucleases. In general, modifications of the 2′ position of ribose decrease an oligonucleotides affinity for the enzyme RNase H, thus favoring the RNAi pathway over the antisense pathway.

Methyl ether and allyl ethers have been used to end-cap the 3′-overhangs of siRNAs to protect them against exonucleases [33,34]. Interestingly, although these bulkier allyl groups do not work well for internal modifications of siRNA, they retain excellent activity when used in anti-miRNAs that are used to down-regulate endogenous miRNA [35].

Backbone modifications including phosphorothioate modification are also commonly used [36]. Besides increasing the hydrophobicity, these modifications also increase resistance to exonucleases and increase plasma protein binding to improve tissue distribution and prevent renal excretion [37]. Phosphorothioate modifications maintain the oligos DNA-like properties and are excellent substrates for the enzyme RNase H [38]. Phosphorothioate containing oligonucleotides also possess low protein binding for serum albumin [39]. Phosphorothioates are rapidly absorbed and distributed following intravenous administration and they have a long elimination half-life (greater than 60 h in humans) [40]. Their clearance is primarily due to metabolic degradation via several nucleases. Modifications to DNA or RNA bases are not widely used in therapeutics, but there is an example where they were shown to increase potency [29].

4. Sample preparation

Bioanalytical techniques begin with sample extraction from a biological matrix. The goal of sample preparation is to isolate the targeted oligonucleotide(s) from interfering compounds. The key lies with maximizing recovery of the analyte while removing unwanted components. Among the many challenges are minimizing sample transfers, minimizing analyte degradation, and avoiding non-specific binding to containers. RNA extractions add additional complexity because these analytes must be protected against RNases. This requires that all glassware, plastic ware, instrument tubing and reagents be RNase free.

One of the simplest approaches to sample preparation is to use dilute and shoot. Using this approach, Chen et al. directly injected plasma and urine samples after diluting with 1% Nonidet P-40 in saline [41]. While the separation and detection limit (0.21 μg/ml) were significantly compromised in this assay, the recovery of the oligonucleotide was reported to be 100%. Bourque and Cohen used an on-line method for preparation of a phosphorothioate DNA [42]. Plasma and urine samples were injected directly on to a strong anion exchange column. A lithium bromide mobile phase was used to wash the serum proteins from the samples and then elute the analyte. The analytes were then fraction collected for subsequent

analysis. This method provided excellent linearity with a rapid 5 min sample preparation time.

Several studies use a proteinase K digestion or methanol precipitation to remove the protein from the biological samples [41,43–46]. These approaches avoid issues with the nonspecific and irreversible binding of oligonucleotide to either solid-phase extraction (SPE) cartridges or many containers. The recovery of oligonucleotides when using the proteinase K digestion with liquid–liquid extraction (LLE) has been reported to be as high as 98% [43,44].

Many of the extraction methods employed in the literature involve phenol/chloroform extractions or modifications thereof [47–49]. These extractions can be time consuming and laborious, sometimes involving six or more steps and three sample transfers [44,46,50]. Other methods use SPE cartridges [51–53]. These significantly reduce sample preparation time, but can still involve multiple sample transfers and time-consuming evaporation steps. Most of the SPE methods employ similar buffer systems as those used in chromatographic separations. Gaus et al. employed a sample extraction strategy where biological samples were purified by anion-exchange HPLC following phenol–chloroform LLE [54]. Ion-exchange peaks were fraction collected followed by C18 SPE for desalting. This study was conducted together with a capillary gel electrophoresis (CGE) assay, therefore the extraction procedure was almost the same as those used in most CGE applications. Dai et al. proposed a much more straightforward sample clean-up procedure for a phosphorothioate DNA [52]. The biological samples were mixed with a loading buffer containing the ion-pair agent, triethylammonium bicarbonate (TEAB) and extracted using a C18 SPE cartridge. The proteins and salts were easily eluted with TEAB and water whereas oligonucleotides were retained on the cartridge due to their increased hydrophobicity and pseudo-neutral properties following ion-pairing with TEAB. The ammonium ion also shields the oligonucleotide from binding with sodium and potassium ions. The recovery of this procedure is reported to be 43–64% depending on the oligonucleotide concentration. The break-through from the SPE cartridge was measured by UV and found to be negligible, indicating that irreversible binding to the SPE support may be the cause for the reduced recovery. A similar method using triethylammonium acetate (TEAA) as the ion-pair agent was employed for the quantitation of a liposome entrapped antisense DNA [55].

The study of quantitation of phosphorothioate DNA by LC–MS conducted by Zhang et al. is by far the most extensive research on biological sample preparation and handling [56]. Various issues have been addressed in this study including chromatographic reproducibility, non-specific binding to containers, enhancing the recovery from a biological sample, and stabilizing the analyte during evaporation. The authors suggest preconditioning the LC column with repeated injections of the analyte to improve the chromatographic reproducibility at lower concentrations. Silanizing glass autosampler vials and using 7 mM triethlyamine (TEA) in 10% methanol was recommended to reduce the non-specific binding of oligonucleotides to sample containers. LLE followed by SPE was performed to increase the recovery. During LLE, 5% ammonium hydroxide was added to the phenol-chloroform to aid the distribution of oligonucleotides to the aqueous phase. The residual organic solvent was removed from the aqueous extract by C18 SPE using a TEA–hexafluoroisopropanol (HFIP) and TEAB buffer. Significant gains in recovery were observed when 100 mM TEA was added to 60% acetonitrile in water as the elution solvent.

RNA sample extraction presents a unique challenge because of the ubiquitous nature of RNases in the environment. All laboratory materials that will be exposed to RNA need to be free of RNases, including sample containers, mobile phase bottles, benches, pipette tips and even water. Traditionally two types of sample extraction are used for RNA, (1) chemical extraction using denaturing agents and organic solvent precipitation and (2) solid-phase extraction by immobilizing the RNAs on a glass support. Very few applications of the determination of RNAs from biological samples have been reported. Beverly et al. reported extraction procedures of a 23-mer siRNA from vitreous fluid samples and retina/choroid samples as part of a stability study from ocular tissues [49]. For vitreous fluid, a one-step chemical extraction was employed by using a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) and 1 mM Tris/EDTA at pH 8.0. The recovery was between 14% and 40% depending on the concentration. A commercially available solid-phase extraction kit was used for retina/choroid sample clean up. Briefly the sample was lysed and homogenized and most of the cellular components and DNA were removed by a mixture of phenol:chloroform (5:1) isoamyl alcohol at pH 4.7. The aqueous layer was then mixed with ethanol to increase affinity toward the glass support and finally the mixture was twice passed through a glass fiber filter. The small RNAs were immobilized on the filter and larger RNAs were washed off. The analyte was recovered by washing the filter with deionized water. However no recovery data was reported for the extraction of the oligonucleotides from these samples.

With increasing demands being made on bioanalytical methods, the need for high sample throughput has become a significant criterion in method development. Many kits are now available for DNA and RNA extraction. These are mostly geared toward PCR applications, but several are also being marketed for small length oligonucleotide applications, especially miRNA. A substantial need exists for simple, high throughput methods for extracting small oligos. The methods must be amenable to a variety of biological matrices including cells and tissues. The primary need is for robust recovery of small amounts of RNA and DNA for pharmacokinetic and pharmacodynamic analysis, as well as, for the study of cellular mechanisms of oligonucleotides.

5. Chromatographic separations

The development of analytical methods for sensitive, quantitative analysis of therapeutic oligonucleotides will be a hallmark for the advancement of this field. Analytical methods must be able to robustly separate, quantify and characterize these biomolecules and their metabolites at levels low enough to allow for intracellular analysis and terminal end-phase kinetics. Another significant challenge lies in separating and identifying the metabolites of DNA aptamers and antisense, as well as siRNAs. siRNAs are double stranded molecules that can be metabolized by nucleases on the 3′ and 5′ end of either strand. This presents a significant separation and characterization challenge. In addition, methods are needed to separate and characterize synthetic DNA used for primers and microarray analysis. For these applications, the samples may contain DNAs that are the same length, but have different base pair combinations. This also applies to pooled siRNAs used in cell culture experiments [57]. Most of the methods reviewed here are amenable to both single stranded DNA (ssDNA) and double stranded RNA. The methods will be divided based on the mechanism of separation. Since DNA and RNA have a strong absorbance at 260 nm, most of the methods are amenable to UV detection and a few methods use fluorescence detection. In addition, there is a dedicated section on mass spectrometric detection focusing on the power of this detection method for low-level quantitation as well as for its ability to identify and characterize oligonucleotides. Specific features that are more amenable to DNA, RNA, or single versus double strand will be highlighted within the individual sections.

