Oligonucleotide Development and Evaluation of Mobile Phase Additives for Electrospray Mass Spectrometry Analysis

By

James Michael Sutton

(Under direction of Michael G. Bartlett)

Abstract

Oligonucleotides are at the forefront of new modalities for improving and tracking human health. To date there have been 10 approved therapeutics by the US FDA and an additional one approved by the EMA bringing the total currently approved oligonucleotides to 11; 7 of the 11 have been approved since 2016. One of the most significant hurdles to getting these therapeutics approved is based on our ability to accurately quantify and track these molecules in patients. In addition, our ability to produce and characterize the purity of these molecules is key to safety, quality and efficacy of these molecules.

In 1997 oligonucleotide analysis took a huge turn with a key finding for ion-pair chromatography of oligonucleotides by using an alkylamine (Triehtylamine acetate, TEAA) and a counterion (Fluorinated alcohol, Hexafluoroisopropanol, HFIP) provide the most sensitive electrospray (ESI) Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The use of ion-pair chromatography is desirable since it takes advantage of the negatively charged phosphate/phosphorothioate backbones of these molecules. Ion-mobility mass spectrometry (IM-MS) has been used to study the size of ions in the gas phase and recent studies have shown that this technique can be utilized for oligonucleotides. IM-MS can also provide an additional degree

of separation in the gas phase allowing separation of isomeric sequences of oligonucleotides unable to be separated by chromatography alone. The highly negatively charged backbone provides other major challenges: severe adduction of both cation and anionic ions, and a large charge state envelope due to the large number of negative charges in the backbone of these molecules. To improve our ability to accurately quality control and improve our analysis of these molecules, I have developed a model for cationic adduction on oligonucleotides when used in combination with alkylamines. I have also compared the stability effects of anionic adduction on oligonucleotides in the presence of the mobile phase consisting of nona-fluorotertbutyl alcohol (NFTB) in conjunction with octylamine (OA). I have shown the potential benefits of using this mobile phase and these anions in conjunction with ion-mobility to study the size of these ions and potentially determine if an oligonucleotide forms a folded state.

Index words: Bioanalysis, Oligonucleotides, Electrospray mass spectrometry , Ion pairing reagents, Alkylamines, Partial least squares (PLS), Bootstrap forest, Cationic adduction, Physiochemical property modeling, Fluorinated alcohols, Ion mobility mass spectrometry, Charge state reduction, Nonafluoro-tert-butyl alcohol (NFTB), Octylamine (OA), Collision cross section (CCS), Nucleic acid CCS calibration, Anionic adduction, Sulfate, Sulfate adduction

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DEDICATION

To Kristina,

For all of your love and support while I finished my degree.

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I would like to thank Dr. Michael Bartlett for accepting me into his lab and mentoring me through the years. I started in the Bartlett lab as an undergraduate and through his mentorship and guidance I decided to go to graduate school and earn an advanced degree. Dr. Bartlett provided me guidance in both my professional and personal life and the decision to continue working for him was easy. I hope that I have earned his trust and respect during my years in his lab and wish his lab the greatest success moving forward. I would like to thank my committee for providing valuable insight to my projects. There are many commitments for a faculty member, and I appreciate all the time they have given me.

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I would like to thank all faculty and staff at the College of Pharmacy that have helped me along the way. I am especially grateful for Mrs. Julie Simmons for holding my hand through complicated paperwork and making sure I stayed on track for my degree.

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CHAPTER 1

INTRODUCTION TO OLIGONUCLEOTIDES

Oligonucleotides are at the forefront of both emerging therapeutic modalities as well as biomarker/disease monitoring. Oligonucleotide regulation of protein expression can be classified into 4 major categories: 1) antisense oligonucleotides (ASOs), 2) small interfering RNAs (siRNAs), 3) microRNAs (miRNAs) and 4) aptamers. ASOs in general block protein translation by mRNA and this can be accomplished by either sterically blocking through binding or by the recruitment of additional enzymes like RNAse H. The siRNA and miRNA are both part of the RNAi pathway, blocking mRNA translation and the major difference between the two is that siRNA are usually dsRNA and miRNA are usually ssRNA. The RNAi mechanism involves the recruitment of the argonaute protein to form the RNA-induced silencing complex (RISC) which leads to mRNA cleavage and protein expression is ultimately blocked (Fire, Xu et al. 1998). ASOs, siRNAs and miRNAs all block the translation of RNA into protein, unlike aptamers which block the protein directly, usually through a steric inhibition mechanism. Aptamers have high binding affinities for their targets because they undergo systematic evolution of ligands by exponential enrichment (SELEX)(Tuerk and Gold 1990, Eaton 1997, Guo, Paul et al. 2008).

Most of these oligonucleotides consist of 3 major parts: the base which consists of the classical adenine (A), thymine (T) or uracil (U), cytosine (C) and guanine (G) ; the sugar which is a 5 constituent ring that differs at the 2′ position and is ribose (2′ -OH) for RNA and is deoxyribose (2′-H) for DNA and the backbone which consists of a phosphate (PO_4^{2-}) linking the sugar rings together to form repeats. Therapeutic oligonucleotides generally have a modified

back bone substituting the phosphorous an oxygen group to a sulfur making it a phosphorothioate, which improves nuclease stability (Eckstein 2000, Eckstein 2014). The bases are generally left unmodified due to their need to complementary bind to mRNA to inhibit the protein translation, however methyl-cytosine is commonly used to decrease immune response in-vitro and increase duplex stability of the complex(Henry, Stecker et al. 2000, Chang, Chang et al. 2016). The ribose sugar is often modified at the 2′ position due to the availability of this proton to assist in the hydrolysis of the backbone of the oligonucleotides. There are several generations of these 2′-ribose modifications starting with methoxy (OMe) and evolving into methoxyethyl (MOE) and finally constrained ethyl which creates a locked nucleic acid (LNA)(Wilson and Keefe 2006, Faria and Ulrich 2008, Mansoor and Melendez 2008, Campbell and Wengel 2011, Juliano 2016, Kim, El Zahar et al. 2020). Chapter 2 provides an in-depth literature review on the classification, properties, modifications of oligonucleotides and a review of sample preparation and bioanalysis of therapeutic oligonucleotides and their metabolites.

The bioanalysis of oligonucleotides with liquid-chromatography mass spectrometry (LC-MS) could not have been made possible without the novel invention of electrospray (ESI-MS) by John Fenn (Fenn, Mann et al. 1989). ESI led the way for biomolecules to be analyzed in the gas phase with greater ease providing an additional tool to study this class of molecules. In 2000, Richard Cole reviewed ESI-MS and describes mechanistic and analytical aspects of ESI that should be considered when developing new ESI methodologies for analytes (Cole 2000). The most controversial in oligonucleotide ionization is tenet 4 “Large molecules are ionized according to a charged residue scenario”. The charge residue model (CRM) states that a charged droplet will transfer its charge to the macromolecule upon reaching its Rayleigh limit and fissioning out (Fernandez de la Mora 2000, Kebarle and Peschke 2000, Nguyen and Fenn 2007,

Hogan, Carroll et al. 2009). Ultimately, these droplets desorb until only one analyte is present and all the charge is transferred onto the analyte as the droplet completely evaporates. Alternatively, the ion evaporation model (IEM) theory states that as the droplet is desolvating and getting smaller the droplet becomes too small for the number of analytes present and the analytes can desorb before the droplet is fully evaporated (Iribarne and Thomson 1976, Fenn 1993). The general consensus is that small molecules are ionized primarily by the IEM and large molecules by the CRM; however, this does not fully explain the behavior of large molecules, and particularly oligonucleotide ionization. The chain ejection model (CEM) is a third competing theory that states for large biomolecules that adapt a compact structure in the liquid phase, one end of the molecule can get charged, and ejected from the droplet dragging the rest of the biomolecule with it and charging the molecule as it passes the charges on the droplet surface (Metwally, Duez et al. 2018). This can explain why unfolded oligonucleotides are highly charged vs folded oligonucleotides which generally carry just a few charges. When ion-pairs are used with oligonucleotides the hydrophilic backbone is covered up and the hydrophobic alkylamines can create a gradient pulling the oligonucleotide to the surface of the droplet and the oligonucleotide can undergo desorption under the CEM if a "tail" of the molecule gets ionized and ejected; if the droplet is sufficiently small then it's possible it will undergo IEM or even CRM.

Oligonucleotides could not be retained on any hydrophobic columns, eliminating reversed phase chromatography as a method of analysis due to their highly charged hydrophilic phosphate backbone. However, in 1997 Apffel, et al. found one of the most effective systems for studying oligonucleotides, was by utilizing ion-pair chromatography with a C18 column utilizing an alkylamine and a fluorinated alcohol counterion (Apffel, Chakel et al. 1997, Apffel, Chakel et

al. 1997). Before the introduction of a fluorinated alcohol ion-pair reversed phase hydrophobic column analysis of oligonucleotides using mass spectrometry was quite limited and suffered from severe signal suppression and ultimately poor sensitivity. Increasing the sensitivity of oligonucleotides focuses on reducing the number of charge states and decreasing spectral complexity by eliminating adduction to the oligonucleotide. Due to the highly negatively charged backbone of the oligonucleotide, cationic adduction poses a large problem for signal suppression in oligonucleotide analysis. Unfortunately, cationic adduction can stem from salting of oligonucleotides for storage, laboratory glassware, plastics, buffers used for the mobile phases (residual ions even in LC-MS buffers) and the LC or MS system themselves. Electrospray ionization also does not occur in a vacuum and often times is done at ambient pressures at room temperature and the gas conditions present are another possible source of contamination. Mobile phase additives have been explored in the past to reduce the cationic adduction with some degree of success (Cheng, Bakhtiar et al. 1994, Muddiman, Cheng et al. 1996, Null, Nepomuceno et al. 2003). Chapter 3 subsequently highlights critical issues in LC-MS analysis pertaining to mobile phase characteristics, appropriate handling of oligonucleotides, mass spectrometry sensitivity, resolution and ionization characteristics and their subsequent application to therapeutic oligonucleotide analysis.

There have been many attempts for different mobile phase systems to improve the detection of oligonucleotides. Triethylamine (TEA) has been the most widely utilized ion-pair reagent for the LC-MS analysis of oligonucleotides (McGinnis, Grubb et al. 2013, Basiri, Murph et al. 2017, Sutton and Bartlett 2019). A host of other ion pair reagents have been studied for the improvement of LC-MS analysis: seven ion-pair reagents were studied by Gaus et al. finding tripropylamine (TPA) having the highest signal(Gaus, Owens et al. 1997),

dimethylcyclohexylamine (DMCHA) was compared to TEA and TEA was found to produce the highest signal (Erb and Oberacher 2014), another study with six alkylamines found DMBA to provide the best sensitivity (Sharma, Glick et al. 2012), another study comparing 13 alkylamines found that the best alkylamine was dependent on the hydrophobicity of the analyte with the best alkylamines being diisopropylamine (DIPA), TPA and diisopropylethylamine (DIEA) (McGinnis, Grubb et al. 2013) and another study used partial least squares modelling of physiochemical properties of alkylamines and sequence of analytes to model best performing alkylamine and found the best to be the order: OA>DBA>HA>TPA>DMBA>DIEA>TEA (Basiri, Murph et al. 2017). Until the partial least squares modelling including physiochemical properties of the alkylamines, method development largely consisted of trial and error of alkylamine choices and concentrations to determine the most sensitive mobile phases. In chapter 4, we model the physiochemical parameters of 17 alkylamine modifiers and their contribution to forming cationic adduction. A qualitative bootstrap model was used to understand which physiochemical properties were most important to forming cationic adduction. We can use this qualitative model to determine ideal physiochemical properties of an alkylamine to promote the least amount of cationic adduction on an oligonucleotide during ESI-MS analysis. In addition, a partial least squares regression model was used to quantitatively predict the amount of cationic adduction on an oligonucleotide in the presence of an alkylamine based on its physiochemical properties. This is desirable because with the qualitative approach we gain insight into properties associated with greater adduction and with the quantitative model we can quickly determine the best alkylamine for our analysis with minimal method development time. Finally, this model was shown to not only work for the ssDNA test set, but for RNA (miR-30c-5P*) and a

phosphorothioate (IONIS 2503). Successful application of the model also helped elucidate that it is likely that the IEM model is the major ionization pathway for oligonucleotides.

Ion-mobility mass spectrometry (IM-MS) is a unique technique that utilizes a chamber filled with a background gas, typically helium or nitrogen, to separate ions by their mass-to-chare (m/z) and size/topology (Clemmer and Jarrold 1997). With appropriate reference materials IM-MS can provide structural information by measuring the arrival time (t_D) of an ion and correlating it to the known collision cross section (CCS, Ω) and ultimately the size and topology the ion(Ruotolo, Benesch et al. 2008). Other structural determination techniques, x-ray crystallography and NMR, can be time consuming and costly to acquire high quality data. Commonly, protein and peptides are used to build these calibration curves due to the well-defined structures of these proteins. Recently, work has been done to create modular calibrant sets with a defined set of both ssDNA and dsDNA to better define the structural implications of oligonucleotides in the gas phase (Lippens, Ranganathan et al. 2016). The oligonucleotide calibrants use molecular dynamic simulations instead of empirical physical data which can be seen as a flaw in the methodology; however, the modular calibrant set was still successfully applied to oligonucleotides to define their m/z and structural properties. IM-MS provides a possible orthogonal technique to quickly identify structural features of oligonucleotides. Recently, a mobile phase consisting of nonafluorotertbutyl alcohol (NFTB) and octylamine (OA) has been shown to promote lower charge state distribution envelopes and that these lower charge states are correlated to hairpins of the oligonucleotides studied. Chapter 5 is dedicated to showing how anionic adduction affects the stability of lower charge state oligos. In this chapter, the stabilizing/destabilizing effects of using anions during IM-MS of oligonucleotides are shown. The (CCS, Ω) are calibrated with IM-MS using both standard proteins and the new

oligonucleotide calibration curve and subsequently compared and contrasted these approaches for obtaining CCS values. The calibration shows that the CCS values of these low charge state oligonucleotides closely match those of a folded or compacted state of these oligonucleotides. At higher charge states, the CCS values are larger indicating that the oligonucleotide has undergone some unfolding or uncompacting.

The culmination of these projects has shed light into the physiochemical properties of mobile phase additives and their effects on oligonucleotide mass spectrometry. The findings of these projects can be applied to therapeutic oligonucleotide analysis and can be implemented in support of pre-clinical method development of emerging therapeutic oligonucleotides.

CHAPTER 2

BIOANALYSIS AND BIOTRANSFORMATION OF OLIGONUCLEOTIDE THERAPEUTICS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY[1]

[1] J Michael Sutton, Jaeah Kim, Noha M. El Zahar & Michael G Bartlett. 2020. *Mass Spectrometry Reviews*. *00*: 1-25. Reprinted here with permission of the publisher.

Table of Contents:

JMS Biography: J Michael Sutton is a 4th year doctoral student at the University of Georgia. He graduated from the University of Georgia in 2016 with a bachelor′s in chemistry with ACS certification and a Bachelors in Pharmaceutical and Biomedical Sciences. He is interested in the analysis and quantitation of therapeutic oligonucleotides utilizing mass spectrometry.

JK Biography: Jaeah Kim obtained her PhD in 2019 from the University of Georgia with a focus on metabolite identification of therapeutic oligonucleotides. She currently works for Biogen working in ADME of oligonucleotide therapeutics.

NMEZ Biography: Noha M El Zahar received her PhD in 2018 from Ain Shams University. She was a Visiting Graduate Research Scholar at the University of Georgia from 2016 to 2018. During her time at the University of Georgia, she developed LC-MS methods to determine degradation products for therapeutic oligonucleotides and also developed new models of chromatographic separations of oligonucleotides. She currently works as a lecturer at Ain Shams University in Egypt.

MGB Biography: Michael G. Bartlett is the Georgia Athletic Association Professor in Pharmacy and Associate Dean for Science Education, Research and Technology at the University of Georgia, College of Pharmacy. Over the last ten years, his laboratory has had broad interest in the development and application of LC-MS methods for oligonucleotide therapeutics and endogenous oligonucleotides as biomarkers for diseases.

Abstract: Since 2016, 8 new oligonucleotide therapies have been approved which has led to increased interest in oligonucleotide analysis. There is a particular
need for powerful bioanalytical tools to study the metabolism and biotransformation of these molecules. This review provides the background on the biological basis of these molecules as currently used in therapies. The article also reviews the current state of analytical methodology including state of the art sample preparation techniques, liquid chromatography-mass spectrometry methods and the current limits of detection/quantitation. Finally, the article summarizes the challenges in oligonucleotide bioanalysis and provides future perspectives for this emerging field.

Key words: Oligonucleotide Therapeutics, Oligonucleotide Sample Preparation, Oligonucleotide Mass Spectrometry, Oligonucleotide Quantitation, Oligonucleotide Metabolite Identification

Acronyms:

mRNA – messenger RNA

OMe – methoxy

MOE – methoxyethyl

LNA – locked nucleic acids

cEt – constrained ethyl

F – fluorine

DNA – deoxyribonucleic acid

RNA – ribonucleic acid

RISC – RNA-induced silencing complex

siRNA – small interfering RNA

PEG – polyethylene glycol

miR (miRNA) – microRNA

SELEX – systematic evolution of ligands by exponential enrichment

kDa – kilodalton

hATTR – hereditary transthyretin-mediated amyloidosis

LC-MS – liquid chromatography – mass spectrometry

EDTA – ethylenediamine tetraacetic acid

SPE – solid phase extraction

GalNAc – N-acetylgalactosamine
TCEP – tris (2-carboxylethyl)phosphine

TEA – triethylamine

LLOQ – lower limit of quantitation

HFIP – 1,1,1,3,3,3- hexafluoro isopropanol

MRM – multiple reaction monitoring

HILIC – hydrophilic interaction liquid chromatography

LOQ – limit of quantitation

PCR – polymerase chain reaction

DBA – dibutylamine

HFMIP – 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol

I Introduction

Small oligonucleotides in the range of 15-25 base pairs in length have emerged as essential biomedical tools for the interrogation and modulation of gene expression in cell and animal models. Therefore, it is not surprising that oligonucleotides have been considered as therapeutic agents with hopes that they would have similar success and allow for specific control of genes in diseases that have been difficult to treat with traditional small molecule therapeutics (Crooke and Bennett 1996, Opalinska and Gewirtz 2002).