6. HPLC methods

HPLC represents a platform that is amenable to high throughput analysis, is relatively inexpensive, easy to use, and robust. It is for these reasons that much work has been done to find separation methods to analyze therapeutic oligonucleotides and their fate in biological matrices. These techniques include ion-exchange chromatography [41] and ion-pair reversed-phase HPLC (IP-HPLC) [58].

7. Ion-exchange chromatography

Ion-exchange chromatography with UV and fluorescence detection is an excellent method for separating charged molecules, and as such, is amenable to large multiply charged oligonucleotides [59]. The separation mechanism for oligonucleotides is achieved with positively charged stationary phases that exchange negatively charged analytes through competition with an increasing gradient of anions in the mobile phase. Typical mobile phases include sodium chloride and sodium perchlorate in Tris or sodium phosphate buffers. These offer excellent selectivity for separating oligos based on length via the number of charges on the phosphate backbone. Sodium perchlorate has been shown to offer selectivity for nucleobase composition. This appears to be due to increased hydrophobic interaction with the stationary phase support [60]. The capability of ion-exchange to separate oligonucleotides from other polar molecules in biological matrices, and its high tolerance for salts, significantly simplifies sample extractions when compared to many other techniques. These unique advantages confer ion-exchange chromatography an indispensable role in the study of the modifications, purification and kinetic analysis of oligonucleotides.

Both strong and weak anion-exchange supports have been used successfully for the separation of DNA [43,44,46,50,60,61] and larger RNAs (>30-mer) [62]. Along with selectivity, ion-exchange allows for various modes of detection, including UV and fluorescence. Arora et al. used a unique fluorescence detection method for quantitative analysis of an antisense morpholino oligomer using a 4×250 mm column [46,50]. Biological samples extracted from plasma and liver tissues were reconstituted with a 5′ fluorescently tagged DNA that was complementary to the analyte. The fluorescently tagged DNA was annealed with the sample. The annealed sample was separated from a 15-mer internal standard and the excess tagged DNA, allowing for quantification of the drug in the biological matrix. This method would not likely be specific for truncated metabolites, because they too, would anneal with the fluorescing primer. The mechanism of separation for this paper is intriguing since it is unclear that morpholino-type oligonucleotides would carry negative charges at the pH of 8 used in this study. Therefore, the separation appears to be employing a significant amount hydrophobic interaction. The paper indicates a UV limit of detection of 40 ng/mL, but no chromatograms showing the limit of detection (LOD) or validation data were provided.

Another study used a unique lithium hydroxide, lithium chloride mobile phase with UV detection at 254 nm on a 4.6×150 mm column for separating analytes. The pharmacokinetics of a phosphorothioate bcl-2 antisense oligonucleotide were determined using a method originally published by Raynaud et al. [43,44,63]. An 18-mer antisense DNA was extracted from liver, kidney, plasma and urine. The analytes were separated using a weak anion-exchange column and a gradient of lithium chloride. The chromatography separated the 18-mer analyte from a 16-mer metabolite and had a reported UV limit of detection of 250 ng/mL. Recoveries for the extraction were reported at 98%, although no validation data was presented.

Analysis of oligonucleotide therapeutics will require separation of the parent molecule from its n-1 metabolites with UV or fluorescence detection. In addition, analysis of pooled oligonucleotides that are the same length, but differ in the composition of base pairs is necessary. This would be useful for analyzing primers, siRNAs and even miRNAs. In Thayer et al., base pair resolution of 21–25 nt unmodified ssDNA is demonstrated using a 4×250 mm column [60]. They also show selectivity for oligonucleotides between 21 and 25 base pairs that have different compositions. They demonstrate that the retention time increases with the pH of the mobile phase as the percentage of guanine and thymine increases in the single stranded oligonucleotides. The changes in retention are based on the ionization of the tautomeric oxygen on guanine and thymine. Higher mobile phase pH is also beneficial for denaturing oligonucleotides allowing for separation as fully linear species. The addition of acetonitrile reduced the retention time of oligonucleotides and altered the pH dependent retention time shifts. Overall, these retention differences can be exploited to separate both same length pooled oligos, as well as parent oligos and their metabolites. Although this is not a biological application, this work shows the selectivity and robustness of the glycidoxyethyl methacrylate based strong anion exchange columns that are specifically designed to be used for oligonucleotide separations. Fig. 4 shows a chromatogram using the same stationary phase on a 2×150 mm column with a NaCl gradient in a Tris buffer at pH 9.0. The chromatogram shows the separation of four double-stranded siRNA 21-mers that have different base pair combinations.

Fig. 5.

Buncek et al. analyzed sequence isomers using two different 4.6×150 mm ion-exchange columns with UV detection and a NaCl gradient at pH 8 [61,64]. They found that both columns resolved the sequence and positional isomers of a 21-mer polyT and polyC modified with A, C, T and G bases in the middle or on the 3′ or 5′ end. They postulate that the orientation of ssDNA and the basicity of the nucleobases in the ion-exchange column affect retention. The negatively charged phosphate groups will be attracted to the stationary phase, while the positively charged bases will be repelled. At pH 9–10, deprotonation of guanine and thymine will contribute to the overall negative charge of the oligo. In addition, secondary conformational changes caused by A–T, G–C base pairing may affect retention. With these mechanistic findings, it is likely that in a true heterooligonucleotide, differences in protonation and hydrophobicity of the bases would contribute to the separation of sequence isomers or oligos that are the same length with different compositions. Neutral pH (6.5–8.0) using $NaClO_4$ as an eluting salt has been used for length dependent separations. The conditions were successfully applied to the quantitative bioanalysis of a 37-mer ribozyme [45].

In considering the separation mechanism for oligonucleotides by ion-exchange chromatography, it is important to realize the complex nature of the interactions between the analyte and the stationary phase. Incomplete coverage of the particles of the stationary-phase by the ammonium groups provides the potential for significant non-ionic interactions to occur between the analyte and the stationary phase. These effects can especially be observed in phosphorothioates, where a chiral center is created when the oxygen atom is replaced by a sulfur atom, creating a mixture of 2^n diastereomers with a range of hydrophobicities [65]. This often causes peak broadening and subsequent loss of resolution due to the impact of these complex interactions with the column. This likely provides the main reason for the less than successful applications of ion-exchange chromatography with phosphorothioate oligonucleotides. To date, there are no reports of resolution between peaks differing by only one base for this class of oligonucleotides.

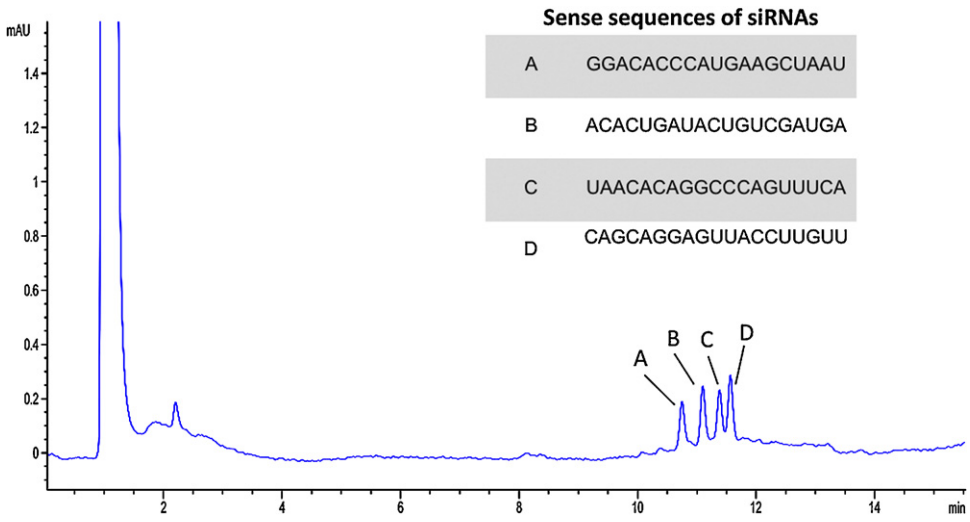

Fig. 4. Chromatogram demonstrating separation of heterooligonucleotides using ion-exchange chromatography with UV detection. Four 21-mer double stranded siRNAs with different base pair combinations were separated using a Dionex DNAPac PA200, 2 × 150 mm column. The sequences of the sense strand are shown. Both the sense and antisense strands also contain UU overhangs on the 3′ end. The mobile phase consisted of an increasing gradient of sodium chloride in a Tris buffer at pH 9.0.