Oligonucleotides face many technical challenges due to their complexity and high molecular weight. However, oligonucleotides possess several unique physiochemical properties which present specific challenges and opportunities for bioanalytical method development. Oligonucleotides are significantly more polar than other biomolecules of similar size due to the high degree of charging of the phosphate backbone. This makes oligonucleotides quite water soluble. However, this hydrophilic nature does present challenges when trying to isolate and chromatographically retain these types of molecules (Metelev and Agrawal 1992, Apffel, Chakel et al. 1997, Thayer, Barreto et al. 2005, Easter, kreoning et al. 2010, Gong and McCullagh 2011, McGinnis, Chen et al. 2012, McGinnis, Cummings et al. 2013, Basiri and Bartlett 2014, Studzińska and Buszewski 2014). Therapeutic oligonucleotides are generally chemically modified to improve their biological stability and increase their *in vivo* half-life. These modifications often make the molecule more hydrophobic and actually modestly reduce the bioanalytical challenge from this class of oligonucleotides when compared to general endogenous oligonucleotides. In general, more hydrophobic molecules can be retained better during sample preparation techniques commonly used for oligonucleotides like liquid-liquid extraction and solid-phase extraction. In reversed-phase chromatography, more hydrophobic

molecules retain more strongly and require a greater amount of organic solvent to elute. The increased organic required can increase sensitivity when using an electrospray source on a mass spectrometer. It should be noted that for phosphorothioates some of the benefits listed above are countered by the 2^n isomers, where n is the number of phosphorothioate linkages, due to decreased chromatographic efficiency and very wide chromatographic peaks (El Zahar, Magdy et al. 2018). The increased sensitivity and increased sample preparation efficiency is still a benefit from adding the phosphorothioate linkage. The structures of some biomolecules are extremely important to their function, larger oligonucleotides often possess complex three-dimensional structures that are critical to their biological function.

IA Classification of Oligonucleotide Therapeutics

Oligonucleotides can be viewed as belonging to four distinct classes based primarily on their mechanism of action: 1) antisense oligonucleotides (ASOs), 2) small interfering RNAs (siRNAs), 3) microRNAs (miRs) and 4) aptamers (Figure 2.1). Each class has distinct physiochemical properties and often requires different sample handling to ensure the stability of the analyte during analysis (Goyon, Yehl et al. 2020).

The antisense mechanism was first demonstrated by Zamecnik and Stephenson who showed that a complementary oligonucleotide sequence was able to reduce protein translation and viral replication (Zamecnik and Stephenson 1978). There are two basic classes of antisense oligonucleotides, which are differentiated by their ability to sterically block protein translation directly and do not promote RNA degradation or by their need to recruit additional proteins such as RNase H (Figure 2.1) (Bennett and Swayze 2010). The antisense oligonucleotides that can act-directly through steric inhibition of their RNA targets have a greater number of available options in terms of the modifications that can be applied to their structures since there is no

enzyme recruitment to worry about inhibiting (Bennett 2019). The main goal is generally simply to maximize the nuclease resistance of the oligonucleotide. However, those oligonucleotides that require the recruitment of other proteins need to be wary that the chemical modifications used to improve nuclease resistance do not interfere with the binding of the recruited enzymes. These needs are typically balanced through the use of gapmers.

The RNA interference pathway was discovered by Melo and Fire in 1998 (Fire, Xu et al. 1998).

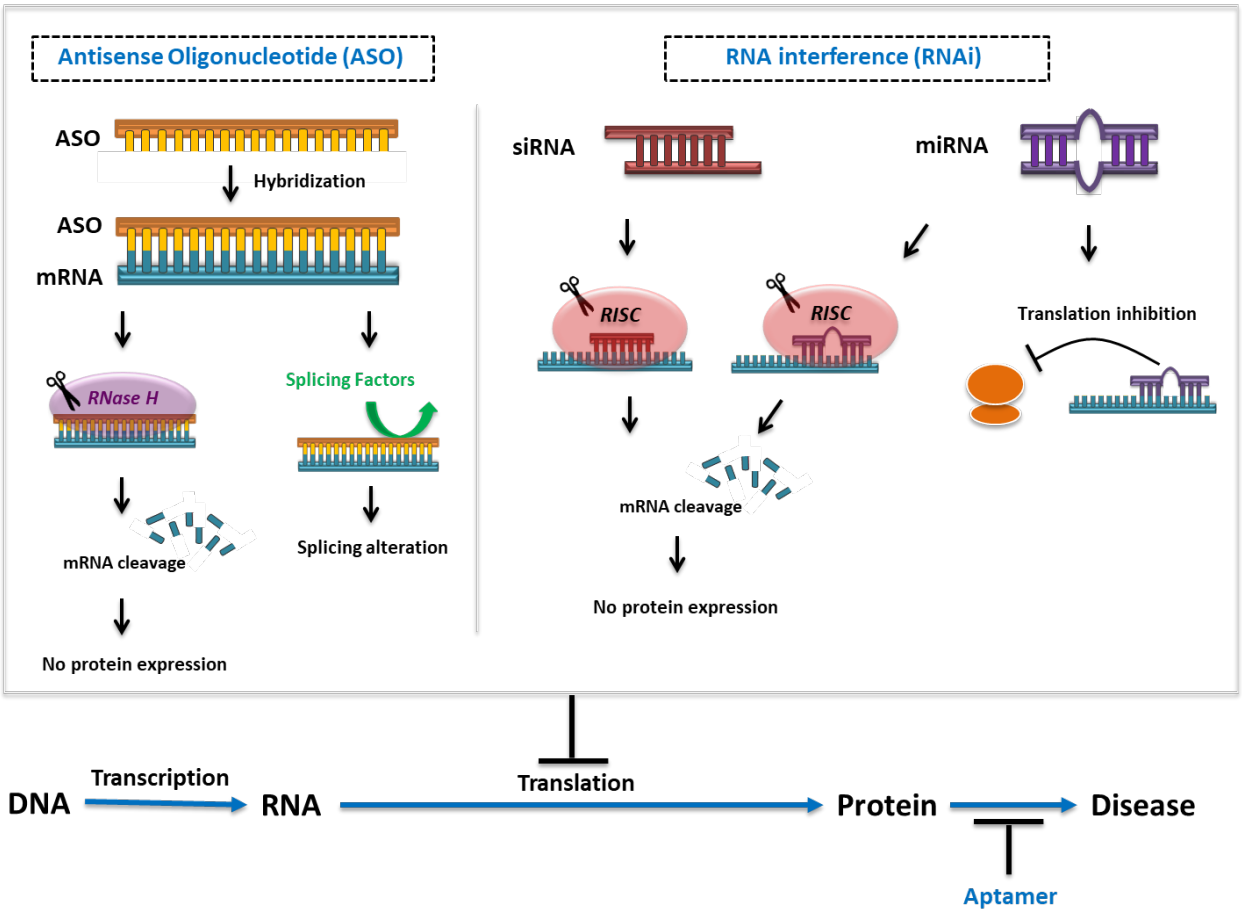

Figure 2.1. Mechanisms of therapeutic oligonucleotides including antisense oligonucleotides (ASOs) and RNA interference (RNAi)

Therapeutically this approach involves using small interfering RNAs which associate with the Argonaute protein to form the RNA-induced silencing complex (RISC), which can then cleave

complementary RNA transcripts (Hammond, Bernstein et al. 2000, Leuschner, Ameres et al. 2006). The RNAi mechanism involves the delivery of a small RNA duplex containing a guide strand and a passenger strand (Figure 2.1). The guide strand is loaded into RISC while the passenger strand is degraded. Modifications of siRNAs need to be quite specific otherwise they will adversely impact the efficacy of these molecules (Weitzer and Martinez 2007). The Argonaute 2 protein only tolerates a minimal number of phosphorothioate modifications at the ends of siRNAs. The 2′ ribose modifications require greater care than antisense oligonucleotides. Fully 2′-OMe or 2′-F modified RNAs have little to no activity (Elbashir, Martinez et al. 2001, Braasch, Jensen et al. 2003, Czauderna, Fechtner et al. 2003). Both of these modifications introduce slight structural distortions. In opposition to these findings, Kraynack and Baker revealed that fully 2′-OMe modified siRNA duplexes were tolerated with comparable activity and potency to the unmodified analog of similar sequence (Kraynack and Baker 2006). Currently the best approach is to use RNAs containing these two modifications in an alternating manner which offsets these structural changes and are well tolerated by RISC and possess excellent activity (Allerson, Sioufi et al. 2005, Lima, Prakash et al. 2012, Foster, Brown et al. 2018). Chemical modification of siRNAs must also take care to avoid causing an innate immune response. This unwanted adaptive immune response has been observed from chemical modifications to improve targeting such as PEGylation (Rusconi 2015).

One promising approach to reducing the problems with innate immune response with siRNA-based therapeutics is to use single stranded microRNAs instead. Since miRs are endogenous, similar effects to siRNA approaches should be possible. Single stranded miRs are similar in structure to antisense oligonucleotides. These oligonucleotides also use the RISC mechanism and therefore have many of the same modification restrictions that exist in siRNA-based

therapeutics (Meister 2013). However, the use of LNA and MOE modifications have been used to improve the activity of an oligonucleotide targeted against miR-122 (Lanford, Hildebrandt-Eriksen et al. 2010, Lennox and Behlke 2011, Hennessy and Moore 2013). In particular a modification pattern that involves two DNAs followed by one LNA has shown promising results *in vivo* for several diseases (Abe, Naqvi et al. 2014). Recently, several companies have completed phase 1 and 2 clinical trials for miR mimetics or anti-miR therapeutics. Regulus Rx has RGLS4326 in phase 1 and RG-012 in phase 2 (Regulus Rx, Press Conferences, 2020). MiRagen has a candidate MRG-201, a microRNA-29b mimetic, in phase 2 clinical trials (Gallant-Behm, Piper et al. 2019). The newest miR mimetic involves delivery in a targeted manner often bundled in bacterial cells and these are called targomiRs (van Zandwijk, Pavlakis et al. 2015).

Aptamers describe oligonucleotides that have been designed to have high binding affinities for either small molecules or biomolecules in a manner similar to protein antibodies (Nimjee, Rusconi et al. 2005). They are produced using repeated rounds of selection using techniques like systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990, Eaton 1997, Ng, Shima et al. 2006, Guo, Paul et al. 2008). Unmodified aptamers have been proposed for the treatment of clotting disorders where only a short duration of action is needed (Rusconi, Roberts et al. 2004, Nimjee, Rusconi et al. 2005) or for treating organs such as the eye where local delivery is possible (Quiram, Hassan et al. 2007). Modifications to the 2′ position using fluorine have been used along with PEGylation to improve the clinical half-life for aptamers (Boomer, Lewis et al. 2005, Da Pieve, Blackshaw et al. 2012). Aptamers have high binding abilities which expands their use into areas such as biosensors, diagnostics and therapeutics (Zhang, Lai et al. 2019).

IB Properties of Therapeutic Oligonucleotides

Oligonucleotides are biopolymers with repeating units composed of three parts, the base, the sugar and the backbone. The differences in the various generations of therapeutic oligonucleotides can be attributed to the alterations to these three portions of the molecule with the intent to improve stability and efficacy. The base is a small organic heterocycle which generally has two types of ring structures, either a pyrimidine or purine (Belmont, Constant et al. 2001, Blackburn, Gait et al. 2006, Sinden 2012, Egli and Saenger 2013). The therapeutic oligonucleotide will have a complementary sequence for its target mRNA transcript. It is generally believed that 12-14 bases are the minimum length needed for a therapeutic oligonucleotide in order to have sufficient binding to inhibit protein production ;however, most are longer. Therapeutic oligonucleotides are normally composed of only the 5 primary nucleobases with the exception of 5-methyl-cytidine, which is often added in place of cytidine to increase duplex stability and to decrease immune response (Henry, Stecker et al. 2000, Chang, Chang et al. 2016). There is currently no evidence supporting modified bases as having a large therapeutic benefit outside the naturally occurring 5-methyl-cytidine.

In therapeutic oligonucleotides, the sugar portion of the structure is often modified from the naturally occurring ribose. This most often happens on the 2′ position hydroxyl group (Faria and Ulrich 2008, Mansoor and Melendez 2008). This group plays a crucial role in the hydrolysis of the phosphodiester backbone and, therefore, removal of this proton is generally found to favorably increase both the chemical and biological stability of oligonucleotides. This originally involved creating methoxy (OMe) derivatives but later evolved to methoxyethyl (MOE) and locked nucleic acids (LNA) including the methylated analog of LNAs which is referred to as a constrained ethyl (cEt) modification (Figure 2.2a) (Wilson and Keefe 2006, Campbell and

Wengel 2011, Juliano 2016, Kim, El Zahar et al. 2020). While these modifications are quite beneficial in terms of increasing the stability of the oligonucleotides, they are disruptive to the activity of RNase H and prevent its typical cleavage of DNA-RNA hybrids. The compromise to balance stability while maintaining RNase H activity was accomplished with the creation of gapmers (Hoke, Draper et al. 1991, Monia, Lesnik et al. 1993). Gapmers have a complementary DNA sequence flanked by 3-5 2′-modified nucleotides on either side of the unmodified DNA sequence (Figure 2.2b) (Kim, El Zahar et al. 2020). One other modification of the sugar that can occur is the substitution of a morpholino group for the ribose. Morpholino containing oligonucleotides function as antisense oligonucleotides by binding to its target RNA sequence and preventing other molecules from being able to interact with this transcript (Iversen 2008, Wan and Seth 2016, Bennett 2019).

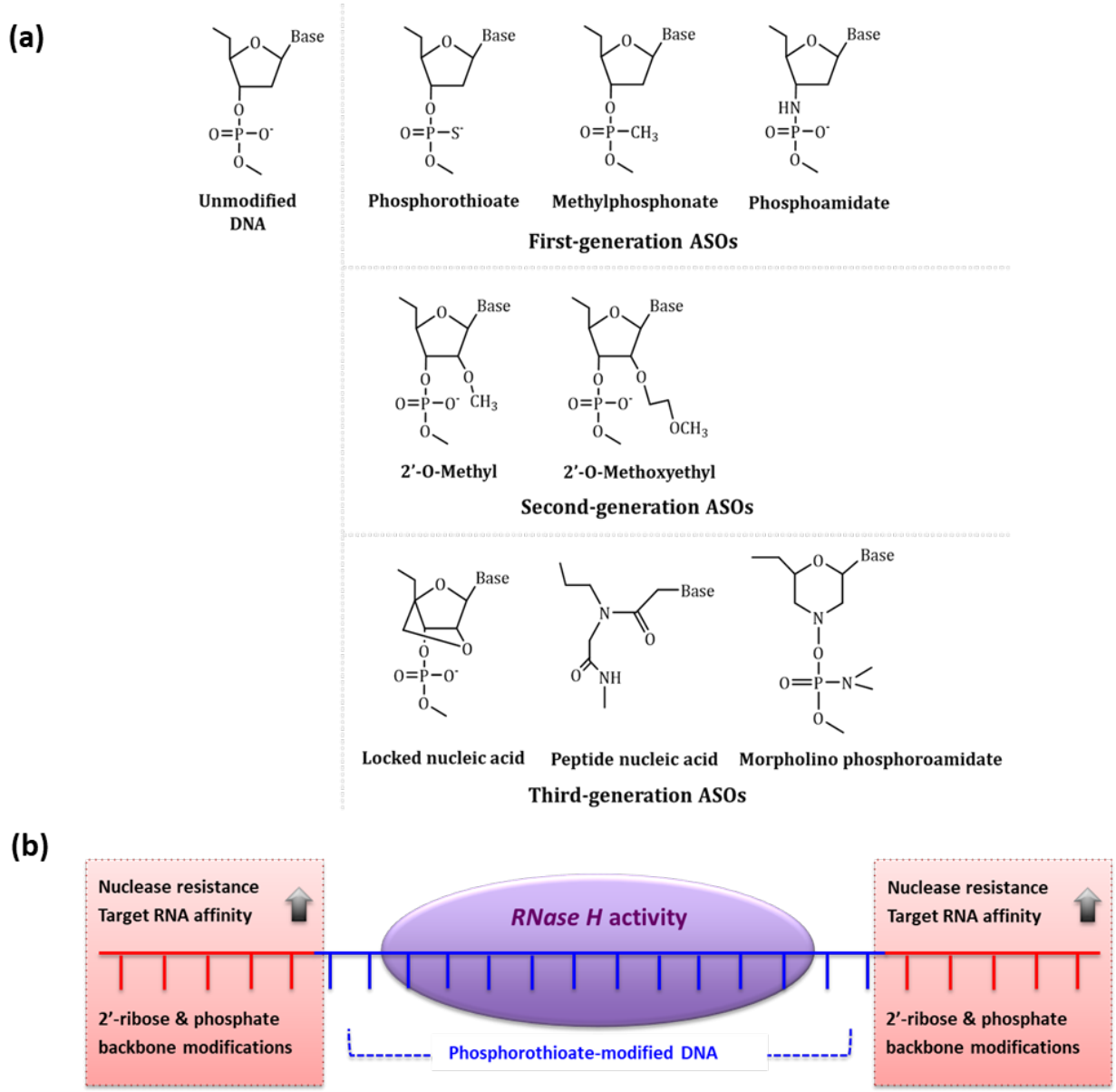

Figure 2.2. (a) Structure of unmodified oligonucleotides and first-, second-, and third-generation ASOs. Reprinted with permission from [Kim et al.] (2020) [Biomedical Chromatography]

(b) Gapmers.