Recently, Thayer et al. demonstrated a new column technology for purification of modified and unmodified synthetic ssDNA and ssRNA [66]. The stationary phase uses porous ion-exchange "nanobeads" that are attached to monolithic polymers on a 4.6 × 50 mm column. The 3 μm pore size is optimized to allow for mass transfer through convection rather than diffusion to improve column efficiency. They demonstrate the semi-preparative purification of 8.25 mg of a 25-mer DNA using a larger version of the column in a single run.

UV is the most commonly used detector with ion-exchange chromatography. UV detection is compatible with the high salt content of ion-exchange mobile phases and provides reasonable detection limits in many methods. With very crude sample preparation, Chen et al. had detection limits of approximately 200 ng/mL from plasma and urine samples using a hand-packed 1 × 20 mm column [41]. Using more rigorous sample preparation methods, detection limits of approximately 50 ng/mL were achieved for a phosphorothioate 37-mer using a 4 × 250 mm column [45]. Since phosphorothioate oligonucleotides have greater band broadening than other types of oligonucleotides, it would be reasonable to expect LOQs as low as 10 ng/mL with unmodified oligos.

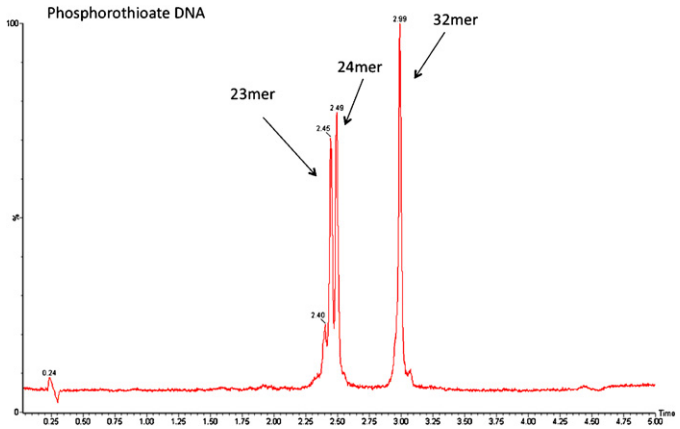

Fig. 5. Full scan total ion chromatogram showing the separation of a 23-mer and 24-mer phosphorothioate DNA and 32-mer poly dT internal standard on a Waters 2 × 50 mm BEH column. The mobile phase consisted of an increasing gradient of 50% methanol in a buffer containing HFIP and TEA.

Fluorescence detection generally requires derivatizing oligonucleotides to improve their response. However, the hydrophobic nature of fluorophores such as fluorescein may compete with charge density dependent separations like ion-exchange chromatography and alter the separation of labeled oligonucleotides. A recent study by Devi et al. separated a 20-mer phosphorodiamidate morpholino dsDNA, a 15-mer dsDNA (internal standard) and a ssDNA primer and found that the elution order of the fluorescein-labeled compounds was reversed relative to the unlabeled compounds [50]. Direct labeling of the 5′ end of an antisense DNA with fluorescein was used in a study of inhibition of gene expression [50,67]. Using fraction collection of the eluent followed by fluorometric analysis, the assay achieved a detection limit of 1 pM for a DNA 20-mer on a 4.6 × 250 mm, column.

A more recent development in oligonucleotide detection is the use of inductively coupled plasma mass spectrometry (ICP-MS). ICP sources do not tolerate high concentrations of organic solvents in the mobile phase; therefore, it would be considered optimal for interfacing with ion-exchange chromatography. ICP-MS provides elemental and isotopic information with a wide linear dynamic range that may exceed six orders of magnitude. Elemental phosphorus from phosphodiester linkages or both elemental phosphorus and sulfur from phosphorothioate linkages can be measured using ICP-MS with good sensitivity [68]. However, since ICP is a destructive ionization mode, it cannot provide additional information about the structure of the analyte.

These examples demonstrate the potential for ion-exchange chromatography in the analysis of oligonucleotide therapeutics. It is realistic to expect that a well-validated procedure with known metabolites could benefit from the high throughput and optimal selectivity of ion-exchange methods. Ion-exchange chromatography with UV detection provides a universal platform that allows for quantification of analytes and their metabolites. However, the identification of new peaks would likely require fraction collection and characterization by MS. Interfacing ion-exchange chromatography with mass spectrometry using the electrospray ionization interface would allow for direct determination of molecular weight and possibly sequencing of oligonucleotides. However, there are significant barriers that will need to be overcome to successfully apply this approach. The electrolytes used for separating oligonucleotides make forming an electrospray plume difficult. The use of an eluent suppressor to remove interfering electrolytes has been used in the past to allow ion-exchange chromatography to interface with mass

spectrometry [69]. However, eluent suppressors limit the selection of mobile phase to species such as hydroxide or carbonate that have not been successful for separating oligonucleotides.

8. Columns and stationary phases for ion-exchange chromatography

Columns for ion-exchange chromatography use both strong anion and weak anion exchange as mentioned earlier. Most reported work was achieved on the 4 × 4.6 mm internal diameter (id) columns [43,44,50,61,64]. Early glycidyl methacrylate polymer based columns have been improved with a glycidoxyethyl methacrylate [60]. The new columns are more stable at higher temperature and pH and exhibit a much improved column lifetime. A monolithic stationary phase with porous ion-exchange "nanobeads" has recently been introduced [66]. Both the methacrylate and monolithic columns are now available in custom 1 mm and capillary sizes as companies move toward supporting nano chromatography applications that have already been so successful in proteomics. As with other column supports, the smaller id columns should provide improved resolution and detection limits although literature references are not available at this writing.

9. Ion-Pair reversed-phase liquid chromatography

Ion-pair reversed-phase liquid chromatography, especially if combined with volatile mobile phase additives, has the potential to be a tremendous advance in the analysis of oligonucleotides. With adequate selectivity, this method could allow for universal UV detection for quantitative analysis, and could more easily be interfaced with mass spectrometry to provide exact mass data and MS/MS characterization. Numerous efforts have been made toward this goal since the first successful chromatographic separation of oligonucleotides in 1978 [70].

Ion-pairing in a reversed-phase system is controlled by a number of factors. The degree of retention of the oligonucleotides is based on the chain length of the n-alkyl ion-pairing (IP) agent and its interaction with the hydrophobic stationary phase [71]. The number of available charges on the biomolecule as well as its secondary structure then governs the interaction with the IP agent. This allows for separations based on the length of an oligonucleotide. However the hydrophobicity of the individual bases can still affect analyte retention. Studies with ssDNA have found that the hydrophobicity of the bases follows the order C < G < A < T [71–73]. The retention difference between heteronucleotides of the same length appears to be based on the sum of the hydrophobicity of all of the base pairs in the sequence. These base pair dependent effects are less apparent with dsDNA because the bases are shielded by the charged backbone [71,72]. Research in new ion-pairing agents has focused on two goals. The first, and most important goal is ensuring that the ion-pairing buffer is compatible with electrospray ionization-mass spectrometry (ESI-MS). These buffers are still useful for UV detection, but the addition of MS detection allows for further characterization of the analytes and potential metabolites. The second goal is to have an IP agent that allows for length-based separations with predictable contributions from the varying hydrophobicity of the bases.

The first chromatographic separations of nucleic acids used TEAA because of its good separation efficiency [70,74,75]. Initially used to purify PCR products and primers, TEAA is an attractive ion-pairing agent because it can be evaporated from a purified sample [71,72]. Typical TEAA concentrations are 100 mM at pH 7 with acetonitrile added to the eluting buffer. UV detection is most often used with this mobile phase. Its use in MS detection is discussed in detail in Section 12.

In one of the few papers that provides linearity data, Huber et al. demonstrated an LOD of 8 ng/mL for a 14-mer ssDNA with a wide linear dynamic range [71]. This separation used a TEAA mobile phase on a 4.6 × 50 column. This paper further showed n-1 separation of phosphorylated poly C and poly Ts ranging from 12 to 30 nucleotides. TEAA has also been used to separate synthetic primers up to 30-mers, as well as long DNA sequences >450-mers [76]. Dickman used a 4.6 × 50 mm column to show that TEAA could be used to separate curved or bent duplex DNAs that have poly A tracks [73]. These curved DNAs have longer retention times in ion-exchange LC that causes them to coelute with higher molecular weight fragments. This paper shows that the curved 378 bp DNA has no effect on analyte retention using TEAA so that dsDNAs can be separated based on length with no contributions from the conformation.