The phosphodiester linkage in the backbone of unmodified therapeutic oligonucleotides is relatively easily hydrolyzed by endo- and exo- nucleases (Smith and Zain 2019). This has led to significant efforts to establish modified backbone chemistries that preserve the activity of the

oligonucleotide while decreasing the rate of chemical and enzymatic hydrolysis of the backbone (Varma 1993, De Mesmaeker, Altmann et al. 1995). In addition, it is imperative to store oligonucleotides at neutral pH because hydrolysis of the phosphodiester linkage can occur under either acidic or basic conditions (Bahr, Aygun et al. 2009). The modifications of the backbone can be broadly classified into two groups. The first include the methylphosphonates and the phosphoramidites which both provide broad resistance to hydrolysis but also dramatically alter the physiochemical properties of the oligonucleotide by removing the charge from the backbone (Dagle and Weeks 1996, Miller, Cassidy et al. 2000, Mansoor and Melendez 2008, Jarver, O'Donovan et al. 2014). The second are the phosphorothioates in which a non-bridging oxygen atom attached to the phosphorous is replaced with a sulfur atom (Eckstein 2000, Eckstein 2014). Phosphorothioate bonds have significantly reduced endo- and exonucleases activity relative to phosphodiester linkages (Sayers, Olsen et al. 1989). However, the repeated introduction of these bonds also creates a chiral center, which is designated as either a “Sp” or “Rp” conformation (Figure 2.3) (Eckstein 1979, Thayer, Wu et al. 2011). This leads to 2^n isomers, where n is the number of phosphorothioate linkages, of the oligonucleotide being generated during its synthesis. These individual isomers have been shown to have different characteristics and functional properties (Lesnikowski 1993, Koziolkiewicz, Krakowiak et al. 1995). However, all Sp or all Rp phosphorothioates have poorer performance than the mixtures of random phosphorothioate containing oligonucleotides (Stec, Cierniewski et al. 1997, Yu, Kandimalla et al. 2000, Wan, Migawa et al. 2014, Iwamoto, Butler et al. 2017). Therefore, precise patterns of Sp and Rp linkages appear to be needed to optimize target binding and RNase H recruitment (Gagnon & Watts, OTS meeting, San Diego, 2014)

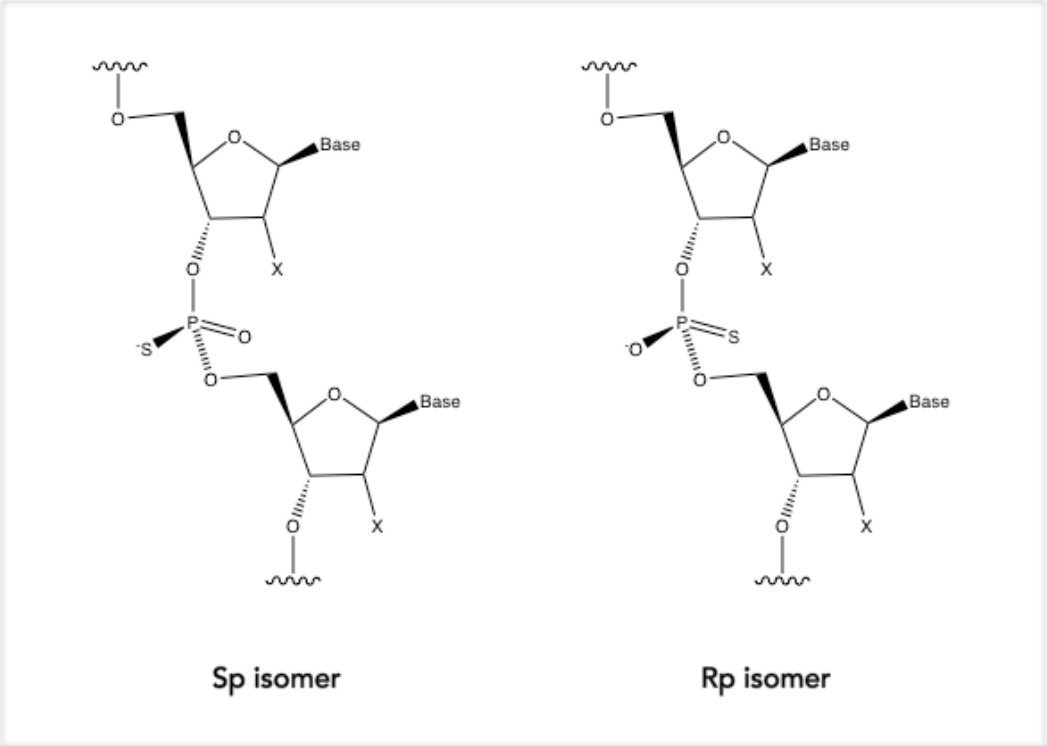

Figure 2.3. Sp and Rp diastereomeric phosphorothioate linkages.

IC Currently Approved Oligonucleotide Therapeutics

There are currently ten oligonucleotide therapeutics that have been approved by the US Food and Drug Administration and Volanesorsen has been approved by the EMA (Ackermann, Guo et al. 2016, Stein and Castanotto 2017, Walsh and Kotz 2018, Paik and Duggan 2019). The first was fomivirsen, also known as Vitravene which was approved in 1998. Fomivirsen is a 21-mer phosphorothioate DNA that employed an antisense mechanism for the targeting of the messenger RNA for the immediate-early (IE)-2 protein in the cytomegalovirus. Inhibition of this protein caused termination of viral replication and was successful in reducing the symptoms of CMV induced retinitis. Fomivirsen is given by injection directly into the ocular vitreous humor on a weekly or biweekly basis (Crooke 1998, Kole, Krainer et al. 2012).

Pegaptanib, also known as Macugen, is an aptamer that targets vascular endothelial growth factor 165. It was approved for the treatment of age-related macular degeneration in 2004. Pegaptanib is a 27-mer that has a 3′-deoxythymidine cap with phosphorothioate linkages to provide increased nucleases resistance. It uses a combination of 2′-ribose protection with the purines all having an –OMe group and the pyrimidines all having a –F group. It also has a 40 kDa PEG group on the 5′-end. Like formiversin, pegaptanib is injected into the ocular vitreous humor once every six weeks (Doggrell 2005, Moshfeghi and Puliafito 2005).

Mipomersen, also known as Kynamro was approved in 2013 for the treatment of homozygous familial hypercholesterolemia. Mipomersen is a 20-mer phosphorothioate 2′-MOE gapmer. The MOE groups are found on the first 5 nucleotides at both the 3′ and the 5′ end of the molecule. Mipomersen is administered by weekly sub-cutaneous injection. This is noteworthy as it is the first approved oligonucleotide therapeutic that is not directly injected into the eye and demonstrates that oligonucleotides can be successfully administered systemically (Gelsinger, Steinhagen-Thiessen et al. 2012, Parhofer 2012).

Eteplirsen, also known as Exondys-51 by Sarepta, was approved in 2016 for the treatment of Duchenne muscular dystrophy (DMD). Eteplirsen is a 30-mer phosphoromorpholidate oligonucleotide. It is the first compound approved using the phosphoromorpholidate backbone chemistry(Lim, Maruyama et al. 2017).

Defibrotide, also known as Defitelio was approved in 2016 for the treatment of severe hepatic veno-occlusive disease that has occurred following chemotherapy or a bone marrow transplant. Defibrotide is a complex mixture of DNAs ranging in size from 9-80 nucleotides with an average size of 50-nucleotides in length. The mechanism of action of defibrotide is currently unknown but it is believed to function by forming a higher order structure likely involving several strands

that allows it to possess unique nuclease resistance (Benimetskaya, Wu et al. 2008, Baker and Demaris 2016, Stein, Castanotto et al. 2016). This is currently the only oligonucleotide therapy where the mechanism of action was unclear at the time of approval. Defibrotide is produced through isolation from porcine intestinal mucosal DNA. The resulting collection of DNA strands is quite complex and have not been amenable to resolution even when using techniques such as capillary electrophoresis.

Nusinersen, also known as Spinraza was approved in late 2016 for the treatment of spinal muscular atrophy (Goodkey, Aslesh et al. 2018, Neil and Bisaccia 2019). Nusinersen is an 18-mer phosphorothioate antisense oligonucleotide that is modified at all of the 2′-ribose position with a MOE group and all of the cytidines contain a 5-methyl group. This is an oligonucleotide with 2′-MOE and 5-methyl cytidine modifications.

Patisiran, also known as Onpattro, was approved August 2018 for the treatment of peripheral nerve disease (polyneuropathy) caused by hereditary transthyretin-mediated amyloidosis (hATTR) in adult patients. The first FDA approved siRNA and the first approval for this particular disease (Adams, Gonzalez-Duarte et al. 2018, Mullard 2018). Patisiran is a 21-mer dsRNA with five - 2′-O-methyluridine modifications, three – 2′-O-methylcytidine modifications and two thymidine modifications on the sense strand. The antisense strand has two- 2′-O-methyluridine modifications and two thymidine modifications.

Inotersen, also known as Tegsedi, was approved in October 2018 for the treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis in adults. Inotersen is a 20-mer phosphorothioate antisense oligonucleotide that is modified at the 2′ ribose position with MOE at the first and last 5 nucleotides making this a 5-10-5 “gapmer” (Ackermann, Guo et al. 2016, Mathew and Wang 2019, Neil and Bisaccia 2019).

Volanesorsen, also known as Waylivra, was approved in 2019 by the EMA for use in conjuction with diet for patients with familial chylomicronemia syndrome (FCS) (Paik and Duggan 2019). Volanersen is an inhibitor of the mRNA of ApoC-III protein which ultimately reduces the triglyceride levels in patients with FCS. Volanersen is a 20-mer 2nd generation 2′-MOE 5-10-5 gapmer with 5′-methyl C modifications (Paik and Duggan 2019).

Givosiran, also known as Givlaari, is an siRNA approved in 2019 for acute hepatic porphyria (Scott 2020). This drug targets aminolevulinate synthase 1 (ALAS1) downregulating ALAS1 mRNA and preventing accumulation of aminolevulinic acid and porphobilinogen. Givosiran is the first approved siRNA with an N-acetylgalactosamine (GalNAc) on the 3′ end of the 21-mer sense strand which allows targeted delivery to the liver. The sense strand has three 2′-F guanines, two 2′-F cytodines and the rest of the bases are 2′-OMe. The antisense strand does not have a GalNAc attached and contains five 2′-F adenines, one 2′-F guanine, five 2′-F uridines and the remaining basese are 2′-OMe.

Golodirsen, also known as Vyondys 53 ,was first approved in December 2019 (Heo 2020). This is the second approval by Sarepta for Duchene muscular dystrophy and is their second approved phosphoromorpholidate oligonucleotide. Golodirsen is a 25-mer phosphoromorpholidate that has been shown to increase dystrophin in DMD patients. Sarepta had particular trouble getting these drugs approved due to the challenges in quantifying dystrophin(Heo 2020).

II Metabolism of Oligonucleotide Therapeutics

When given intravenously, unmodified oligonucleotides are quickly degraded within a few minutes. The major metabolism of oligonucleotides is due to nucleases (Kole, Krainer et al.

2012). Nucleases are hydrolases that degrade oligonucleotides by typically adding water across either the 5′ or 3′ side of the phosphodiester bond. Nucleases can be either a protein, RNA or DNA and can use water, deoxyribose, inorganic phosphate or an amino acid side chain to conduct the nucleophilic attack required to break the phosphodiester bond(Yang 2011, Liu, Yu et al. 2017). While the chemical reaction catalyzed by this class of enzymes is quite simple, nucleases have quite strict substrate specificity. Therefore, there is significant variety in the structure and mechanism of action among these enzymes. Nucleases are broadly categorized using several different nomenclatures (Eun 1996, Mishra 2002, Rittié and Perbal 2008). The first are endo- and exonucleases which describe whether the phosphodiester linkage that is hydrolyzed is on a terminal nucleotide (exo) or is an internal linkage (endo). Many are categorized by their general substrate specificity as either a DNase or an RNase; however, there are many nucleases that can hydrolyze both major types. The final way to categorize nucleases is based on whether they require zero, one or two metal ions for their activity. The role of metals is important when considering *in vitro* experiments where metal scavengers are often added to inhibit nucleases. However, this approach is not practical *in vivo* due to the ubiquitous use of metals for a wide range of biologically critical reactions (Blackburn, Gait et al. 2006, Bian, Pan et al. 2011, McGinnis, Chen et al. 2012).

Most of our early understanding of the metabolism of therapeutic oligonucleotides comes from the seminal studies of Crooke which focuses largely on phosphorothioates (Crooke, Graham et al. 2000). In general, the metabolism of phosphorothioate-containing therapeutic oligonucleotides increases with length up to the 12-mer level and then remains constant as they become larger. They are primarily metabolized by 3′-exonucleases but there has also been metabolism noted from 5′-exonucleases and endonucleases. These first generation

phosphorothioates are primarily metabolized by exonucleases. This caused the rise of the second-generation therapeutics to contain gapmers which block the exonuclease activity. These gapmers are instead initially metabolized by endonucleases from the center of the strand and then exonucleases can further metabolize them.

More recently, a triantennary N-acetyl galactosamine moiety has been added to several therapeutic oligonucleotides to improve targeting to hepatocytes by using the asialoglycoprotein receptor (Prakash, Graham et al. 2014, Shemesh, Yu et al. 2016, Husser, Brink et al. 2017) (Figure 2.4). The addition of this carbohydrate creates the opportunity for additional metabolism of therapeutic oligonucleotides. The sequential loss of each of the three N-acetylgalactosamine groups has been observed along with the partial loss of the aminohexyl linker. The loss of the carbohydrate targeting moiety appears to be quite rapid as there have not been any observed metabolites resulting from exo- or endonuclease activity that still contain any of the N-acetylgalactosamine groups.

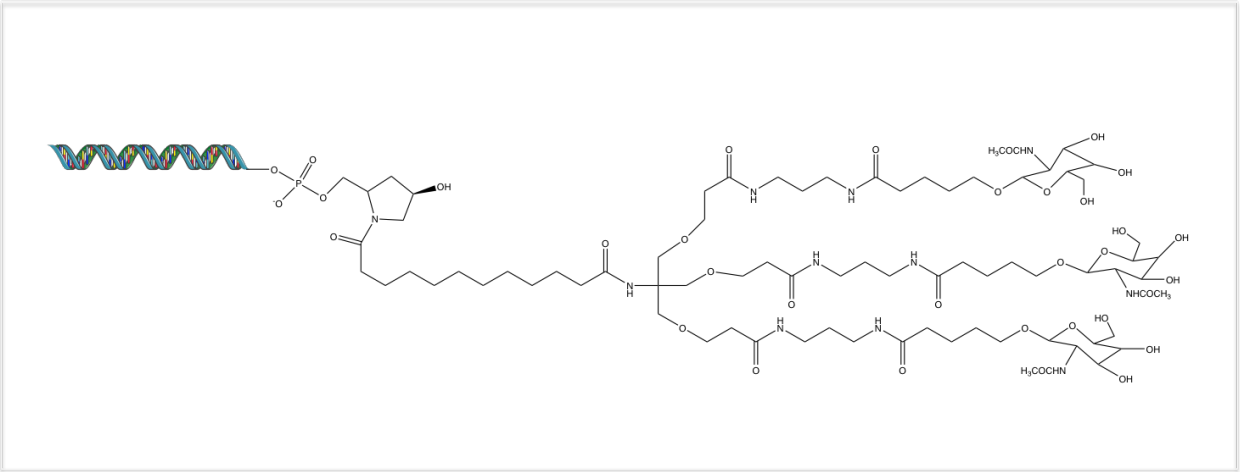

Figure 2.4. Structure of GalNAc modified therapeutic oligonucleotide

III Bioanalysis of Oligonucleotides and Their Metabolites

Since therapeutic oligonucleotides are intended to alter a biological target, it is imperative to be able to accurately determine their concentration in biological samples. There is also a need to determine the metabolites that are produced *in vivo*. Metabolites have a potential to have activity and could cause off-target toxicity. Identifying the metabolites early in a study may help researchers determine if there is potential for off-target toxicity which would be important to understand when interpreting the results of preclinical and clinical studies. The tissue distribution of these compounds will also be important. Many therapeutic oligonucleotides either have targeting moieties built into their structures or will be formulated using delivery strategies that should increase their concentrations within specific organs (Ahn, Bones et al. 2010).

IIIA Sample Preparation Techniques

There have been many approaches used successfully to isolate therapeutic oligonucleotides and their metabolites prior to LC-MS analysis (Figure 2.5) (Bartlett, Kim et al.). They can be divided into several different categories. These approaches cover a range of general sample preparation strategies but they each have particular caveats that are specific to this class of biomolecules.

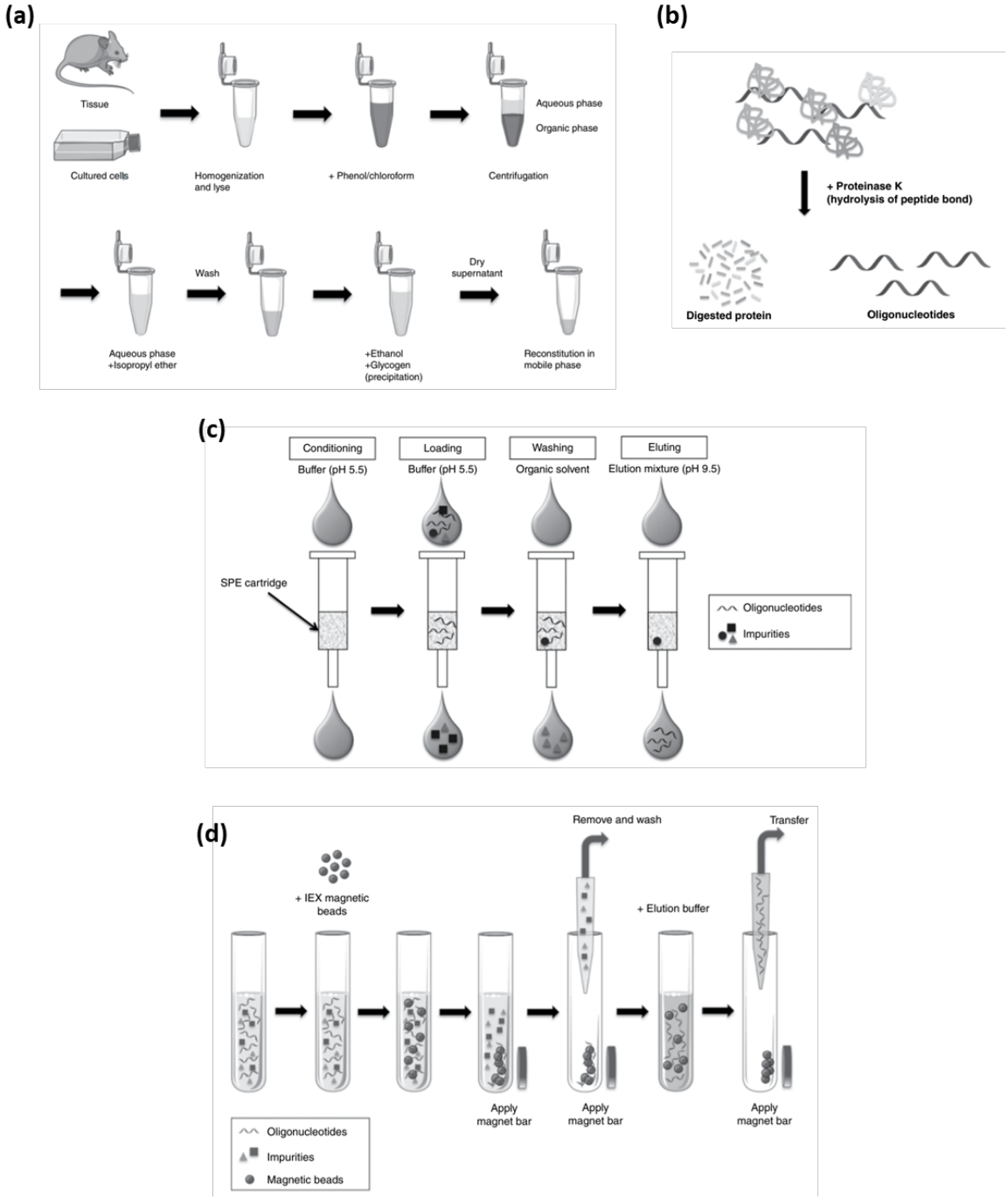

Figure 2.5. Sample preparation techniques for LC-MS bioanalysis of oligonucleotides. (a) Trizol extraction. (b) Proteinase K digestion. (c) Solid-phase extraction. (d) Magnetic bead extraction method.

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IIIA1 Trizol Extraction

The trizol extraction was developed in 1987 by Chomczynski and Sacchi as a simple one step method for the isolation of RNA (Chomczynski and Sacchi 1987) (Figure 2.5a). It was modified in 1993 by Chomczynski to allow for the isolation of either DNA or RNA. This approach is widely used as a sample preparation strategy to isolate oligonucleotides from cell culture and tissues. The biological sample is mixed with phenol and guanidine isothiocyanate. This disrupts membranes releasing intracellular oligonucleotides while maintaining their solubility. After solubilization, chloroform is added and allowed to separate forming a clear upper aqueous layer containing RNA, siRNA and therapeutic oligonucleotides and a red colored lower organic layer containing DNA (Chomczynski 1993, Simms, Cizdziel et al. 1993, Chomczynski and Sacchi 2006, Kotorashvili, Ramnauth et al. 2012). There may also be a small interphase layer, depending on the nature of the biological sample, which may be added to the lower organic layer as it also contains some DNA. The aqueous layers can then be separated from the organic/interphase layers. The RNA can be isolated from the aqueous layer by precipitation using isopropanol while the DNA can be isolated from the organic layer (with or without the interphase layer added) by precipitation using ethanol. The precipitates can be washed to remove any impurities and then resuspended in the initial mobile phase for subsequent LC-MS analysis. The purpose of any sample preparation method is to remove the analyte from endogenous matrix. In this trizol extraction, it should be noted that the proteins move into the organic phase and since oligonucleotides generally have very high protein binding affinities this extraction highlights the importance to separate the oligonucleotides from the proteins.

The trizol extraction has been widely used for decades and provides a reliable isolation technique for a wide variety of oligonucleotides (Meng and Feldman 2010). In general, relatively clean extracts are expected with recoveries around 70% for most oligonucleotides in the range of 10-30 bases in length (Chen and Bartlett 2013). However, for therapeutic oligonucleotides it is not always clear if they will distribute into the upper or lower layer. Phosphorothioate-containing oligonucleotides are more hydrophobic but will still end up in the aqueous layer, unless they are significantly hydrophobic in which case they could be in the organic layer. Less modified siRNA or miRNA type therapeutics also tend to distribute into the upper aqueous layer. It is therefore important to check both layers when this approach is used for a new molecule to avoid any unforeseen complications.

There are many other additives that can be used to improve the trizol extraction process such as chaotropic agents, metal scavengers or solubilizing agents (Müller, Werner et al. 1998, Tan and Yiap 2009, Ali, Rampazzo et al. 2017). Some of these are well known but many of them are proprietary in nature. It is also possible to use trizol kits to isolate the protein fraction which will partition with the DNA but can be selectively precipitated away from the DNA. It is important to recognize that, depending on your experimental needs, it may be possible to use additives that will enhance the recovery of one of these three fractions but that this improvement will likely come at the expense of the other two fractions.

IIIA2 Proteinase K Digestion

Another successful sample preparation for the isolation of oligonucleotides from biological samples involves the use of proteinase K digestion (Figure 2.5b). One of the strengths of this approach is that it greatly reduces the number of sample transfer steps that are involved in sample preparation (McGinnis, Cummings et al. 2013, Basiri, Sutton et al. 2019). Since it has

been previously noted that non-specific adsorptive losses become quite significant at lower concentrations of oligonucleotides, processes that can minimize this problem are attractive. The simplicity of this approach also facilitates automation for the processing of larger batches of samples.

This approach removes proteins through enzymatic digestion. Proteinase K is a general protease that will reduce all proteins by cleaving after hydrophobic or aromatic amino acids (Morihara and Tsuzuki 1975). It is necessary to maintain physiological pH and temperature during the digestion to maintain optimal activity of proteinase K and also keep the quick digestion time that makes this approach attractive. Similarly, to the trizol extraction method, there are several additives that greatly improve the quality of this approach. For example, dithiothreitol can be used to reduce protein disulfide bonds and increase the rate that proteinase K can hydrolyze proteins with more complex structures. Similarly, guanidinium chloride can be used to denature proteins and also increase the rate of protein degradation (Jeanpierre 1987). To help preserve oligonucleotides during the proteinase digestion, compounds that inhibit nucleases activity can be added such as ethylenediamine tetraacetic acid (EDTA) which can sequester metal atoms that are critical co-factors for several families of nucleases (Dattagupta, Fujiwara et al. 1975, Hilz, Wiegers et al. 1975). When well optimized, proteinase K digestion can be accomplished in three hours (McGinnis, Cummings et al. 2013, Basiri, Sutton et al. 2019).

One of the great advantages of the proteinase K digestion is that it is a quite generic approach that can be readily applied to many different types of oligonucleotides including therapeutic oligonucleotides. The recovery for oligonucleotides using the proteinase K digestion has been shown to be greater than 90%. Another advantage of this approach is that it could also be paired with other sample pretreatment strategies such as solid-phase extraction or on-line column

trapping similar to how protein precipitation is often used in conjunction with liquid-liquid extraction or solid-phase extraction in small molecule bioanalysis (Bourque and Cohen 1993, Chen, Qian et al. 1997, Raynaud, Orr et al. 1997, Chen and Bartlett 2012, Kotorashvili, Ramnauth et al. 2012, Shimizu, Jinno et al. 2012).

IIIA3 Solid-Phase Extraction

Solid-phase extraction has been a consistently successful sample preparation strategy across all types of analytes (Figure 2.5c). Therefore, it is not surprising that it has had success with oligonucleotides as well (Dai, Wei et al. 2005, Johnson, Guo et al. 2005, Chen and Bartlett 2012). Solid-phase extraction methods typically use weak-anion exchange (WAX) or hydrophilic-lipophilic balanced sorbents. Both of these packing materials are able to effectively exploit the highly polar and charged properties of oligonucleotides.

In general, the loading of oligonucleotide samples occurs following buffering to a pH around 5.5 which allows for strong retention of the analyte. Having such strong retention allows for the removal of other endogenous analytes using relatively strong solvents such as a 1:1 mixture of a pH 5.5 buffer and an organic solvent such as acetonitrile or methanol. In order to elute the compounds, the pH of the buffer must be increased to 9.5 and used as a 1:1 mixture with an organic solvent mixture of acetonitrile and tetrahydrofuran. Once the sample is eluted from the SPE cartridge, the elution solvent is evaporated and the sample can be reconstituted to the LC-MS mobile phase prior to injection (Chen and Bartlett 2012).

Analyte recovery using SPE approaches generally range from 60-80% (Chen and Bartlett 2012). In addition, SPE is easily automatable and can be scaled for high-throughput by using 96-well plates. One of the general difficulties for SPE is that it is often subject to non-specific adsorptive losses due to the substantial surface area contained within the cartridges. This can result in

difficulty in using it for trace analysis. It should also be noted that SPE is often used in combination with the phenol-chloroform extraction and ethanol precipitation (Zhang, Lin et al. 2007, Deng, Chen et al. 2010).

IIIA4 Hybridization Extraction with Magnetic Beads

Magnetic beads have become widely used in sample preparation for the isolation of analytes (Berensmeier 2006, Ye and Beverly 2011) (Figure 2.5d). One of the major advantages of magnetic beads is their ability to facilitate the washing and isolation steps of sample preparation due to their ability to retain analytes throughout these processes. This reduces the number of sample transfer steps and can therefore reduce analyte losses due to non-specific adsorption. To date magnetic beads have been used in two different manners, targeted and non-targeted. In targeted applications a capture strand that contains 12-15 complementary bases of the analyte sequence is biotinylated. This allows for it to be used in conjunction with streptavidin magnetic beads to isolate a specific complementary sequence from complex samples. To date this approach has been applied to both mRNA, miRNA and therapeutic oligonucleotides (Albretsen, Kalland et al. 1990, Levison, Badger et al. 1998, Berensmeier 2006, Cantara, Crain et al. 2011, Ye and Beverly 2011, Kim, Basiri et al. 2019, Sips, Ediage et al. 2019). For therapeutic oligonucleotide assays, there is evidence that this approach may bias against the recovery of closely related species such as shortmers or other metabolites (Kim, Basiri et al. 2019). However, if it is not necessary to recover metabolites this is certainly a powerful approach. The other real challenge of this approach for bioanalysis is that it is so selective that it makes the use of an internal standard difficult. This particular problem has been addressed recently by Basiri and co-workers who showed that it was possible to actually use the biotinylated capture strand as an internal standard with excellent performance (Basiri, Sutton et al. 2019).

The other approach is more generic in nature and involves the use of magnetic ion-exchange beads (Ye and Beverly 2011). When using anion exchange the retention of the analytes is improved by the addition of a weak acid to the sample to maximize the negative charge on the phosphodiester backbone. The beads can be washed to remove residual biological material using an acidic solution, which will ensure that the analyte remains bound to the beads. The wash can be improved by the addition of a small amount of a non-ionic surfactant such as polyoxyethylenesorbitan (Tween) which will help to solubilize some of the more hydrophobic biological material. It is critical not to use ionic surfactants such as sodium dodecylsulfate or Triton X-100 as these may competitively remove the oligonucleotides from the surface of the magnetic beads. Once the samples have been washed the oligonucleotides can then be eluted using a high concentration of ammonium chloride. The approach using magnetic ion-exchange beads also allows for facile introduction of an internal standard to the sample prior to sample preparation since the isolation mechanism does not discriminate oligonucleotides other than the analyte.

In general, the use of magnetic beads has great potential for sample preparation whether it is used in a targeted or non-targeted format. This approach minimizes the number of sample transfer steps, which will decrease analyte loss as a result of non-specific adsorption. Also the recoveries using magnetic beads are quite reasonable with reports between 80-95% (Ye and Beverly 2011).

IIIA5 On-line Trapping Columns

Another approach that reduces the number of sample transfers and also provides opportunities to concentrate the analyte prior to LC-MS analysis has been the use of trapping columns. This type of approach involves the use of a C_{18} trapping column, which allows for the effective removal of

lower molecular weight compounds from the samples prior to LC-MS analysis. For oligonucleotides it was first introduced by Isobe and co-workers (Isobe, Yamauchi et al. 2002, Nakayama, Yamauchi et al. 2015, Yamauchi, Nobe et al. 2016). A cell contains RNAs over a wide range of sizes from only a few bases to more than 10,000. Therefore, they proposed to fractionate the samples in order to group their RNAs by their relative size. One of their fractions contained microRNAs which are similar in size to therapeutic oligonucleotides. They then injected this fraction onto a trapping column to remove the lower molecular weight components of the sample. Once this was accomplished the trapping column was back flushed onto a capillary LC column for separation.

Similarly, Hussar and co-workers recently used this approach with a GalNAc modified therapeutic oligonucleotide (Husser, Brink et al. 2017). In this case they performed SPE on the sample and then injected a large aliquot of the final solution from the SPE experiment onto a capillary trapping column. The trapping column was then back flushed onto a capillary column for analysis. This approach allowed for the determination of several metabolites including the loss of the GalNAc targeting moieties and several shortmers from both the 3′ and 5′-end of the molecule.

Many of the methods listed for these techniques actually use a combination of sample preparation techniques for oligonucleotides. It is not uncommon to have a liquid processing step (LLE or digestion) followed by a solid phase extraction (SPE or magnetic beads) and can even be followed up by on-line analytical techniques like the trapping column.

IIIB Key Considerations for Sample Preparation of Therapeutic Oligonucleotides

As with all sample preparation procedures there are several critical elements that are specific to oligonucleotides and will require specific attention in order to develop methods that will have

high performance. As has been stressed several times already, oligonucleotides are particularly susceptible to non-specific adsorption (Zhang, Lin et al. 2007, Deng, Chen et al. 2010, van Dongen and Niessen 2011). There are two specific ways that the adverse impact of this challenge can be minimized. The first is through the use of sample containers that are designed to minimize interactions with oligonucleotides. This requirement can be met by selecting sample tubes that are made from materials that possess a low zeta potential. This means that their surfaces will not have a substantial degree of charging, which will minimize ionic interactions with the oligonucleotides. The second is minimizing the total number of steps in the sample preparation process which will reduce the number of different surfaces that the analyte would be exposed to during the isolation from the biological matrix. Plasma protein binding is another major concern surrounding the sample preparation of these molecules in biological matrices. Oligonucleotides exhibit a high degree (≥85%) of plasma protein binding (Yu, Kim et al. 2007, Basiri and Bartlett 2014, Geary, Norris et al. 2015). This binding must be disrupted in order for the oligonucleotide to be fully extracted from the samples and analyzed. Sample additives can be used to disrupt the proteins, like dithiothreitol, without the need for a full sample digestion.

As mentioned during the discussion on metabolism, nucleases are the major source of degradation of oligonucleotides. However, it is often not appreciated that nucleases are found in secretions from the skin. Therefore, the analyst needs to avoid contact with all of the supplies used in the sample preparation and analysis of the analyte. The inadvertent introduction of nucleases into the experiment can significantly reduce the level of analyte recovery and also degrade method precision and accuracy. Therefore, choose materials that are certified as nuclease free. If you are able to do so, it is wise to autoclave materials (including buffers and

other solutions), clean the lab bench with bleach and have a dedicated lab coat for RNA work to minimize the potential for nuclease interference with results.

The choice of anticoagulant can have a significant adverse effect on the analysis of oligonucleotides. It has been observed that heparin can cause a substantial interference with the analysis of oligonucleotides (Bandres, Bitarte et al. , Korfmacher 2009, Kotikalapudi and Patel 2015). This is likely due to the similarity of the physical properties that it shares with oligonucleotides such as being highly negatively charged, similar molecular weight and also being quite water soluble. Therefore, choosing a lower molecular weight anticoagulant such as EDTA is likely a better choice. It has been used successfully in several bioanalytical assays for oligonucleotides.

The sulfur atom in the phosphorothioate linkage of oligonucleotide backbone is prone to oxidation. The sample preparation step that has shown the highest propensity for accelerating this reaction is evaporation. Evaporation steps are commonly used following either the Trizol extraction procedure or solid-phase extraction to exchange solutions with a high organic content for the initial mobile phase of the LC-MS analysis. Therefore, analysts need to be wary of identifying metabolites as oxidation products as they may be artifacts of sample preparation. The addition of antioxidants such as Tris (2-carboxylethyl)phosphine (TCEP) have been shown to greatly reduce this phenomena (Chen and Bartlett 2012, Erb and Oberacher 2014).

Bioanalytical methods that aim to quantitate therapeutic oligonucleotides and their metabolites will generally use an internal standard to improve method reliability. Since stable isotope labeled oligonucleotides are not readily available, analogs are generally used. In most cases the internal standard should be a little larger than the analyte of interest, as the metabolites will mostly be smaller than the parent drug. These metabolites will therefore mostly elute at shorter retention

times and having the internal standard in the same retention window will complicate the identification of these often trace level metabolites. In addition, it is beneficial to use the internal standard concentration near the top of the calibration curve. At this concentration it will occupy non-specific binding sites and reduce adsorptive losses for the analytes (Chen and Bartlett 2012, Chen and Bartlett 2013, McGinnis, Cummings et al. 2013).

Each of the issues described in this section need to be evaluated before beginning oligonucleotide method development. If not well controlled, these challenges will substantially reduce the performance of methods. The various sample preparation options described in this section can all be impacted by these options and, therefore, it is critical to be aware of them and not rediscover them during method development and validation.

IV LC-MS Bioanalytical Methods

Over the past twenty years many methods for the bioanalysis of oligonucleotides have been reported. The methods can generally be grouped into two categories; 1) those that focus on quantitation of the therapeutic and possibly some of the metabolites and 2) those that focus on the isolation and identification of metabolites of therapeutic oligonucleotides.