TEAA has also been successful in the separation of RNAs. In an excellent application for miRNAs, Dickman and Hornby used a commercially available capillary column with a TEAA mobile phase to concentrate miRNA from total RNA extracted from HeLa cells [77]. *Let-7* miRNA was spiked into total extracted RNA. The separation allowed for the miRNA to be enriched and separated from the total RNA fraction. This is an excellent proof of concept for research applications involving miRNA research and one of the few oligo applications using a commercially available capillary column.

Gilar et al. did an extensive study of retention prediction with ssDNA using 4.6 mm id columns of varying length [76]. They presented a mathematical model to predict the retention of heterooligonucleotides using 100 mM TEAA at pH 7. Using 39 different heterooligonucleotides, they show the successful application of this model by predicting the mobile phase strength required to elute the oligonucleotides. The experimental data further showed that the hydrophobicity of C and G is less than that of A and T, confirming earlier findings [70–72,76].

TEAB has also been used in a number of applications for the separation of single stranded and duplexed DNA. This mobile phase was developed because of its compatibility with MS detection. A thorough overview of this work is available in a review by Huber and Oberacher [78]. The first report of TEAB for the separation of oligos was in 1999 by Huber and Krajete using capillary columns [79]. They showed separation of 8–40-mer ssDNAs. They also showed that TEAB showed improved resolution over TEAA for smaller oligonucleotides ($p(dT)_{12}$ and $p(dT)_{13}$), but similar resolution for larger oligos (>30-mer).

Oberacher et al. used capillary columns to successfully separate 21-mer ssDNA primers of the same length and different composition, as well as, a dsDNA primer product [80]. They proposed that electrostatic interaction of the duplex was the retention mechanism for dsDNA, whereas the hydrophobic interaction of the bases were responsible for the separation of the ssDNA. The duplex could be analyzed for base substitutions by raising the column temperature above the melting temperature (Tm) of the oligo. At this temperature, the single strands were easily resolved and characterized. This same paper introduced butyldimethylammonium bicarbonate (BDMAB) as an ion-pairing agent. They compared the separation efficiency of BDMAB to both TEAB and TEAA. Because BDMAB is a more hydrophobic IP agent, higher concentrations of acetonitrile can be used for elution allowing for greater sensitivity when interfaced with MS detection. Greater details on this mechanism are presented in Section 12 of this review.

Holzl et al. also used BDMAB for RNA separations on capillary columns. Interestingly, they added EDTA to the sample preparation to reduce the cation adduction that is often observed with MS detection. A synthetic unmodified 55-mer RNA was prepared with a 900-fold molar excess concentration of EDTA. The chromatography easily separated the EDTA and allowed the detection of the desalted

55-mer. They showed separation of synthetic 21-mer RNAs from their failure sequences down to a 7-mer, although coelution of some of the n-1 sequences was observed. This paper also gives the only example of on-line MS sequencing of a 32-mer.

Bothner et al. used diisopropylammonium acetate and acetonitrile with a 0.5 × 150 mm column to separate modified oligos [81]. Both phosphorothioate oligos, and methylphosphonate oligos were investigated. Both of these modifications produce chiral centers creating enantiomers with varying hydrophobicities, which makes chromatographic separation more difficult. An oligo containing 50% methylphosphonates along with its n-1 and n-2 metabolites were analyzed. Although not fully chromatographically resolved with UV detection, MS detection with selected ion monitoring allowed for the determination of each metabolite. The same result was found with a phosphorothioate oligonucleotide. This is an excellent early example of investigations of modified oligos that are very challenging to analyze.

Hexylammonium acetate (HAA) has also been used as an ion-pairing agent [82]. Introduced recently by McCarthy et al., HAA separation of unmodified duplex RNA from excess single strands was demonstrated using UPLC with a 2.1 × 50 mm column. They also used this mobile phase for semi-preparative purification of synthetic RNA. They noted minor on-column melting of duplex RNA at ambient temperatures and found that this IP agent afforded reasonable MS detection.

10. Hexafluoroisopropanol/TEA buffers

Apffel et al. made a significant contribution toward oligonucleotide chromatography with the introduction of the buffering system containing HFIP and TEA [83,84]. Methanol is used as the organic eluent because acetonitrile is only miscible in HFIP in very small amounts. This buffer allows for excellent selectivity and is compatible with MS detection. With HFIP in the mobile phase, TEA becomes less soluble and, therefore, more likely to bind to the stationary phase allowing for a more stable layer of ion-pairing agent. Overall this makes the separation mechanism predominately an ionic interaction [76]. This separation mechanism is highlighted in the determination of phosphorothioate oligonucleotides where the peak broadening effects caused by the different hydrophobicities of phosphorothioate enantiomers is eliminated [85]. Because TEA has far greater solubility in methanol, as the concentration of the methanol increases, TEA is desorbed from the stationary phase, and the ion-paired analytes are eluted [76]. The sensitivity of the adsorption of TEA to the stationary phase, and the elution with increasing methanol concentration is evident in the shallow gradients that are used in these applications. The HFIP/TEA mobile phase is most effective at 400 mM HFIP and 16.3 mM TEA, a far lower concentration than the 100 mM optimum TEAA concentration [85]. The lower concentration of TEA improves the ionization efficiency compared with TEAA. This lower concentration also lessens competition of TEA with other cations so increased adduction is possible with this mobile phase. These optimized concentrations of TEA and HFIP allow for increased separation efficiency and enhanced signal intensity for MS detection [85]. Additionally, this mobile phase allows for improved length based separation because there is less dependence on the hydrophobicity of the bases as compared with other IP agents [76].

There are a number of examples of HFIP/TEA mobile phase separations of both synthetic and biological oligo applications. Synthetic oligos are used in many applications ranging from primers for PCR and genotyping, to therapeutic DNA and RNA. Synthesis byproducts include failure and mismatch sequences, as well as, oligos with cyanoethyl groups that were not completely cleaved during the final deprotection reaction. HFIP/TEA has been used for quality control of SPE purified primers and Taqman probes with excellent separation of the synthetic byproducts [72,85]. The separation also allows for purification and quality control of modified and unmodified therapeutic oligos and their failure sequences [85].

By far, the most difficult oligo separations are those from a biological matrix. Even with the extensive sample preparations described earlier, there are still impurities that must be separated from the sample. These impurities can have detrimental effects on reproducibility, column lifetime, and may cause significant ion suppression in ESI-MS.

There are a few examples where duplex RNA is separated from its single strands. This degree of separation allows for complex analysis of siRNAs in both formulations and biological systems. The RNA interference mechanism ends with the guide strand in the RISC complex, so ultimately, a method should be able to separate and detect this single strand. Beverly et al. separated the duplex from the single strands to determine the ocular metabolism of an siRNA directed against a vascular endothelial growth factor (VEGF) receptor [49]. This siRNA was modified by end-capping the sense strand with abasic residues, and substituting two uracils with phosphorothioate linked thymines on the 3′ antisense strand. Although the chromatography did not separate the n-1 duplex RNA, MS detection of the duplex allowed the identification of metabolites based on their molecular weight. Denaturing chromatography confirmed the identification by allowing MS detection of the single strands and their metabolites. This paper represents the most thorough analysis of siRNA and its metabolites *in vivo*. Two other papers by Beverly and co-workers also showed separation of the duplex from the single strands for siRNA modified with both 2′ fluoro and 2′ O-methyl groups on the ribose [48,86]. siRNA and their metabolites were extensively characterized in urine, rat and human serum as well as liver microsomes [48,86].

Validation of analytical methods is required for all assays that test therapeutics in humans. Validation describes the sensitivity, reproducibility and robustness of a method. In one of the few oligo papers to describe such data, Murugaiah et al. use an HFIP/TEA separation to analyze a liposomal formulation containing two different duplex siRNAs containing 2′-O-Me and phosphorothioate modifications [87]. The chromatography separates all four single strands. This allows for quantitative analysis of the dosage form with UV detection with no sample preparation. Validation data showed excellent linearity and reproducibility for all four single strands. Although the lower limit of quantitation (LLOQ) is very high at 10 μg/mL, this assay represents the first demonstration of the analysis of a liposomal formulation with two different siRNAs.