IVA Quantitation of Therapeutic Oligonucleotides

A method was developed to determine a 15-mer that was used to target Raf-1 as a potential anti-cancer agent (Johnson, Guo et al. 2005). The 15-mer contained phosphorothioate linkages in the first linkage at the 5′ and 3′ end of the molecules but the remaining twelve linkages were phosphodiesters. For sample preparation they compared solid phase extraction using either C18 or hydrophilic lipophilic balance cartridges to simple protein precipitation using acetonitrile with 0.1% TEA. The use of protein precipitation or solid phase extraction was found to depend on the species and tissue that was being assayed, but the majority of the samples were tested using

protein precipitation. The separation was then completed using a polymeric reversed-phase column. The mobile phase consisted of trimethylamine and acetonitrile, and by using a gradient they were able to elute the analyte and a sequence analog internal standard within 4 minutes. The LLOQ for this method is not particularly low and ranged between 25 for plasma to 500 ng/mL for kidney homogenate. Since the oligonucleotide still contained a number of phosphodiester linkages it was not surprising that it was only detectable following IV injection for approximately 6 hours in monkeys and 24 hours in mice. There was no mention of metabolites being observed but since the analytes had a relatively low retention factor they may have been in the system void volume.

The first comprehensive bioanalytical method for the quantitation of a therapeutic oligonucleotide and its metabolites was developed by Dai and co-workers (Dai, Wei et al. 2005). Their method used solid-phase extraction with hydrophilic lipophilic balance cartridges to isolate the therapeutic oligonucleotide and 3 shortmers (n-1 → n-3) originating from the 3′ end of the molecule from plasma. The separation was conducted using a C18 column with the TEA/HFIP mobile phase. The absence of any signals corresponding to metabolites originating from the 5′-end of the molecule allowed for the conclusion that metabolism was primarily a result of 3′-exonucleases. The LLOQ for the method was 100 ng/mL from plasma, which allowed for the measurement of the analytes for up to 8 hours following an IV dose.

The first major advance in the quantitative bioanalysis of a therapeutic oligonucleotide and its metabolites using LC-MS was the method developed by Zhang and co-workers at Pfizer (Zhang, Lin et al. 2007). This method combined a Trizol extraction with solid-phase extraction to isolate an antisense phosphorothioate deoxynucleotide and seven shortmers. The method was validated from plasma from 5 ng/mL to 2000 ng/mL. This manuscript dealt with many of the central issues

of oligonucleotide bioanalysis such as non-specific adsorption, oxidation of the phosphorothioate linkages upon evaporation and optimization of the concentrations of TEA and HFIP to enhance method sensitivity. The seven shortmers that were used in the assay were all chromatographically resolved from the full length therapeutic oligonucleotide; however, they ranged in size from n-4 to n-6 from either the 3′ or 5′ end of the molecule. Therefore, this assay did not evaluate the more common n-1 shortmer metabolites.

A similar approach was taken by Deng and co-workers who combined a variation of the Trizol extraction using dichloromethane and phenol with solid phase extraction to analyze a 24-mer phosphorothioate deoxynucleotide (Deng, Chen et al. 2010). The separation was conducted using a C18 column with a TEA/HFIP mobile phase. They also observed several shortmers including n-1, n-2 and n-3 from the 5′ end of the molecule. It was also possible that they observed n-1 from the 3′ end of the molecule but since both ends of the molecule contained thymine and were unable to be chromatographically resolved it was not conclusive. Following intravenous injection, they were able to observe the intact therapeutic oligonucleotide for up to 5 hours and the three shortmers for 1-3 hours.

The first bioanalytical method to quantitate an antisense therapeutic oligonucleotide containing locked nucleic acid modifications was conducted by Turnpenny and co-workers (Turnpenny, Rawal et al. 2011)They also used a combination of liquid-liquid extraction followed by solid-phase extraction for sample preparation. Their recovery was 90% from both plasma and tissues. For their separation they used a monolithic column, which allowed for greater throughput than any method to date with a similar LLOQ to the methods of Zhang and Deng (Zhang, Lin et al. 2007, Deng, Chen et al. 2010).

The first bioanalytical method to employ a nontraditional sample preparation scheme for the bioanalysis of a therapeutic oligonucleotide was by Ye and Beverly who used ion-exchange magnetic beads in a 96-well plate format (Ye and Beverly 2011). Using this approach, they were able to recover greater than 80% of the two strands of their siRNA. They were also able to observe metabolites originating from both the 3′ and 5′ end of the antisense strand. However, they observed greater metabolism from the end of the molecule containing fewer hydrogen bonds and proposed that for double stranded therapeutics this is an important predictive indicator for metabolism.

The first method that was reported for quantitative bioanalysis for a therapeutic oligonucleotide that employed solid-phase extraction without needing an initial Trizol extraction was reported by Chen and Bartlett (Chen and Bartlett 2012). In this method they were able to isolate both the full length 24-mer phosphorothioate deoxynucleotide and its 3′ n-1 metabolite using this approach. The key was the introduction of a lysis buffer to disrupt protein binding by the analytes prior to the solid-phase extraction step. The separation was completed using a bridged ethylene hybrid C18 column using diisopropylethylamine and HFIP as the mobile phase. The recovery and LLOQ of this method was similar to that of Zhang et al (Zhang, Lin et al. 2007).

Chen and Bartlett had another interesting advance in sample preparation when they were able to combine the classic Trizol extraction with ethanol precipitation to isolate a 24-mer phosphorothioate deoxynucleotide and its 3′ n-1 metabolite from plasma (Chen and Bartlett 2013). The separation was carried out using a bridged ethylene hybrid C18 column using the diisopropylamine (DIPA)/ 1,1,1,3,3,3-hexafluoro -2-propanol (HFIP) mobile phase. However, the substitution of ethanol in place of methanol in the mobile phase, along with the new sample

preparation approach, allowed for a 4-fold increase in the LLOQ relative to their previous method.

Hemsley and co-workers have developed the first LC-MS method that was able to detect pg/mL levels of an oligonucleotide (Hemsley, Ewles et al. 2012). They applied it to the determination of an unmodified DNA so, while this would not qualify as a therapeutic oligonucleotide, the use of on-line solid-phase extraction for the sample preparation is particularly noteworthy. This allowed for a larger initial loading and resulted in quantitation from 50 pg/mL to 20,000 pg/mL.

Ewles and co-workers developed a LC-MS method for the quantitation of the 18-mer phosphorothioate oligodeoxynucleotide, Trabedersen, along with six of its metabolites (Ewles, Goodwin et al. 2014). The metabolites that were detected included n-1, n-2 and n-3 from both the 3′ and 5′ end of the molecule. The sample preparation for this molecule uses Trizol extraction followed by solid-phase extraction and the recovery was around 75% for all analytes. One notable feature of this method is the ability to determine two isobaric metabolites and thereby demonstrate the importance of accounting for the major metabolites as they may potentially interfere with other shortmer MRM channels. In general this has to do with the significant overlap of major fragment ions among the intact therapeutic and its metabolites. The most abundant fragment ions for oligonucleotides will not be sequence specific (Figure 2.6) (Kim, Basiri et al. 2019). These ions are the deprotonated bases or phosphorothionate-ribose monomers and polymers that often do not have any nucleobases attached.

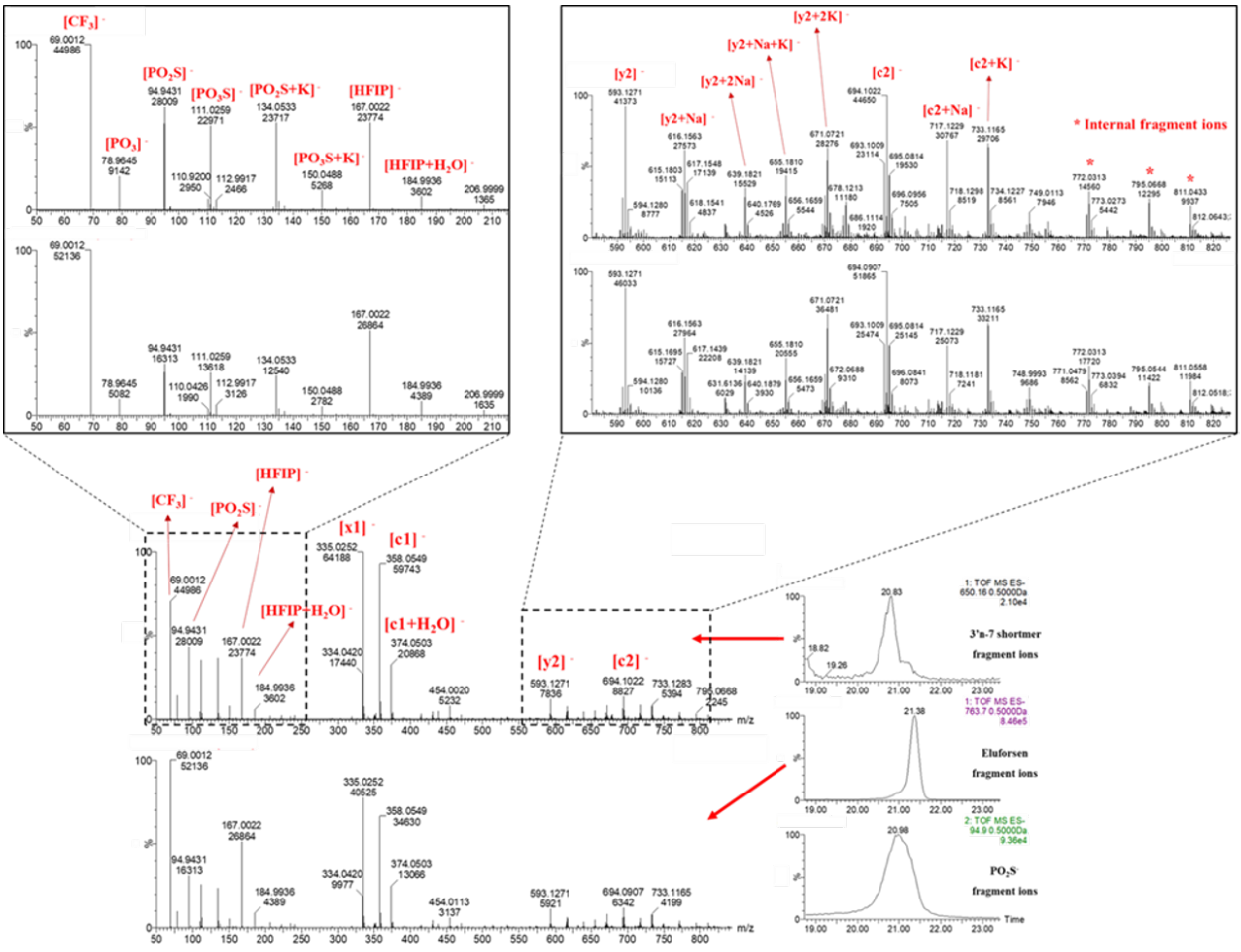


Figure 2.6. An example of fragmentation patterns of a full-length oligonucleotide and its 3′-end n-7 metabolite. Reprinted with permission from [Kim et al.] (2019)

The first LC-MS method for the bioanalysis of a phosphorodiamidate morpholino, 20-mer Radavirsen, was recently accomplished by Meng and co-workers (Meng, Zhang et al. 2017). They employed solid-phase extraction for sample preparation. Because of the presence of the phosphorodiamidate linkage in the backbone, the LC-MS analysis was conducted in positive ion mode over the range from 5 ng/mL to 1,000 ng/mL.

Hydrophilic interaction liquid chromatography (HILIC) is another method that can be used to analyze therapeutic oligonucleotides. There are only a handful of published results surrounding this methodology for application to oligonucleotides (Alpert 1990, Holdsvendova, Suchankova et

al. 2007, Easter, kreoning et al. 2010, Gong and McCullagh 2011, Easter, Barry et al. 2013, Studzińska, Łobodziński et al. 2017, Lobue, Jora et al. 2019). Currently the most significant challenge facing this approach the reduced sensitivity when compared to ion-pair chromatography. Lobue and coworkers have shown that for their method the ion-pair method is more sensitive for 3 out of 4 oligonucleotides present, including a phosphorothioate (Lobue, Jora et al. 2019). Another challenge is that HILIC may have greater difficulty achieving similar chromatographic resolution to ion-pair chromatography since the mechanism of retention for different column chemistries is currently still under investigation (Gong and McCullagh 2011, Li, Lynen et al. 2012, Easter, Barry et al. 2013, Gong 2017, Studzińska, Łobodziński et al. 2017, Lobue, Jora et al. 2019). Studzinska provides the greatest insight into how column chemistries impact HILIC performance comparing several columns indirectly (Studzińska, Łobodziński et al. 2017). There is also the issue about the salt concentration used in HILIC and needing compatible salts at compatible concentrations for LC-MS. However, there are other major advantages of HILIC due to the lack of ion-pair, which could be beneficial in true unknown metabolite studies. HILIC does not use ion-pairs which could interfere and adduct to true unknown metabolites making the analysis more complicated. The most significant advantage in using HILIC is the ease in switching between methods (especially going between positive ion and negative ion modes) since the alkylamines and fluoroalcohols generally cause significant ion suppression in positive ion electrospray. This challenge can also be addressed by segregating the solvent lines within the LC system or by dedicating LC systems to oligonucleotide analysis. Overall, this is a promising new area that may continue to provide viable options to ion-pair chromatography (Li, Lynen et al. 2012, Gong 2017).

Recently a study was published testing the impact of oligonucleotide modification on chromatographic retention and MS sensitivity (Kaczmarkiewicz, Nuckowski et al. 2019). The resulting study showed that as you increase hydrophobicity of the therapeutic oligonucleotide you increase the resulting retention when amine ion-pairs are utilized in reversed phase chromatography. The authors obtain LOQ's from 0.15-0.56uM.

The first LC-MS method for direct quantitation of a microRNA was by Basiri & coworkers quantitating miR-451 from rat, mouse and human plasma and comparing the results to real time-quantitative PCR (polymerase chain reaction) (Basiri, Sutton et al. 2019). The authors utilize a proteinase k digestion followed by extraction of miR-451 by magnetic beads. The separation was carried out on a C18 column with dibutylamine (DBA) and 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP). It was shown that this DBA/HFMIP mobile phase generated a higher MS signal intensity when compared with the traditional triethylamine (TEA) and hexafluoroisopropanol (HFIP). This is the first reported use of HFMIP over HFIP in the mobile phase for oligonucleotide analysis. The authors report an LOQ of 0.5 ng/mL up to 200 ng/mL and that the endogenous concentrations of miR-451 in rat and human plasma are 0.912 ng/mL and 1.17 ng/mL, respectively. There are other studies involving mass spectrometry of microRNA but none have a satisfactory LOQ for bioanalysis (Katoh, Hojo et al. 2015, Nakayama, Yamauchi et al. 2015, Studzińska and Buszewski 2018).

IVB Metabolite Identification for Therapeutic Oligonucleotides

The first study of the metabolism of a therapeutic oligonucleotide was conducted by Gaus and co-workers on several phosphorothioate oligodeoxynucleotides (Gaus, Owens et al. 1997). Their studies were conducted in rats and involved analysis of plasma, liver and kidney samples. They observed shortmers from both the 3′ and 5′ ends of the molecules but the metabolite distributions

had some organ specificity. They observed 3′ shortmers, 5′ shortmers and shortmers arising from combined losses from both ends in the kidney. The 3′ shortmers were the predominant species suggesting that 3′ exonuclease activity was the major pathway of metabolism in this organ. Interestingly, 5′ shortmers were mostly observed in the liver with lower levels of shortmers arising from losses at both the 3′ and 5′ end also being observed. This suggested that 5′ exonuclease activity was the major contributor to metabolism in the liver.

Griffey and co-workers also evaluated the metabolism of a 20-mer phosphorothioate oligodeoxynucleotide (Griffey, Greig et al. 1997). The compound was dosed in pigs and metabolites were assessed in kidney homogenate following fractionation using ion-exchange sample preparation. As opposed to the previous study in the rat, in the pig kidney the metabolites mostly appeared as 3′ shortmers with losses from 1 to 8 nucleotides observed. This raises the possibility that the metabolism of phorphorothioate oligodeoxynucleotides may be species dependent, as well as organ dependent.

One of the most extensive studies on the metabolism of therapeutic oligonucleotides was conducted by Crooke and co-workers (Crooke, Graham et al. 2000). They evaluated more than 20 different sequences of phosphorothioate oligodeoxynucleotides, as well as two gapmers containing 2′-ribose propyl modification following incubation in rat liver homogenate. The metabolites were isolated from the liver homogenate using sequential anion exchange and reversed-phase solid phase extraction cartridges. The compounds were relatively stable with an initial loss of 20-40% of the compound within the first 2 hours of incubation followed by a dramatic slowing of the rate. After 8 hours, most of the phosphorothioate oligodeoxynucleotides had not reached 50% loss of the intact therapeutic oligonucleotide. This study showed that smaller phosphorothioate oligodeoxynucleotides were metabolized faster; however, for

compounds with 12 or more nucleotides the rate of metabolism no longer varied directly with length. Based on the data it is possible that there may be some sequence effects on the rate of metabolism for the longer oligonucleotides. There does appear to be a stabilizing effect of purines on metabolism, although this effect did not hold in all cases but should certainly be considered as a contributing factor. This study was the first to directly examine oxidation of the phosphorothioate backbone. While the conversion of a single phosphorothioate to a phosphodiester was observed, the levels did not significantly change from the amount that was present at time zero. Therefore, they concluded that oxidation of the backbone is not a major metabolic pathway in the rat liver. The study of the two gapmers showed the expected increase in stability for these compounds when compared to the phosphorothioate oligodeoxynucleotides. Interestingly, they observed metabolites resulting from endonuclease cleavages from the regions of the oligonucleotide that were not protected by the 2′-O-propylation.

Several studies have evaluated the metabolism of therapeutic siRNAs using LC-MS. Beverly and co-workers evaluated the ocular metabolites of a siRNA that he was targeting the VEGF receptor 1 for the treatment of macular degeneration (Beverly, Hartsough et al. 2006). The samples were in ocular fluid and were prepared using a commercial kit that was similar to a Trizol extraction. They observed shortmers originating from both the 3′ and 5′ ends of the molecule. The sense strand was protected by having an abasic residue at first position at both the 3′ and 5′ ends of the molecule. Interestingly, only metabolites from the 5′ end of the molecule were observed for this strand. There was no n-1 shortmer present, demonstrating that the abasic residue was providing the expected nuclease resistance. They observed n-2 through n-11 from the 5′ end with the exception of n-4 and n-5. It should be noted that these both fell during a stretch of the sequence of three purines in a row and likely indicated the increased stability

imparted by these nucleobases. The antisense strand did not have the terminal abasic residue and metabolites were observed from both the 3′ and 5′ ends of the molecule. Interestingly, the metabolism of the antisense strand proceeds from n-1 through n-4 from the 3′ end of the molecule but because of the significant number of purines at the 5′ end metabolism from this end does not begin until n-6.

Zou and co-workers examined the metabolism of a siRNA duplex targeting the hepatitis B virus. They evaluated metabolism in serum and liver microsomes from rat and humans (Zou, Tiller et al. 2008). The metabolites were isolated using a combination of liquid-liquid extraction and solid-phase extraction prior to LC-MS analysis. This particular study was quite interesting because of the wide variety of modifications that were present in the sample including 3′ and 5′ terminal abasic groups, 2′-fluoro groups, 2′-O-methyl groups and 2′-deoxyribose. These sequences were also somewhat unusual in that they did not contain phosphorothioate backbones but rather had the more natural but less nuclease resistant phosphodiester linkages. This study also employed an orbitrap mass spectrometer and used the high resolution and exact mass capabilities of this instrument to improve the determination of the metabolites as seen in figure 2.7. In all of their studies all of the metabolites were observed to originate from the 3′ end of either strand. From human serum they only observed metabolism on the antisense strand with n-1 through n-5 shortmers observed. However, this is interesting as these residues contained both 2′-fluoro and 2′-O-methyl modifications which did not prevent metabolism. The combination of the abasic terminal group and the 2′-fluoro groups appears to prevent metabolism of the sense strand. The results from rat serum were identical for the antisense strand but there were three metabolites observed as a result of endonucleases in the sense strand at n-7, n-9 and n-10 from the 3′ end. For the human liver microsomes nearly the opposite of serum was observed with no

metabolism of the antisense strand observed and n-4 through n-11 shortmers observed for the sense strand. The results of the rat liver microsomes were similar with n-3 through n-12 shortmers observed for the sense strand and only n-3 and n-4 shortmers were observed for the antisense strand. In the case of the antisense strand the therapeutic oligonucleotide has two 2′-O-methyl groups on the first two linkages from the 3′ end that appear to have significantly greater nuclease resistance.

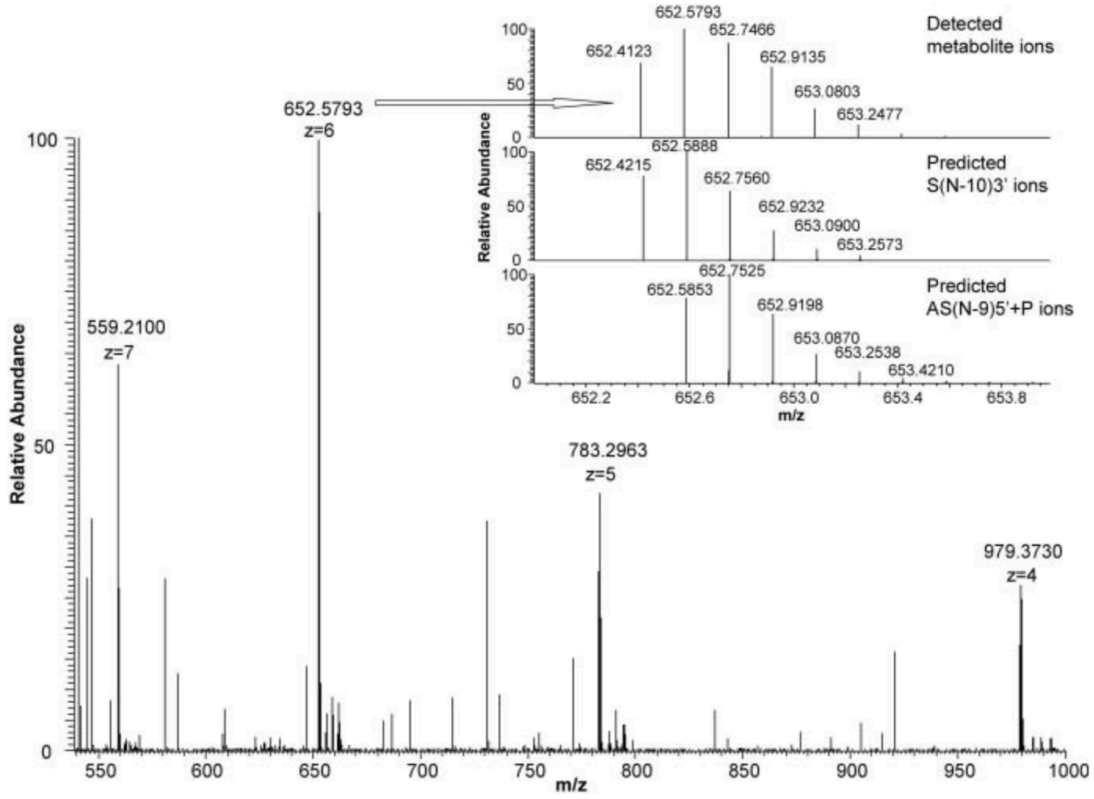


Figure 2.7: A chromatogram highlighting the performance of high resolution and exact mass Reprinted with permission from [Hochman] (2008) [Wiley]

McGinnis and co-workers evaluated the metabolism of a 21-mer siRNA duplex containing DNA overhangs to improve stability in cell culture (McGinnis, Cummings et al. 2013). The siRNA

was used to target the enzyme calcium independent phospholipase A_2ß. Sample preparation involved digestion of the biological material using proteinase K followed by direct LC-MS analysis of the remaining solution. The DNA overhangs appeared to protect those regions of the sense and antisense strands. However, metabolites were observed originating from both the 3′ and 5′ ends of both the sense and antisense strands with greater metabolism observed from the end of the double strand containing fewer hydrogen bonds between the two strands.

Recently, the use of triantennary GalNAc conjugation for improved liver targeting of antisense oligonucleotides has been reported (Nair, Willoughby et al. 2014). The metabolism of a therapeutic oligonucleotide containing this targeting moiety has been recently reported by Husser and co-workers (Husser, Brink et al. 2017). In this study they evaluated a 13-mer and 16-mer each with the GalNAc targeting moiety but containing different sequences. For the isolation of the metabolites they used solid-phase extraction with hydrophilic lipophilic balance cartridges. To enhance the detection of the metabolites they used a trapping column, which allowed for a larger injection volume along with capillary chromatography. For both sequences they observed the loss of the triantennary GalNAc targeting moiety along with shortmers. For the first sequence they observed n-1 through n-3 from both the 3′ and 5′ ends of the molecule. For the other sequence they only observed metabolism from the 3′ end of the molecule. This was perhaps due to the presence of three purine bases on the first three positions of the sequence at the 5′ end of this molecule that were not present in the other sequence.

Eluforsen, a 33-mer phosphorothioate with 2′-O-methyl ribose modificaitions was granted orphan drug designation and fast-track designation. Kim, et al. conducted a study looking at the metabolites in mouse liver homogenate, mouse liver and lung and monkey liver and lung (Kim, Basiri et al. 2019). They report using SPE over magnetic bead pulldown due to the advantage of

capturing more metabolites. With SPE combined with a proteinase K digestion protocol, they report being able to capture 3′ n-1 through n-8, as well as 5′ n-1 through n-8. They report using DMCHA and HFIP as a suitable mobile phase for their analysis. Perhaps most importantly, they show that there is a difference between in vitro and in vivo metabolism of this oligonucleotide. In vitro generates no 5′ metabolites while the in vivo samples contain both 5′ and 3′ metabolites. Reversir, a trivalent GalNac modified siRNA, was studied by Li & coworkers in monkey liver, plasma and urine (Li, Liu et al. 2019). The authors employed SPE using clarity OTX cartridges with an evaporation and reconstitution concentrating step at the end. The study showed that metabolism of Reversir was reported as an n-1 with loss of the GalNac modification and an n-3 with the loss of the GalNac. However, a novel deamination was determined to be the most abundant metabolite which consisted of a 2′-O-methyl-adenosine to a 2′-O-methyl-inosine in the monkey liver but not in the urine or plasma. This was the first report of an A-to-I RNA editing metabolite.

IVC Therapeutic oligonucleotides softwares for mass spectral data analysis

Different algorithms have been implemented for the data collection, sequencing, processing and analysis of LC-MS and LC-MS/MS profiles of oligonucleotides and their metabolites (Nakayama, Yamauchi et al. 2015). Determination of the nucleotide composition based on the mass of the oligomer and the masses of the four canonical ribonucleosides was one of the first attempts for computational analysis (Pomerantz, Kowalak et al. 1993). In 1996, McCloskey and coworkers introduced the first computer algorithm for oligonucleotide sequencing (Ni, Pomerantz et al. 1996). They next developed The Mongo Oligo Mass Calculator with an accompanying tool Oligo Composition Calculator for the analysis of genetic mass spectrometric data with various functionalities (Rozenski, Crain et al. 1999). They also developed

Oligonucleotide Sequencer (SOS) for the de novo determination of short nucleic acid sequences (Rozenski and McCloskey 2002). The COMPAS algorithm was developed for comparative sequencing of DNA fragments (Oberacher, Wellenzohn et al. 2002). The Links and MS2Links programs were developed by Young and coworkers to interpret the MS and fragmentation data generated from modified nucleic acids (Kellersberger, Yu et al. 2004). GeMS is another software for synthetic gene design, many of which exceeded 5 kb in length, developed by Jayaraj and others at KOSAN Biosciences (Jayaraj, Reid et al. 2005). GNU polyxmass is a software developed by Filippo Rusconi for mass spectrometric simulations of biopolymers as oligomers (Rusconi 2006). GenoMass is a software for the identification of oligonucleotide DNA adducts (Liao, Chiu et al. 2007, Liao, Shen et al. 2009). A software package with two algorithms OMA and OPA was developed by Nyakas et al. for the analysis of raw mass spectral data from MS and MS^E experiments of native and modified nucleic acids (Nyakas, Blum et al. 2013). The RNA Mass Calculator (RNA MC) is a program used to calculate theoretical masses for unmodified and modified oligonucleotides (Yang, Leopold et al. 2013). Moreover, various computer programs (Oberacher, Mayr et al. 2004, Kretschmer, Lavine et al. 2010, Oberacher and Pitterl 2011) have been utilized for automated de novo sequencing of MS/MS data. For RNA mass spectrometry data analysis, several algorithms have been developed such as RNA mass mapping (RMM) (Matthiesen and Kirpekar 2009), Ariadne (Nakayama, Akiyama et al. 2009), RoboOligo (Sample, Gaston et al. 2015), RNAModMapper (Yu, Lobue et al. 2017) and NucleicAcidSearchEngine (NASE) softwares (Wein, Andrews et al. 2020).

V Conclusions & Future Perspectives

VA Conclusions

When developing an LC-MS method for the determination of oligonucleotides and their metabolites there are a number of factors which should be considered during method development. Below are brief overviews of many of these key considerations.

- Non-specific adsorption is a more significant cause for reduced sensitivity for this class of molecules. Therefore, it is wise to minimize the number of sample transfer steps in the method and also to avoid filtration of samples. One strategy that has been effective in minimizing this effect is the introduction of a relatively high concentration oligonucleotide into the sample. This oligonucleotide may also serve as the internal standard in addition to its role in occupying non-specific adsorption sites.
- Sample preparation will likely require a combination of the approaches outlined in the sample preparation section. The most common approach used to date has involved the use of a Trizol extraction prior to solid-phase extraction.
- The metabolism of oligonucleotides has been observed to primarily arise due to exonuclease activity resulting in shortmers. It is difficult to *a priori* predict whether these metabolites will arise solely from the 3′ or 5′ end of the molecule or potentially both. The presence of several purine residues in a row does appear to diminish metabolism in these regions of the molecule.
- The metabolism of oligonucleotides does not appear to be consistent between the blood and various organs and therefore metabolism studies should be conducted in each specific target organ and should not be expected to be the same as what is observed in the liver.

VB Future Perspectives

There are still many advances to be made in the field of oligonucleotide analysis. Sample preparation is one of the major challenges. New non-adsorptive materials could be designed to prevent binding to benefit oligonucleotide and even protein/peptide analysis. In addition, there has not been a lot of work detecting oligonucleotides outside plasma, liver and ocular fluid. This is partially due to the lack of specific targeting of these molecules. Future studies looking at oligonucleotides that partition to specific tissues will be necessary as more GalNAc, cholesterol, and other conjugated oligonucleotides are explored. These uniquely modified oligonucleotides will provide challenges as they become potential therapeutics.

Oligonucleotides suffer from poor sensitivity due to being analyzed in the negative ion mode, due to the negative ion mode causing an envelope of charges and an additional envelope of cationic adducts (Muddiman, Cheng et al. 1996, Sutton and Bartlett 2019). In addition, most of these therapeutic oligonucleotides are dosed infrequently (2-3 weeks to months apart) due to their relatively long half-lives. This makes analysis of these molecules for pharmacokinetic or toxicity studies difficult at these later time points due to the low concentration left in tissue/plasma. Instrumental and methodological advancements for increased sensitivity would make analyzing these oligonucleotides much easier. One simple method of improving the sensitivity of these molecules would be to analyze them in the positive mode as adducts. It could be possible to use a zwitterionic ion-pair or mobile phase additive that can adduct the phosphate backbone and be ionized in the positive mode. In theory this could lower detection limits for these molecules.

A recent development by Li & coworkers suggested that the classic Alkylamine/Fluoroalcohol (TEA/HFIP or similar) ion-pairing mechanism used to separate oligonucleotides is not as well

understood as the field once thought (Li, El Zahar et al. 2018). Classically these alkylamines would electrostatically bind to the negatively charged backbone of the oligonucleotides and the hydrophobic chain of the alkylamine would interact with the C18 column. The fluoroalcohol is utilized as a counter-ion and is more important for the MS sensitivity than the LC separation. However, high resolution transmission electron microscopy images suggest that these alkylamine/fluoroalcohol pairs create micelles or have the potential to create micelles. This suggests another mechanism of separation during routine analysis of these molecules. There is also a pH effect that can dramatically affect LC retention. High concentrations of HFIP are often employed to improve MS sensitivity. HFIP causes the pH of the mobile phase to be lower and if the pH of these mobile phases isn't controlled, then the retention time increases dramatically due to the increase of acid (HFIP pKa ~7.97) Basiri, van Hattum et al. (2017) but when the mobile phase is pH adjusted then the differing concentrations of HFIP don't dramatically affect retention. There is a balance to satisfy the LC separation but not use mobile phase modifiers that decrease the method sensitivity. Better LC separation occurs at lower pH but many acids are not MS compatible. A better understanding of these mobile phases and their contribution on fundamental electrospray processes in conjunction with their chromatographic impact is needed. Another exciting field for oligonucleotides is the recent interest in aptamer technology. Due to the binding nature of aptamers the secondary structure of these oligonucleotides will be extremely important for their function. Ion-mobility has recently been used to separate isomeric RNA (Takebayashi, Hirose et al. 2013). Other ion-mobility work has been done with g-quadruplexes and even a small amount with phosphorothioates that looks at collision cross section and relates this back to the size and shape of the oligonucleotide (Wang and Patel 1993, Baker, Bernstein et al. 2006, Mazzitelli, Wang et al. 2007, Arcella, Portella et al. 2012), Basiri,

van Hattum et al. (2017). Ion-mobility could become an important tool for additional degrees of separating oligonucleotides and even molecular biological studies of the secondary structure. This could be especially useful if microRNA becomes a leading therapeutic candidate and the synthetic microRNA is not modified enough to be completely separated by chromatography. Ion mobility provides that extra degree of separation that could be utilized over a high resolution instrument if the mass of the endogenous RNA and the synthetic RNA are very close. Currently a lot of focus is on the secondary structure of proteins, but it should be relatively easy to translationally apply some of this work to oligonucleotides. This could provide quick and robust methods for secondary structure analysis, aptamer binding studies and even quality control of finished aptamer products (if secondary structure confirmation was required). Finally, the recent advances in software for analyzing oligonucleotides has advanced the field significantly but has some drawbacks with different functionalities in each software package. It would be convenient and easier for newer oligonucleotide scientists if there was a user-friendly compilation of these functionalities in one software package.