The HFIP–TEA mobile phase has been used for the quantification of oligonucleotides with varying success with respect to sensitivity and specificity. Dai et al. used 100 mM HFIP and 8.6 mM TEA as a mobile phase with a 2.1 × 50 mm column to successfully separate an 18-mer phosphorothioate antisense ssDNA from six metabolites, including a 3′ n-1 sequence. 3′ and 5′ single nucleotide deletions were also clearly distinguished from each other by comparing product ion mass spectra. All of the metabolites were quantified together with the parent compound [52]. The sensitivity was limited (LOQ = 100 ng/mL) possibly due to the over-simplified sample clean-up. Several other studies have attempted to improve method sensitivity using the HFIP–TEA buffer system. In one study, the organic portion of the mobile phase with HFIP–TEA consisted of mixtures of acetonitrile and methanol instead of the more commonly used 50% methanol in water, for the determination of the liver metabolism of a phosphorothioate DNA oligonucleotides [52]. Possible rationale for this solvent choice is the reported higher ionization efficiency of acetonitrile when used as a sheath liquid when analyzing oligonucleotides by capillary LC–MS. Zhang et al. provided a comprehensive report on the sample preparation and quantitative analysis of a phosphorothioate oligonucleotide in rat

plasma using a 2.1 × 50 mm column [56]. This paper has one of the lowest detection limits for quantitation of DNA or RNA at 5 ng/mL. Deng et al. followed this with a similar method that obtained a LLOQ of 4 ng/mL [53]. Both of these methods used tandem mass spectrometry for detection.

11. Columns and stationary phases for reversed-phase applications

Most of the successful methods using reversed-phase liquid chromatography use either a porous C18 stationary phase or a pellicular or monolithic poly(styrene-divinylbenzene) (PS-DVB) stationary phase. Huber and Oberacher use the PS-DVB stationary phase to make monolithic 0.2 × 60 mm capillary columns [78]. They reported extraordinary sensitivity for both UV and MS detection of oligos in the femtomole and attomole range [79,88–90]. PS-DVB monolithic nanocolumns are now available commercially, although no literature reports on quantitative analysis are currently available. Other companies are also beginning to produce their oligonucleotide columns in capillary sizes. This will no doubt be important to increase separation efficiency and detection limits mirroring improvements seen in proteomics [91].

Silica based and polymeric based C18 columns have also been used for oligo separations. Selectivity for these large biomolecules appears to improve with smaller particle size [76]. Slow mass transfer of oligos in the stationary phase can contribute to peak broadening. A smaller particle size shortens the diffusion path and improves separation for these slowly diffusing molecules [73,92]. Both elevated temperatures and lower flow rates also enhance the diffusion process. Polymeric columns appear to be more stable to higher temperatures than conventional silica based columns.

A recent paper by Easter et al. introduced oligo separations on a hydrophilic interaction liquid chromatography (HILIC) column (2 × 150 mm) with detection by inductively coupled plasma mass spectrometry [68]. HILIC columns use water as the strong eluent and allow for separations without the use of IP agents. Although the chromatography could only separate the full-length unmodified oligo from the n-5 oligo, the novel use of ICP-MS to measure the phosphate backbone as phosphorous oxide (*m/z* 47) is noteworthy. This method could also be used for phosphorothioate oligos by measuring the sulfoxide and phosphorous oxide. Detection limits for a dT_{30} were 0.336 ng/mL. Further work on improving separations could make this technique an excellent choice for quantitative analysis of oligos.

12. Mass spectrometry

Mass spectrometry detection with electrospray ionization provides the most accurate and thorough characterization of oligonucleotides. The easiest mass spectral measurement is the determination of molecular mass, which can be used to confirm the identification of synthetic by-products and metabolites. The molecular mass alone is a significant piece of information as demonstrated by Pomerantz et al. who applied a simple algorithm that determined the base compositions of oligonucleotides arising from T1 RNA digests [93]. While molecular weight can indicate composition and even highlight potential modifications in the oligonucleotide's structure, more detailed analysis is required for confirmation.

One approach to confirm the presence of modifications in an oligonucleotide is to digest the oligo into its nucleosides and then use LC–MS to determine the presence of any novel structures [94]. In source fragmentation of the glycosidic bond yields ions that are diagnostic of the base and sugar portions of the nucleoside and allows for further localization of the modification in to these

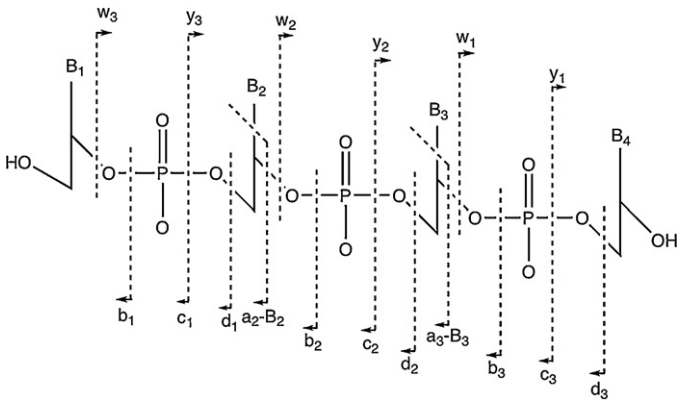

Fig. 6. The fragmentation of DNA produces predominantly a-Base (a-B), b, and d ions while RNA produces predominantly c ions from the 5′ end. Fragmentation from the 3′ end produces predominantly w and y ions for DNA while RNA produces predominantly y ions. The d and w ions are produced by loss of water [98].

portions of the structure. Sequencing of smaller oligonucleotides or partial sequencing of larger oligonucleotides can be accomplished using nuclease digestion. In this case, aliquots are taken from the digestion reaction at fixed time intervals and the molecular weight of the remaining oligonucleotide is measured. When used with either a 3′ or a 5′ specific nuclease, approximately 3–4 bases from each end can be determined using this approach [95]. In two recent papers, Farand and co-workers used digestion of siRNAs for sequence confirmation of oligos modified with 2′-F, 2′-O-Me, and abasic residues. Their methods allow for the de novo sequencing of both strands of highly modified siRNAs [96,97].

However, full characterization of oligonucleotides using MS/MS is often required to absolutely confirm sequence or to place a modification within a sequence. The nomenclature for sequencing oligonucleotides using MS/MS was proposed by McLuckey and co-workers [98,99] and is patterned after the widely used Roepsdorf-Fulman nomenclature for peptide sequencing (Fig. 6). The fragments arise from cleavages along the phosphodiester backbone resulting in a series of ions that can be used to determine the order within an oligonucleotide. In DNA, the major series of ions are the w ions and the a-Base (a-B) ions, while in RNA the major ions are the c/y series. For DNA, w ions are used to determine sequence in the 3′ to 5′ direction while a-B ions are used to determine the sequence in the 5′ to 3′ direction. In some cases, other specific ion series such as the y ions and the c ions may also be useful for sequencing DNA [100]. RNA sequencing uses the y ions to determine the sequence in the 3′ to 5′ direction while the c ions are used to determine the 5′ to 3′ direction. While oligonucleotides up to the 50-mer level have been successfully sequenced using FT-MS and MS^n [101], it is difficult to fully sequence oligonucleotides above the 25-mer level with only a single stage of MS/MS in most mass spectrometers. This size limitation is due to inefficient transfer of energy into such large molecules, and also to the secondary fragmentation and the resulting complexity of the mass spectrum. Modifications to the backbone or to the nucleobases can alter (sometimes radically) the fragmentation behavior of oligonucleotides [102,103]. While this can sometimes make fully sequencing modified oligonucleotides challenging, it often highlights the positions of the modifications. The modifications that have the greatest change in the fragmentation are those that occur in the nucleobases. These modifications alter the gas-phase acidity or electronegativity of the nucleobase, which is considered to be a critical factor in the formation of both the w and a-Base sequence ions [99,104]. Since modifications of the backbone, sugar or nucleobase are intrinsic features of an oligonucleotide there is little, outside of derivatization that can be done to improve mass spectral fragmentation behavior [105]. Therefore,

efforts should be turned toward maximizing signal intensity from the source; however this requires a thorough understanding of the factors influencing ionization of oligonucleotides during their transfer from the solution-phase into the gas-phase.

Electrospray ionization has emerged as the method of choice for the determination of oligonucleotides by mass spectrometry. Two theories have emerged to explain the mechanism of the transition of molecules into the gas phase by electrospray ionization. [106–110] Dole originally proposed the charged residue model (CRM), which states that electrospray ionized droplets undergo a series of coulombic explosions as the solvent droplet evaporates and the charge repulsion within the droplet equals the surface tension (the Rayleigh limit). This ultimately leads to a droplet that contains one molecule of solute, which then becomes a free gas phase ion as the last of the solvent evaporates. Iribarne and Thomson proposed the ion evaporation model (IEM). In this model, the droplet evaporates and approaches the Rayleigh limit. As the Rayleigh limit is reached, the field strength of the ions on the surface of the droplet become strong enough to overcome solvation forces and ions are ejected into the gas phase. Desolvation continues and more ions are ejected as the Rayleigh limit is repeatedly approached until the droplet is completely desolvated.