Table 2.1: Summary of Sample Preparation for Therapeutic Oligonucleotides

Technique	**Recovery**	**Complexity**	**Time Required**	**Specificity**
Trizol Extraction	~70% (10-30 bases)	Simple	1-2 hours	Broad
Proteinase K Digestion	>90%	Simple	2-3 hours	Broad
Solid-Phase Extraction	60-80%	Simple	3-4 hours	Broad
Magnetic Bead Extraction	80-95%	More Complex	5-6 hours	Broad or Very specific
On-Line Trapping Column	-	More Complex	2-3 hours	More Specific

Table 2.2: Summary of Sample Prep and LC conditions of Therapeutic Oligonucleotides

Oligo	**Sample Prep**	**LC-MS Instrument**	**Mobile Phase**	**Column**	**LLOQ**
15-mer with 5′ & 3′ Phosphorothioate ; remaining 12-mer phosphodiester (Johnson, Guo et al. 2005)	SP C18 or SP HLB with Protein Precip: ACN w/ 0.1% TEA	Agilent 1100 HPLC - AB Sciex API 4000 triple quadrupole	A: 10 mM TEA 95:5 ACN:H_2O pH 10 B: MeOH	Polymeric Reversed-Phase column (PRP-1 25mm x 2.3mm, 1.9µm PS)	25-500ng/mL (mouse plasma, monkey plasma, mouse: heart, kidney, liver, lungs, spleen homogenate)
18-mer phosphorothioate and 3′ n-1 to n-5 (Dai, Wei et al. 2005)	SP HLB cartridges	Shimadzu HPLC system - Finnigan LCQ Ion Trap	A: H_2O w/ 100mM HFIP/8.6mM TEA pH 8.35 A: MeOH with 100mM HFIP/8.6mM TEA pH 8.3	C18 (Xterra MS18 50mm x 2.1mm, 2.5 µm PS)	100ng/mL (rat and human plasma)
24-mer Antisense phosphorothioate deoxynucleotid	Phenol/Choloroform LLE w/ SP HLB cartridges	Shimadzu HPLC system - AB Sciex API 3000 and API	A:H_2O w/ 1.7mM TEA/100mM HFIP pH 7.5 B: MeOH	C18 (Hypersil GOLD 50mm x 2.1mm, 1.9µm PS)	5-2000ng/mL (rat plasma)

e and 7 shortmers (n-4 to n-6) (Zhang, Lin et al. 2007)		5000 triple-quadrupole			
24-mer phosphorothioate deoxynucleotide & 5′n-1 to n-3 & 3′n-1 (Deng, Chen et al. 2010)	LLE using phenol/dichloromethane w/ SP HLB cartridges	Shimadzu HPLC system - AB Sciex API 4000 triple-quadrupole	A:H_2O w/ 2.85mM TEA/100mM HFIP pH 7.8 B: MeOH	C18 (Phenomenex Genimi 150mm x 4.6mm, 5µm PS)	4.0 ng/mL (rat plasma)
16-mer and 120mer Antisense LNA phosphorothioate (Turnpenny, Rawal et al. 2011)	LLE using phenol/chloroform with SP plates (high-throughput)	Agilent 1100 HPLC - AB Sciex API 4000 QTRAP linear ion trap	A: TEA/HFIP: MeOH: H_2O 0.25:4:5:90.75 pH 8 B: MeOH:Buffer A 9:1	Monolithic column (Phenomenex Onyx C18 2 mm x 50mm)	10-25ng/mL (CD-1 mouse matrices liver, kidney, plasma)
5-21mer modified Therapeutic Oligos (siRNA)	Ion-exchange magnetic beads w/ 96 well plate (high-throughput)	Waters UPLC - Waters SYNAPT HDMS qTOF	A: H_2O w/ 200mMHFIP/ 8.5mM TEA B: MeOH	Bridged ethylene C18 (Waters BEH 2.1mm x 50/100mm ,	50-1000ng/mL (human serum)

(Cantara, Crain et al. 2011)				1.7µm PS)	
24-mer phosphorothioate deoxynucleotide & 3′ n-1 (Chen and Bartlett 2012)	C18 SP extraction	Waters Acquity UPLC - Waters SYNAPT G2 HDMS qTOF	A: H_2O w/ 15.7mM DIEA/50mM HFIP B: 50:50 H_2O:ACN w/ 15.7mM DIEA/50mM HFIP	Bridged ethylene C18 (Waters BEH 2.1mm x 100mm , 1.7µm PS)	10-1,000 ng/mL (rat plasma)
24-mer phosphorothioate deoxynucleotide & 3′ n-1 (Chen and Bartlett 2013)	LLE w/ phenol/chloroform w/ EtOH PPT	Waters Acquity UPLC - Waters SYNAPT G2 HDMS qTOF	A: H_2O w/ DIEA/HFIP B: 50:50 H_2O:EtOH w/ DIEA/HFIP *Varying DIEA/HFIP concentrations	Bridged ethylene C18 (Waters BEH 2.1mm x 100mm , 1.7µm PS)	2.5ng/mL (rat plasma)
15-mer Unmodified DNA (Hemsley, Ewles et al. 2012)	On-line SP weak anion exchange (WAX) extraction	Waters Acquity UPLC - AB Sciex API 5000 triple quadropole	A: H_2O w/ 15mM TEA/400mM HFIP B: 50:50 H_2O:MeOH 15mM	C18 Column (Waters acquity OST 50mm x 2.1mm, 1.7µm PS)	50-20,000 pg/mL

			TEA/400mM HFIP		
18-mer phosphorothioate deoxynucleotide (Trabedersen) & 5′ & 3′ n-1 to n-3 (Ewles, Goodwin et al. 2014)	Phenol/Choloroform LLE w/ SP HLB cartridges	Waters Acquity UPLC - AB Sciex API 5000 triple quadropole	A: 100:1:0.1 v/v/v H_2O:HFIP:TEA B: 100:1:0.1 v/v/v MeOH:HFIP:TEA	Bridged ethylene C18 (Waters BEH 2.1mm x 100mm , 1.7µm PS)	100-600 ng/mL (human, monkey and rat plasma)
phosphorodiamidate morpholino 20-mer Radavirsen (Meng, Zhang et al. 2017)	SP HLB plate extraction	Shimadzu HPLC system - AB Sciex API 5000 triple quadropole	A: 1% formic acid in H_2O B: MeCN C: 1% formic acid in H_2O, MeOH, MeCN	C18 column (Metasil AQ 50mm x 2mm)	5-1,000 ng/mL (human plasma)
24-mer phosphorothioate & various modified RNA (Lobue, Jora et al. 2019)	None	Thermo Scientific Ultimate 3000 - Waters SYNAPT G2 HDMS qTOF	HILIC A: 70:30 H_20:ACN B: 30:70 H_2O:ACN IP A: 8mM	HILIC: 2x150mm, 5 µm PS PEEK SHodex HILICpac VN-50 IP:	HILIC: 583.3µ g/mL IP: 1120.55 µ g/mL

			TEA:200mM HFIP in H_2O B: 8mM TEA:200mM HFIP in 1:1 H_2O:MeOH	2x150mm, 5 µm PS Phenomenex LUNA C18	
Various modified ASO's (Kaczmarkiewicz, Nuckowski et al. 2019)	LLE w/ phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v)	Shimadzu LCMS 8050	A: 5mM amine/150mM HFIP B: MeOH	Kinetex C18 Kinetex C8 Kinetex F5 Syncronis aQ	167.1-768.66 µg/mL
22-mer RNA (miR-451) (Basiri, Sutton et al. 2019)	Proteinase K digestion followed by magnetic bead extraction	Waters Acquity UPLC - Waters SYNAPT G2 HDMS qTOF	A: 15mM dibutylamine, 25mM 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol B: 95% MeOH	Waters Acquity UPLC BEH C18 column (1.7 µm, 1.0 x 100 mm)	0.5ng/mL

Table 2.3: Summary of Sample Prep and LC conditions of Metabolite Identification

Oligo	**Sample Prep**	**Mobile Phase**	**Column**
2x 20-mer phosphorophioate oligodeoxynucleotides (Gaus, Owens et al. 1997)	Proteinase K digestion w/ LLE Purified w/ Poros HQ/M anion exchange column desalted w/ SPEC C18 columns	A:H_2O w/ 5mM tripropylamine B: 3:2 2-propanol/ water w/ 20mM tripropylamine	ODS PVA-C18 (1mm x 100mm, Keystone Scientific)
20-mer phosphorothioate oligodeoxynucleotide (Griffey, Greig et al. 1997)	LLE phenol/ chloroform Purified w/ Poros HQ/M anion exchange column desalted w/ Poros II R/H column	0.4M HFIP pH 7.0 w/ trimethylamine and a linear gradient of 20-60% MeOH over 45 min	Intertsil 5 µm C18 (150 x 1.0mm, Micro-Tech)
20x 6-20 - mer phosphorothioate oligodeoxynucleotide, 2 gapmers (Crooke, Graham et al. 2000)	SPE w/ strong anion exchange column (J&W Scientific) desalting w/ Isolute C18(EC) column (International Sorbent Technology)	A:H_2O w/ 5mM tripropylamine B: ACN w/ 20mM tripropylamine	Poros R2/H (2.1 x 30mm, Perspective Biosystems)
21 & 23 mer siRNA (Beverly, Hartsough et al. 2006)	Ocular fluid- LLE Retina/choroid - modified procedure using Mirvana RNA extraction kit (Ambion Inc.)	A:H_2O w/ 200mM HFIP & 8.15mM triethylamine pH 7.9 B:50:50 H_2O:MeOH w/ 200mM HFIP & 8.15mM triethylamine	XTerra MSC18, 3.5 µm (2.1× 150 mm, Waters)

23-mer siRNA (Zou, Tiller et al. 2008)	Phenol/Choloroform LLE w/ SP HLB cartridges	A:H_2O w/ 100mM HFIP & 1.7mM triethylamine pH 7.5 B:MeOH	XBridge OST C18 column 2.5 µm (2.1 x 50 mm, Waters)
21-mer siRNA duplex containing DNA overhangs (McGinnis, Cummings et al. 2013)	Proteinase K digestion followed by a modified Trizol extraction (LLE w/ phenol/cholorform)	A:H_2O w/ 10mM DIPA & 25mM HFIP B:50:50 H_2O:MeOH w/ 10mM DIPA & 25mM HFIP	Acquity BEH C18 1.7 µm (2.1 x 50mm)
13 & 16-mer GalNAc moiety AS oligonucleotides (Husser, Brink et al. 2017)	SPE w/ OASIS HLB cartridges	A: H_2O/MeOH/HFIP/DIPEA 90/10/1/0.1 B: H_2O/MeOH/HFIP/DIPEA 10/90/1/0.1 (98mM HFIP & 5.8mM DIPEA)	Trapping: Xbridge BEH C4 5 µm (300 µm x 50mm) Analytical: Xbridge BEH C18 5 µm (300 µm x 50mm)
33-mer phosphorothioate w/ 2′-O-methyl ribose (Kim, Basiri et al. 2019)	Proteinase K digestion followed by SPE w/ Clarity OTX or magnetic beads	A: 15mM DMCHA:25mM HFIP in 5% MeOH B: 95% MeOH	Phenomenex Clarity 2.6-µm Oligo-XT 100 Å, 2.1 × 100-mm

Table 2.4: Summary of MS Conditions for Metabolite Identification

Oligo	**Instrument**	**MS Parameters**	**Metabolites**
2x 20-mer phosphorophioate oligodeoxynucleotides (Gaus, Owens et al. 1997)	5989 quadrupole MS (Hewlett Packard)	Negative Ion Mode *m/z* 550-1200	3′ & 5′ metabolites ranging n-1 to n-9 (rat plasma, liver & kidney)
20-mer phosphorothioate oligodeoxynucleotide (Griffey, Greig et al. 1997)	LCQ quadrupole ion trap (Finnigan MATT)	Negative Ion Mode *m/z* 600-1850	3′ & 5′ metabolites ranging n-1 to n-8 (pig kidney)
20x 6-20 - mer phosphorothioate oligodeoxynucleotide, 2 gapmers (Crooke, Graham et al. 2000)	Finnegan LCQ quadrupole ion trap mass spectrometer (ThermoQuest Corporation)	Negative Ion Mode *m/z* 600-1200	3′ & 5′ metabolites of various sizes (rat liver)
21 & 23 mer siRNA (Beverly, Hartsough et al. 2006)	Sciex API 365 triple quadrupole mass spectrometer (Applied Biosystems)	Negative Ion Mode *m/z* 800-1900	3′ & 5′ metabolites ranging n-1 to n-11 (rabbits eyes - ocular fluid, retina/choroid)
23-mer siRNA (Zou, Tiller et al. 2008)	Finnigan LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific)	Negative Ion Mode *m/z* 500-2000	3′ metabolites ranging n-1 to n-12 (human and rat serum and microsomes)

21-mer siRNA duplex containing DNA overhangs (McGinnis, Cummings et al. 2013)	Synapt G2 HDMS qTOF (Waters)	Negative Ion Mode *m/z* range not stated	3′ & 5′ metabolites ranging n-1 to n-4 (human PC-3 prostate cancer cells)
13 & 16-mer GalNAc moiety AS oligonucleotides (Husser, Brink et al. 2017)	Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific)	Negative Ion Mode *m/z* 80-1800 Resolution 80-1800	3′ & 5′ metabolites ranging n-1 to n-7 & loss of GalNAc (rat hepatocyte)
33-mer phosphorothioate w/ 2′-O-methyl ribose (Kim, Basiri et al. 2019)	Synapt G2 HDMS qTOF (Waters)	Negative Ion Mode *m/z* range not stated	3′ and 5′ metabolites, mouse liver & lung, monkey liver & lung

CHAPTER 3

CURRENT STATE OF OLIGONUCLEOTIDE CHARACTERIZATION USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY: INSIGHT INTO CRITICAL ISSUES[2]

[2] J. Michael Sutton, Guilherme J Guimaraes, Vidya Annavarapu, William D. van Dongen and Michael G Bartlett. 2020. *Journal of the American Society for Mass Spectrometry*. 31(9). 1775-1782.

Abstract:

As interests increase in oligonucleotide (OGNs) therapeutics, there has been a greater need for analytical techniques to properly analyze and quantitate these biomolecules. This article looks into some of the existing chromatographic approaches for oligonucleotide analysis, including anion exchange, HILIC, and ion pair chromatography. Some of the key advantages and challenges of these chromatographic techniques are discussed. Colloid formation in mobile phases of alkylamines and fluorinated alcohols, a recently discovered analytical challenge, is discussed. Mass spectrometry is the method-of-choice to directly obtain structural information about oligonucleotide therapeutics. Mass spectrometry sensitivity challenges are reviewed including comparison to other oligonucleotide techniques, salt adduction and the multiple charge state envelope. Ionization of oligonucleotides through the charge residue model (CRM) and the ion evaporation model (IEM) are analyzed. Therapeutic oligonucleotides have to undergo approval from major regulatory agencies, the impurities and degradation products must be well characterized to be approved. Current accepted thresholds for oligonucleotide impurities are reported. Aspects of the impurities and degradation products from these types of molecules are discussed, as well as optimal analytical strategies to determine oligonucleotide related substances. Finally, ideas are proposed on how the field of oligonucleotide therapeutics may improve to aid in future analysis.

Introduction:

Oligonucleotide (OGNs) therapeutics are an exciting new class of treatments for human disease, with more than ten now approved and dozens more in clinical trials. They operate through a variety of molecular mechanisms designed to interfere with the processing of genetic

information. Oligonucleotide therapeutics may be composed of either a single antisense strand or can be double stranded with both sense and antisense strands. The individual strands tend to be between 13-30 nucleotides, although there is no maximum size. To increase their resistance to catabolism and avoid *in vivo* cleavage at the phosphodiester linkage, improve distribution and also to increase their potency, there are a wide variety of chemical modifications introduced into these structures. These modifications can occur on the base, sugar or the phosphate backbone. In order to enhance the stability of oligonucleotides upon administration to the body, the phosphate groups in the backbone are often replaced by phosphorothioate (PS) groups or morpholino moieties (Tamm, Dorken et al. 2001, Li, El Zahar et al. 2018). Methylations of the bases (particularly cytosine) and modification of the 2' hydroxyl position of the ribose can also increase nuclease resistance (Wilson and Keefe 2006, Mansoor and Melendez 2008, El Zahar, Magdy et al. 2018):

- 2′-fluoro-nucleic acids
- 2′O-Me-nucleic acids (OMe)
- 2′-O-(2-methoxy) ethyl (MOE)-nucleic acids
- locked nucleic acids at the 5′ and 3′ ends
- morpholino oligonucleotides where the ribose sugar moiety is replaced with morpholine rings and the anionic phosphodiester linkage is replaced with nonionic phosphorodiamidate groups

One of the more recent strategies has been the addition of carbohydrate targeting groups onto one of the ends of an oligonucleotide strand. All of these potential chemical alterations result in a significant degree of complexity in the final structures of these molecules. These

molecules can also have higher-order structure. In the case of aptamers, this is part of their main mechanism of action. Due to these features, oligonucleotide therapeutics are highly specific and have significant potency toward their targets. The impact of secondary structures are generally minimized during analysis by using elevated chromatographic temperatures to prevent the formation of double strands, loops or hairpins.

Oligonucleotides are commonly analyzed through hybridization techniques such as ELISA (enzyme-linked immunosorbent assay and PCR (polymerase chain reaction) (Wang, Meng et al. 2013). These techniques have greater sensitivity over mass spectrometry (MS), but they lack in specificity. Mass spectrometry is required to analyze impurities, degradants and other biological/chemical modifications that are invisible to hybridization techniques. Mass spectrometry techniques often utilize liquid chromatography (LC) on the front end of the analysis to separate the unique molecules from one another. Historically, the mobile phases for LC alkylamine and acetate counterion resulted in unsatisfactory sensitivity. Recently, advances in the mobile phase to include alkylamines and fluorinated alcohol counterions have resulted in significant improvements in sensitivity. However, these new mobile phases suffer some mechanistic drawbacks.This critical insight aims to review and propose alternative retention mechanisms involved in LC-MS analysis of oligonucleotides, to evaluate current challenges involved in their MS analysis, and to report optimal approaches to determine oligonucleotide impurities. The current status of accepted thresholds for oligonucleotide impurities are discussed.

Ion-Pair Chromatography of Oligonucleotides:

Ion-pair reversed phase chromatography (IP-RP) has become the LC method-of-choice for OGNs. Mobile phases used for chromatographic separation of oligonucleotides initially

consisted of combinations of alkyl amines with corresponding carboxylic acid counter ions (Fritz, Belagaje et al. 1978). These organic carboxylic acids were superseded by hexafluoroisopropanol (HFIP) which had ideal physiochemical properties for use in LC-MS assays. It is quite volatile and also due to its pKa is ionized at the high pH values typically used and therefore serves well as a counter ion. Following this change there has been significant research done with respect to the choice of the alkylamine. The various alkylamines confer differing amounts of hydrophobicity to the oligonucleotides. Therefore, the extent of chromatographic retention can be modulated by varying the alkylamine used. To this day, the combination of alkyl amines and fluoroalcohols remain the most widely used mobile phase composition. While there are many choices of ion pairs, it has been proposed that the best choice is highly dependent on the composition of the oligonucleotides being studied. The best choice of fluoroalcohol appears to be dependent on the choice of alkylamine (Basiri, van Hattum et al. 2017).