Recently, Nguyen and Fenn explored the ESI of biomolecules in the presence of water vapor [110]. They found that adding a polar solvent vapor to the bath gas increased the abundance of desolvated ions. They proposed that this was due to the condensation enthalpy released as solvent vapor molecules bound to the droplet surface. This causes solute ions to be sputtered from the droplet surface. Their findings indicate that the IEM model more closely explains the ESI behavior of biomolecules.

The IEM also more fully supports the differences in ionization efficiency observed when different ion-pairing agents are considered. There are several significant factors that impact the transition of ionized oligos from charged electrospray droplets into the gas-phase. The volatility and density of the mobile phase components, the proton affinity of the ion and counter ion, and the surface tension of the droplet are critical to the electrospray desorption process. The concentration and pH of the mobile phase are also important factors. In order for an ion to leave the droplet, there must be sufficient repulsion energy for the ion to break through the surface tension. Lower droplet surface tension is found with higher organic solvent concentrations. However, higher organic solvent concentrations will impair the chromatographic separation. The challenge is to find the appropriate ion-pairing agent, both ion and counter ion, and balance this with sufficient organic concentrations to lower surface tension to allow for sufficient separation while improving ionization efficiency.

Triethylamine is the most common ion-pairing agent used for oligonucleotides. Extensive studies have looked at the impact of the acidic counter ion on signal intensity. When considering the desolvation of the droplet, the boiling point of the ion and counter ion play an important role. Experimental evidence has shown that TEAA gives lower signal intensity than HFIP/TEA or TEAB [79,83,84,90,92]. TEA has a lower boiling point than acetic acid (89 °C vs. 118 °C). When TEAA is used with ESI, the TEA evaporates more rapidly than the acetic acid. This raises the pH in the droplet leading to lower ionization efficiency likely due to competition for ionization between the acetic acid and the oligo. TEAA is also less hydrophobic than other IP agents. It requires less organic modifier so the surface tension of the droplet is higher, which again, causes lower ionization efficiency.

When TEAB or BDMAB are used for ion-pairing, the volatility of the counter ion, bicarbonate, is far greater than both ion-pairing agents. Huber and Krajete showed that, with the addition of an acetonitrile sheath liquid, TEAB gave up to 7-fold higher signal intensity than TEAA with equivalent chromatographic performance [79]. In a separate paper, Huber and co-workers found that a BDMAB mobile phase increased sensitivity even more than TEAB. BDMAB is significantly more hydrophobic than TEAA or TEAB. This allows for a higher concentration of acetonitrile to elute the oligo. This higher organic concentration reduces the surface tension providing up to 10 times greater signal intensity than TEAB [111].

The conductivity of the counter ion can also play a role in the ionization. Huber and Krajete did an interesting study in which they tested various counter ions with TEA. They used acetate, bicarbonate, formate, and chloride in solutions containing 20% acetonitrile at pH 8.9. They found that the volatility of the counter ion did not correlate with increased signal intensity [79]. The acetate counter ion gave the best signal intensity under these conditions. They inferred that the increased conductivity of the counter ion suppressed the oligo signal through competition for ionization.

Ionization efficiency must also be balanced with charge state reduction. Charge state reduction allows for greater signal intensity over fewer m/z values, and this can increase detection limits. In general, the charge state is reduced when protons remain associated with the phosphodiester backbone leaving fewer negative charges. This process occurs during the transfer of ions to the gas phase as well as within the gas phase. Muddiman et al. proposed a mechanism for charge state reduction in which the hydrogen bound proton is shared in a dimer between the phosphodiester bond and the counter ion [112]. Because of the higher proton affinity of the backbone, the counter ion is lost as a neutral on entry, as well as, within the gas phase. In general, the charge state is reduced with the addition of acids with a lower p*K*a. Figs. 7 and 8 illustrate the desorption and ionization of oligonucleotides in an HFIP/TEA mobile phase. These figures offer a visual model of how the mechanisms established by Muddiman, Fenn, and the IEM model might look with this mobile phase.

The HFIP/TEA mobile phase developed by Apffel et al. has been the most widely used in biological applications measuring oligos [23,48,49,52,53,56,113]. Gilar et al. optimized the buffer composition of HFIP/TEA to 400/16.3 mM to maximize separation efficiency and mass spectral signal intensity for ssDNA [92]. They found that HFIP/TEA gave superior signal intensity over TEAA. As stated earlier, Huber and Krajete found that TEAB with an acetonitrile sheath liquid increased the signal intensity up to 7-fold compared with TEAA [79]. The direct comparison of the signal intensity of HFIP/TEA and TEAB or BDMAB has not been published, but it is apparent that these mobile phases all provide improved signal intensity over TEAA. The addition of HFIP also shifts the charge states to higher values and does not produce the charge state reduction seen with TEAB and BDMAB. This indicates that the proton affinity of HFIP is higher than bicarbonate [48,89]. One advantage of HFIP is its very low boiling point (bp = 58.2 °C). Apffel et al. proposed that this allows HFIP to evaporate in the gas phase, raising the pH of the droplet toward 10 [83,84]. As the pH increases, TEA dissociates from the backbone and the oligo is desorbed in to the gas phase. The lower p*K*a of HFIP (9.3) also allows for rapid evaporation because it is not significantly dissociated at the typical pH used with these buffers. Ionization of HFIP does cause background interference at low m/z values (<500 m/z) as indicated in Fig. 8, but this does not typically interfere with the charge state distributions from therapeutic oligos (19–24-mers).

Recently, Ivleva et al. offered additional work using HFIP/TEA with the separation of phosphorothioate and LNA siRNAs using UPLC and a 2.1 × 50 mm column with MS^E characterization [114]. MS^E characterization is achieved by alternating between low and elevated (MS^E) energy in the collision cell allowing for on-line sequencing. The low energy data provide molecular weight information about the analytes. The elevated energy data provide ion fragmentation information for all of the analytes. This provides an advantage over MRM detection because all of the ions are

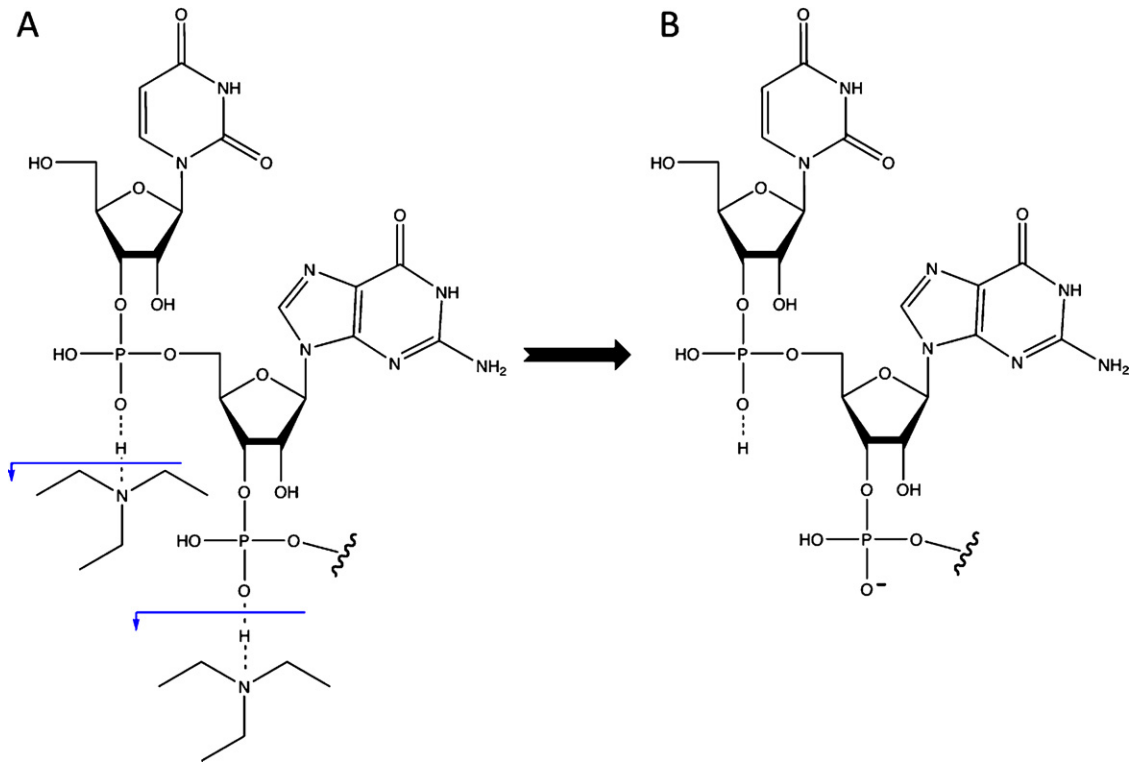

Fig. 7. Proposed mechanism for ionization of oligonucleotides in an HFIP/TEA mobile phase. A. The positively charged TEA molecules are hydrogen bound to the phosphate backbone in solution. B. As the ions are desorbed, TEA evaporates as a neutral, leaving the proton bound to the oligonucleotide backbone. TEA might also desorb as the protonated positive ion leaving a negative charge on the backbone.

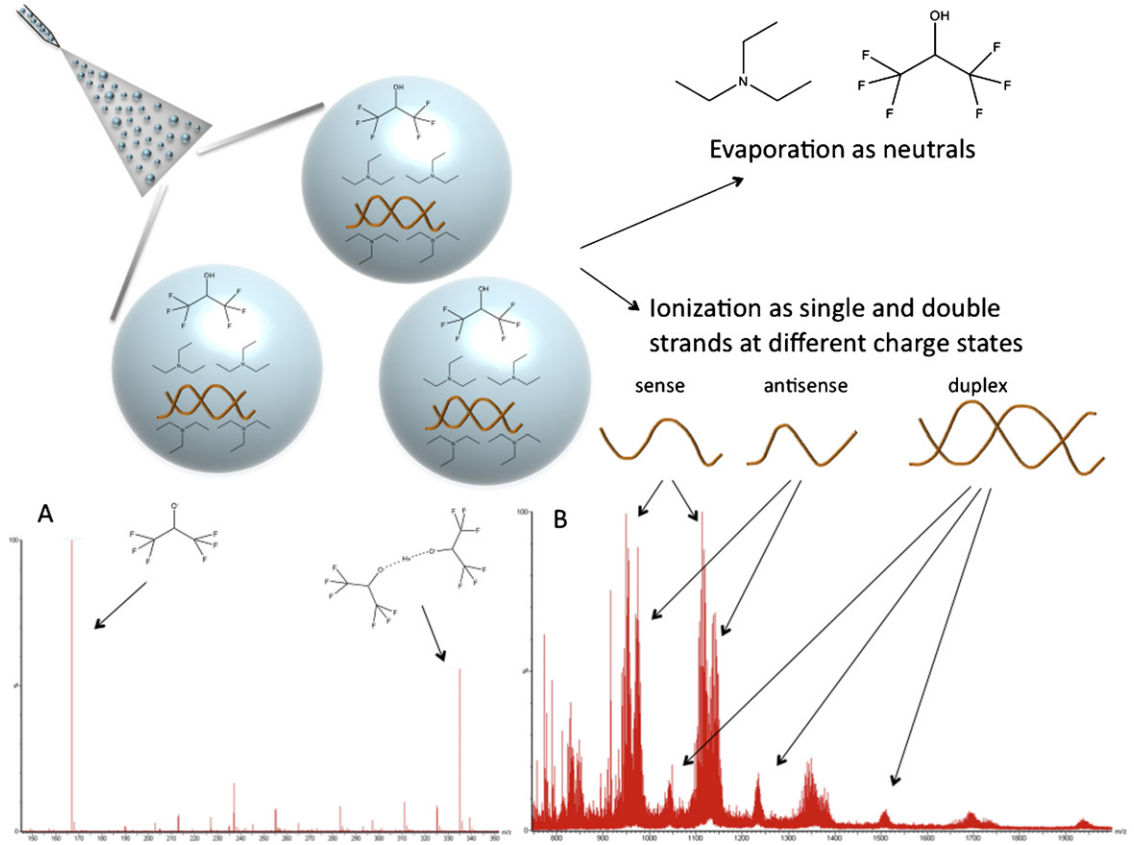

Fig. 8. Illustration of the ionization of siRNA in an HFIP/TEA mobile phase. As the ions are desorbed, both TEA and HFIP can evaporate as neutrals. Ionization of these molecules produces positively charged TEA, which is not detected. A. Panel A shows HFIP, plus an HFIP dimer, which are detected at m/z 167 and 335 respectively. B. The mass spectrum in panel B shows that siRNA can be detected as the duplex, or be denatured in the ESI process to produce the sense and antisense strands.

fragmented and detected, not just selected ions. Therefore, if new peaks arose in a chromatogram, MS^E would be able to analyze these without alterations to the MS method. The authors used this method to characterize modified synthetic siRNA and its failure sequences and by-products as well as metabolites from an *in vitro* hydrolysis procedure. They showed that MS^E characterization of siRNAs gave superior and more rapid performance over an existing MS/MS method for structural isomers as well as metabolites. They also noted that MS^E gave a superior signal to noise ratio, which may improve detection limits. Nikcevic et al. demonstrated separation and detection of low level impurities from the synthesis of phosphorothioate oligos [115]. In addition to separating critical synthesis impurities, this paper offers a comparison of reversed-phase columns as well as a detailed explanation of expected synthetic impurities and their origin.

Detection limits will continue to be the greatest challenge with MS detection. Zhang and co-workers have demonstrated validated detection limits of 5 ng/mL and 4 ng/mL for a single stranded phosphorothioate DNA oligomer using a 2.1×50 mm column with MRM detection [53,56]. Castleberry and Limbach used a proteomics technique with a 1×150 mm column to determine the relative quantitation of tRNAs in a mixture [116]. In this novel quantitation method for oligos, tRNAs were isotopically labeled with nucleases in ^{16}O or ^{18}O water. The resulting mixtures were analyzed and the relative quantitation of the tRNAs determined with detection limits in the femtomole range. This technique might be relevant to the burgeoning field of miRNA biomarkers where relative quantities can be indications of disease states.

Still, these low detection limits and quantitation have not been reproduced with dsRNA. The ultimate challenge remains to obtain the simultaneous separation and determination of the siRNA single strands and the duplex while providing superior ionization efficiency and signal intensity. Investigation must continue to find the balance between chromatographic performance and solution conditions necessary to obtain high signal intensity. The advent of nanospray technologies and smaller columns will likely play a role in increasing sensitivity along with further improvements in mass spectrometers.

13. Matrix assisted laser desorption ionization (MALDI)

Matrix-assisted laser desorption/ionization (MALDI) analysis has also been used to characterize oligonucleotides for quality control purposes. Because of the negative charge of the backbone, a neutral matrix, 6-aza-2-thiothymine (ATT), has been used to analyze ssDNA and duplex RNA and DNA [117,118]. Duplex DNA is more difficult to stabilize than RNA. Various additives such as cobalt (III) hexamine and ammonium citrate have been used to stabilize the DNA duplex during the desorption process [119,120]. Duplex RNA appears to be more stable in the desorption process. Bahr et al. have used MALDI for quality control of synthetic siRNAs [121]. They analyzed the purity and correct annealing of siRNAs using ssRNA as an internal standard. They were able to determine the relative quantity of duplex and single strands using ATT and diammonium hydrogen citrate. Bahr et al. further explored the de novo sequencing of siRNA with MALDI [122]. They applied a simple fast method using acid hydrolysis followed by the characterization of the mass ladders to identify modified sequences containing both phosphorothioate and 2′-MOE modifications.

14. Metabolite characterization using mass spectrometry

In addition to the quantification of parent oligonucleotide drugs, structural characterization of unknown metabolites plays an important role in the development of therapeutic oligonucleotides. As with all drug classes, the quantification and characterization of metabolites and their potential activity is required for regulatory approval. Identification of the active metabolites of therapeutic oligonucleotides can provide more accurate information about their potency and potential side effects. Characterization of the metabolite profiles can shed light on the metabolic processing of the oligonucleotides. Comparison of the metabolism pattern of oligonucleotides with different modifications facilitates an understanding of stability. The combination of liquid chromatography with mass spectrometric analysis of therapeutic oligonucleotides and their metabolites can provide extensive structural information. In general, the most common metabolites arise from exonuclease and endonuclease activity producing chain shortened metabolites of the parent compound. Chromatographic separation of these metabolites decreases the complexity of the mass spectrum that results from multiple overlapping oligonucleotides and also from potential interferences from the biological matrix. Separation of metabolites can also increase detection limits due to less competition for ionization among species. This section will provide a brief review metabolite characterization in the literature. Lin et al. have written a more extensive review [113].

Most of the metabolites of therapeutic oligonucleotides can be directly sequenced by gas-phase fragmentation due to their limited chain length. Sequencing oligonucleotides above 25 bases is difficult due to secondary fragmentation patterns that make interpretation of the mass spectrum difficult. Additional enzyme digestion may therefore be necessary before MS analysis [123]. In general, a-B and w ions are the most abundant in the MS/MS spectra of DNA oligonucleotides and c and y ions are the most abundant in the MS/MS spectra of RNA oligonucleotides. However, different modifications have various impacts on the fragmentation pattern of the analytes. For example, Ni et al. observed that b ions and y ions are significantly more abundant in phosphorothioate containing oligonucleotides relative to those with phosphodiester backbones. This suggests that these ions may be more useful for constructing "mass ladders" for phosphorothioates [124].

Computational algorithms greatly facilitate the use of mass spectral data to identify and confirm the sequence of a given oligonucleotide [125]. Initially oligonucleotide sequencing involved manual interpretation of mass spectra by comparing a table of calculated *m/z* values corresponding to expected peaks present in a given mass spectrum [54]. One useful algorithm was developed to generate lists of DNA and RNA base compositions for a given molecular mass, mass tolerance and backbone composition in a metabolism study of an antisense phosphorothioate [47]. With the wider application of oligonucleotides in laboratory and clinical settings, internet based computer programs have become available to assist with mass spectra interpretation. The MONGO algorithm developed by McCloskey and co-workers is available from the website of University of Utah. This algorithm is capable of calculating the molecular mass, *m/z* values of an electrospray series and the masses of potential fragment ions arising from a selected precursor ion. However, this program supports few modifications to the oligonucleotide structure. The SOS algorithm later developed by the same group, allows users to define any combination of base, sugar or backbone modifications to the parent structure and modify and rebuild alternate sequences [126].

Even though many of the instrumental and interpretation tools available today were not available in the early stage of oligonucleotide therapeutic discovery and development, a great amount of information on oligonucleotide metabolic pathways and degradation patterns has been generated and accumulated over the past 20 years. Antisense phosphorothioate DNAs have been the most extensively studied therapeutic oligonucleotides including their *in vitro* and *in vivo* metabolism as well as tissue distribution. The

in vitro metabolism of a 21-mer phosphorothioate oligonucleotide was studied following incubation with liver homogenate [8]. The 3′-exonuclease activity was found to be predominant while 5′-exonuclease activity and endonuclease activity were also observed. The *in vivo* metabolism of a 20-mer phosphorothioate DNA (Isis 2302) was studied in pig kidneys. The parent drug and its metabolites were fractionated by anion-exchange chromatography and desalted prior to LC–MS analysis. For most metabolites a-B ions and w ions are the most diagnostic ions observed from fragmentation. Only 3′ chain shortened metabolites were observed in fraction B, which contained several metabolites (N + 1 to N – 3). For metabolites of shorter chain length, fragmentation of the parent ion provides useful information on how many nucleotides are cleaved and from which end. The fragment ions also helped to identify a metabolite with an adducted iron atom, which otherwise could have been misinterpreted. Small amounts of depurination from the 3′-end were also observed. Interestingly, N + 1 metabolites were also observed in fraction B but no definitive structural information was obtained for these. Similar findings are also seen from the *in vivo* metabolism study of two other phosphorothioate oligonucleotides [54]. The most abundant metabolites resulted from 3′ degradation. The loss of one adenine base was also observed in liver metabolism. The metabolite profile from kidney and liver tissues displayed distinctive patterns. 3′ metabolites had relatively high peak signal areas when compared to 5′ metabolites observed from kidney. The sum of the area of all metabolites from the kidney was significantly higher than in liver. Degradation from both the 3′ and 5′ termini were observed in the liver. A 5′ N – 2 metabolite showed nearly twice the area when compared to that of the 3′ N – 2 metabolite in the liver. No 5′ N – 1 metabolites were found in the liver tissue.

Dai et al. characterized the metabolites of a bcl-2 antisense phosphorothioate DNA G3139 in human and rat plasma and urine with the assistance of the SOS program [52]. Six major metabolites were generated from 3′ metabolism. It is worth noting that since the base on both the 3′ and 5′ ends were thymines, the deconvoluted mass spectra of the precursor ions did not provide enough information to rule out either one of the metabolites. The researchers obtained tandem mass spectra of the standard of both metabolites and compared the w series and a-B ions with the unknown metabolites and assigned it as the 3′ N – 1 chain shortened sequence. All other metabolites were easily identified using deconvoluted mass spectra and their sequences were verified using the SOS program.

Metabolite characterization of double stranded oligonucleotides can be more complicated due to the presence of both single stranded and double stranded metabolites. In addition, the double stranded ion species can dissociate in the electrospray source, which will further complicate the identification process unless sufficient chromatographic separation was achieved prior to ionization. Beverly et al. studied the *in vitro* and *in vivo* metabolism of an siRNA in urine and from ocular samples [48,49]. 5′ to 3′ degradation profiles were both observed in the urine sample spiked with the sense and antisense strands. This was likely due to the abasic protecting group on the 3′ end of the sense strand and the phosphorothioate linkages on the 3′ end of the antisense strand. Interestingly, there were no N – 1, 2 or 3 cleavage products observed from the 5′ direction. The author proposed a mechanism by which the endonucleases jumped the protected abasic site to start the cleavage, and the exonucleases took over the degradation from there. Different cleavage sites on the phosphate backbone were also observed among different metabolites. The degradation of the antisense strand started later in the time course because of the O-methyl group at the 2′ location of the ribose on each purine base on this strand. This slower onset of degradation was important for therapeutic reasons as the antisense strand would directly interact with the mRNA. The duplex exhibited much better stability when compared to the single strand in urine. Due to the denaturing effects of the ESI process, the signal of both sense and antisense strands were observed in the mass spectrum of the duplex. The author pointed out that single strands formed from the denatured duplex could reanneal, and give false information on duplex metabolites. This issue was addressed by spiking single stranded metabolites (5′ N – 3 sense strand) at twice the concentration of the duplex and analyzing the result. The mass spectrum did not contain a mass corresponding to a duplex containing the N – 3 metabolite, indicating that annealing between the full length and metabolite strands had not occurred. In the *in vivo* metabolism study of the duplex, siRNA sequences shorter than N – 2 on one strand are all unambiguously identified, including 3′ N – 3 and 3′ N – 5 antisense strands binding to a full length sense strand, respectively, and a 3′ N – 5 antisense strand binding to a 5′ N – 5 sense strand both containing terminal phosphates. However, a sequence of N – 2 antisense strand binding to a full length sense strand could not be assigned as a 3′ chain shortened metabolite or a 5′ end one with a terminal phosphate with enough resolution (0.05 Da difference).

15. Conclusion

The analysis of oligonucleotides will be a driving force behind the successful laboratory and clinical applications of these biomolecules. The work presented here highlights the successes and challenges in this area. Both RNA and DNA therapeutics will benefit from methods that can quickly and successfully isolate, separate, quantitate and characterize the parent drug and its metabolites. The validation of these methods will be critical for regulatory approval including precision, accuracy, and recovery from biological matrices. Methods will need to be validated for the characterization of synthetic therapeutic oligonucleotides and for the identification and characterization of the metabolites formed *in vivo*. Therapeutic oligonucleotides have many unique challenges. However, it is unclear if these challenges require specific validation criteria. In the absence of any clarifying statements from regulatory agencies the default will be to follow the existing guidance for small molecule method validation. While there are a few examples where methods have been validated in this manner, it is unclear what size oligonucleotides will be capable of routinely achieving the same rigorous criteria that are applied to small organic therapeutics.

Research in RNomics continues to demand improved methods to identify and characterize RNAs. Approximately 90% of the genome sequence is transcribed into RNA, and only 1.5% of this is translated in to proteins. This leaves a substantial number of non-coding RNAs that have not been identified [91,127,128]. There are considerable needs for precise, robust methods to identify miRNAs as biomarkers and to further define the mechanisms of siRNAs and other small RNAs.

In the future, research needs to focus on the development of more salt tolerant methods to allow ion-exchange chromatography to become more compatible with mass spectrometry. This may involve the development of two-dimensional separations such as ion-exchange chromatography followed by reversed-phase chromatography. Similar to trends in proteomics, studies need to be carried out using capillary LC and nanospray mass spectrometry to achieve better sensitivity for both ion-exchange and ion-pairing methods. Simpler sample extraction procedures with high recovery also need to be developed. Although biological approaches such as RT-PCR and hybridization methods are still the most prevalent methods used in pre-clinical and clinical studies of oligonucleotide therapeutics, the separation and identification ability of LC–MS methods provide obvious advantages, especially in support of pharmacokinetic and metabolism studies.

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