One of the unique challenges of using LC-MS to analyze oligonucleotides is the stability of the mobile phases. However, this is only hinted at in most studies which usually note the need to remake mobile phases, often daily. This is somewhat unexpected because this has not previously been noted when using alkylamines for ion-pair chromatography. In fact, the tributylamine mobile phase, lacking HFIP, used for oligonucleotide impurity analysis by Roussis et al. at Ionis appears to be quite stable (Roussis, Pearce et al. 2019). It appears that the combination of the alkylamine and fluoroalcohol creates an outstanding, yet transient environment for conducting high quality LC-MS.

When alkylamines are used in combination with fluorinated alcohols, the general principle of separation has been that the alkylamine acts as an ion-pairing agent by both

shielding the negatively charged phosphate backbone making the analyte more hydrophobic and modifying the stationary phase to introduce an additional ionic retention mechanism. The fluoroalcohol has also been noted to improve the distribution constant of the oligonucleotides between the mobile phase and stationary phase (Apffel, Chakel et al. 1997). Recent studies have revealed that this mobile phase is far more complex than has been previously understood. Studies by Li et al. have shown that at relatively low concentrations of the alkylamine (below 10 mM) there is a linear increase in retention with increasing alkylamine concentration (Li, El Zahar et al. 2018). However, between 20-100 mM there is a massive decrease in analyte retention. The change in retention was also found to correlate with the solution pH. The higher the pH the lower the retention. Solution pH adjustments using formic acid were found to restore analyte retention. While this is useful for demonstrating the impact of pH on retention, formic acid along with many other common organic acids, cause massive ion suppression in LC-MS of oligonucleotides. Therefore, there appears to be a need to find ways to adjust mobile phase pH while maintaining high electrospray desorption efficiency. The current solution to this issue has been to use a relatively low concentration of alkylamine (15-25 mM) with 100 mM of the fluoroalcohol. Fluoroalcohols such as HFIP and HFMIP have a pKa around 8, which means they behave as weak acids and have a positive impact on the overall pH of the mobile phase.

Proposed Ion-Pair Chromatographic Micelle Mechanism:

Further analysis of the alkylamine / fluoroalcohol mobile phase system was conducted using transmission electron microscopy, which observed that there are micelles or colloids formed as the ion pairing reagent's concentration and the solution temperatures are raised (El Zahar, Magdy et al. 2018). The positive charge on these alkylamine-containing colloids appear to

decrease the interaction between the oligonucleotides and the stationary phase. It does appear that the fluoroalcohol plays a role in colloid formation, but the mechanism of this interaction is not well understood. However, the aging of the mobile phases appears to be well connected to the time dependent formation of these colloids and therefore methods to eliminate this issue would be of significant value.

There are many common practices that are frequently used in the making of mobile phases. A few of the important ones include preparing them fresh, checking the pH value and using clean containers and lab equipment. Apart from these, it has been seen that lower pH values have less micelle formation, making the mobile phase more stable and prevent an unwanted loss of retention (Li, El Zahar et al. 2018).

Considerable care is required in choosing the types of solvents and additives to be used in mobile phases; this diminishes the chances of ion suppression of the additives/solvents that have a relatively high proton affinity. Studies at Ionis have suggested that removing the fluoroalcohol from the mobile phase has eliminated some of these issues. (Roussis, Pearce et al. 2019). This suggests that segregation of fluoroalcohols from alkylamines may in fact prove beneficial since the highest sensitivities are obtained when using optimized alkylamines and fluroalcohols. The role of the organic solvent has also not been fully explored with respect to mobile phase optimization. To date most studies have used methanol, since the fluoroalcohols have the greatest solubility in this solvent. Earlier work by Chen et al. showed that using ethanol provided twice the LC-MS response of methanol and work by Moderna has used acetonitrile (Chen, Mason et al. 2013, Jiang, Yu et al. 2019). It is unclear how using these solvents might impact the formation of micelles or colloids and perhaps this is part of the reason that these solvents have shown promise in limited use (El Zahar, Magdy et al. 2018).

Alternative Chromatographic Approaches:

Alternative chromatographic methods such as HILIC and anion exchange have been used in the analysis of oligonucleotides. HILIC methods provide different selectivity and in some applications, have shown similar performance to IP-RP methods (Lobue, Jora et al. 2019). HILIC methods are trying to address one of the major limitations of IP-RP methods which is eliminating the need for the alkylamines, which create significant carry-over ion suppression if there is a need to run in positive ion mode. However, to date, the HILIC conditions to surpass the performance of IP-RP methods have yet to be found. Even under the current state of knowledge, HILIC methods should still be pursued since its selectivity may be better suited for a particular application. Overall, HILIC methods for oligonucleotide analysis remains new with a myriad of unexplored possibilities that could make HILIC an appealing alternative to IP-RP methods.

Anion exchange methods have shown some of the most efficient separations of oligonucleotides. Results showing baseline separation of four 21 mer oligonucleotides defy the idea that anion ion exchange works purely as a function of the number of charges and bases contained within an oligonucleotide (McGinnis, Cummings et al. 2013). While this method provides great separation, the mobile phase is incompatible with mass spectrometry due to the abundant use of non-volatile salts. There have been studies showing the implementation of two-dimensional analysis where the use of a trap column allows for the mobile phase to be exchanged, therefore enabling mass spectrometry implementation (Husser, Brink et al. 2017, Ahmad, Blasko et al. 2018). Overall, there appear to be yet unexplored secondary interactions with polymeric ion exchange phases that may be capable of being further exploited for even greater separation power (Biba, Jiang et al. 2013, El Zahar, Magdy et al. 2018).

IP Method Optimization - Impact on Mass Spectrometry Performance:

Optimization of ion-pair chromatography has a direct impact in the mass spectrometry performance. Historically, reversed phase ion-pair utilizing a base (alkylamine) and acidic counter ion are used for oligonucleotide analysis. The incompatibility of acetate counterion with MS is the driving factor for the switch to fluoroalcohol based mobile phase combinations becoming the most popular mobile phases for oligonucleotide analysis. Acetate mobile phases provide better chromatographic resolution when compared to HFIP, but the decreased MS signal is a major drawback. Systematic optimization of fluoroalcohol and alkylamine combination have shown to increase sensitivity and even reduce the amount of cationic adduction (Chen and Bartlett 2013, Chen, Mason et al. 2013, McGinnis, Grubb et al. 2013, Basiri, Murph et al. 2017, Basiri, van Hattum et al. 2017, Sutton and Bartlett 2019). Matching the hydrophobicity of the alkylamine and the oligonucleotide being analyzed increased the MS sensitivity significantly (Basiri, Murph et al. 2017, Basiri, van Hattum et al. 2017). However, there are many other sensitivity challenges.

Mass Spectrometry Sensitivity Challenges:

Sensitivity is a challenge in utilizing mass spectrometry for oligonucleotide analysis. The best methods can accurately quantitate down to high picogram/low nanogram levels when compared to other hybridization techniques (ELISA, PCR, fluorescence) which can do high femtogram/low picogram levels. The advantage of mass spectrometry in comparison to these hybridization techniques, is the specificity of the measurement (Wang 2013). Hybridization techniques typically require amplification or an indirect measurement unlike mass spectrometry

which measures the oligonucleotide directly. The advantage of this is that amplification or indirect measurements can be skewed by measuring full length oligonucleotides as well as impurities & metabolites. For example, PCR & ELISA often utilize hybridization of a complementary strand but if there is a missing base on the strand that does not inhibit binding, then the oligonucleotide minus base is included in the measurement for the full-length oligonucleotide. In mass spectrometry this would not be the case because the mass of the oligonucleotide minus the base will be less than the full-length oligonucleotide and it is easier to differentiate what is being measured.

Electrospray ionization is used for oligonucleotides because of the ability to "softly" ionize biomolecules. However, electrospray creates a charge envelope that distributes total ion signal of the full-length oligonucleotide over at least 10+ charges which is again subdivided into isotopic distributions. Moreover, poly-anions like oligonucleotides, show a tendency to exchange the H^+ of the phosphate or phosphorothioates groups of the oligonucleotide backbone for Na^+ and K^+ which adds additional challenge for sensitive ESI-MS analysis of oligonucleotides (van Dongen and Niessen 2011, Basiri, Murph et al. 2017, Sutton and Bartlett 2019). Having multiple exchangeable H^+ in the OGN backbone leads to $[M–mH+mAlkali–nH]^{n–}$ exchange ions in negative-ion mass spectra. As a result of the H+–Alkali+-exchange, the multiply-charged oligonucleotide ions will be further dispersed among multiple cation-containing species with a m/z 22/n difference for sodium exchange and an m/z 38/n difference for potassium exchange (with n being the charge state of the ion). Taking the multiple charge, the isotopic distribution and the alkali exchange together means that the MS signal is distributed over at least 50+ channels.

Mass Spectrometry Ionization Considerations:

Ionization in electrospray for oligonucleotides has had a lack of clarity in regard to the mechanisms involved (Kebarle and Peschke 2000, Nguyen and Fenn 2007). It is generally accepted that the charge residue model (CRM), which states that there is one analyte per droplet until the droplet is so small that the biomolecule is ionized, is the mechanism of ionization (Fernandez de la Mora 2000, Hogan, Carroll et al. 2009). competing theory is the ion evaporation model (IEM) that states before a droplet gets too small for one analyte per droplet the analyte is desorbed (Iribarne and Thomson 1976, Fenn 1993) The chain ejection model (CEM) states that for an unfolded analyte one "tail" of the analyte can be charged and ejected from the droplet. The analyte is gradually ejected and through H^+ migration charged to be in equilibrium with the droplet, while simultaneously leaving the droplet (Metwally, Duez et al. 2018). The CEM may explain why unfolded oligonucleotides are highly charged upon ionization versus folded oligonucleotides. Oligonucleotides contain hydrophilic phosphate groups that tend to associate with water in the interior of the droplet, which is why the leading theory is the CRM; however, when you add an ion-pair you create an environment where the hydrophilic portion of the ion-pair interacts with the phosphate and the hydrophobic end of the ion-pair tends to move towards the droplets surface (Chen, Mason et al. 2013, Basiri, Murph et al. 2017, Sutton and Bartlett 2019). It is therefore plausible that if enough of the ion-pair is at the edge of the droplet, then the oligonucleotides could be close to the edge of a droplet and be desorbed via the IEM or through the CEM. Therefore, it is highly probable that desorption of oligonucleotides employs a combination of ionization mechanisms. Improved understanding of ways to advantageously exploit these mechanisms is a significant challenge which must be addressed to continue to improve ionization efficiency and therefore method sensitivity. One possibility to increase

sensitivity is increasing the amount of organic present, like with HILIC chromatography or organic vapor assisted electrospray (Weng, Liu et al. 2017). The organic is known to play an important role in the desorption of oligonucleotides by changing the organic or increasing the concentration of organic present (Chen, Mason et al. 2013).

MS Impurity/Related Substances Challenges:

Mass Spectrometry is the method-of-choice to directly obtain structural information from therapeutic oligonucleotides and its impurities. It is also indispensable for determining impurity levels. As several modified full-length impurities, the n-1 deletion and n+1 addition sequences (see also below at section synthesis impurities) cannot be baseline separated chromatographically, it is a challenge to quantitate these impurities at 0.2% (Table 3.1).
To distinguish these impurities with molecular masses close to its parent compound, high resolution mass spectrometry is essential. A classic example of this is the deamination impurity (see also below under section synthesis impurities) that converts the amine ($-NH_2$) group from either a cytosine base or a 5-methyl cytosine to a carbonyl and removes the double bond on the nitrogen and adds a hydrogen, which converts the base to uracil or thymine, respectively. This deamination is only a 1 dalton (Da) difference in mass.

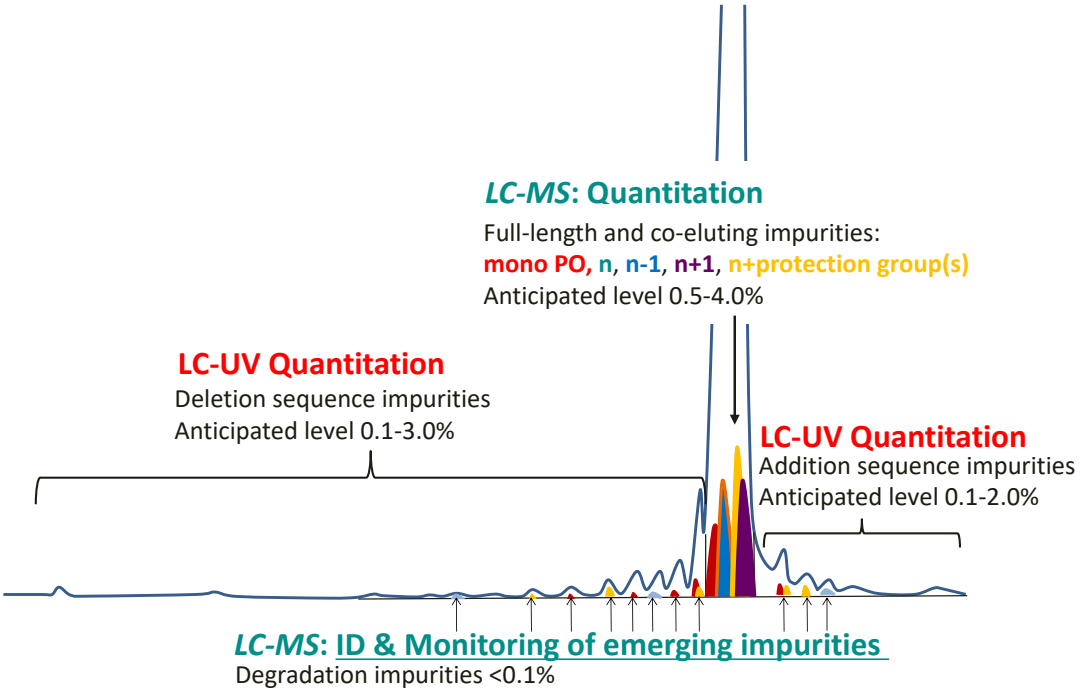


Figure 3.1: Analytical strategy to comprehensively determine related substances of therapeutic oligonucleotides using LC-UV-MS.

Optimal analytical strategy to determine oligonucleotide related substances:

Presently, the combination of IP-HPLC-UV-MS is required to obtain a full overview of all related substances of phosphorothioated oligonucleotides as illustrated in Figure 3.1. LC-UV (large blue outlined peak) is sufficient to determine deletion sequence impurities up to n-2, addition sequence impurities from n+2 and their related emerging impurities, LC-MS (colorful peaks inside the large blue UV peak) is required to determine the co-eluting impurities of the parent compound (n), i.e. mono PO, n, n-1, n+1, n+protection groups.

Table 3.1: Reporting, identification and qualification threshold for oligonucleotide impurities according to ICH guidelines, EU guidelines and leading oligonucleotide researchers.

	Reporting threshold	**Identification threshold**	**Qualification threshold**
ICH Q3A and Q3B	0.05%	0.10%	0.15% or 1 mg
Impurities in peptides European Pharmacopeia (Ph. Eur) 2034	0.1%	0.5%	1.0%
Oligonucleotide Community			
Capaldi (Capaldi, Teasdale et al. 2017	0.2%	1.0%	1.5%

Table 3.2: Concentration determination of ICH Q3A thresholds for oligonucleotide impurities from the given ICH 0.15% for small molecule vs two different length oligonucleotides

Impurity qualification threshold (Q3A)				
Type of molecule	MW (Da)	%	mg	uMol
Small molecule	700	0.15	1.0 mg	1.43
Oligonucleotide 18-mer	7000			0.14
Oligonucleotide 30-mer	11000			0.09

Reporting Thresholds for Related Substances of Therapeutic Oligonucleotides:

Currently there is no generally accepted reporting threshold for oligonucleotide therapeutics. Capaldi, et al. cautiously use a reporting threshold for related substances of therapeutic oligonucleotides based on small molecule and peptide drugs (Capaldi, Teasdale et al. 2017). As listed in Table 3.1, for most small-molecule drugs, the reporting threshold is set to 0.05% (ICH Q3A and Q3B) and for impurities in peptides set at 0.1% (Pharmacopeia). For setting the reporting threshold, it must also be taken into account that most oligonucleotide impurities comprise a mixture of several components, so the reporting threshold applies not to a single impurity, but rather to a group of several related components. Therefore, when the decision is made to disregard an impurity, the

individual components are present at only a fraction of the reporting threshold (Capaldi, Teasdale et al. 2017). Moreover, it is important to realize that therapeutic oligonucleotides have a relatively high molecular weight and thus, in comparison to small molecules, much less molecules per weight unit are present, as illustrated in Table 3.2. Based on the facts that related compounds are mixtures of relatively high molecular weight, a reporting threshold of 0.2% was considered to be attainable in the majority of cases (Capaldi, Teasdale et al. 2017).

Table 3.3: General scheme for oligonucleotide synthesis with possible side product impurities and their type of related substance

Step in synthesis	**Side product**	**Type of related substance**
Deprotection DMT	n-x (n-1, n-2, *etc.*)	Deletion sequences
	depurination	full length
Coupling	n+x (n+1, n+2, *etc.*)	Addition sequences
	impurity in amidite	+PS
Capping	n-x	Deletion sequences
Sulfurization	PO	full length
	DMT phosphonate	longmers (n+x)
	n-x	Deletion sequences
Final cleavage and deprotection	protecting group remaining (CNEt)	Addition sequences
	incomplete cleavage linker (Unylinker)	+PS

Synthesis Impurities:

Related substances of therapeutic oligonucleotides originate from the synthetic route and its chemical transformations, starting materials and its impurities and degradation routes (Capaldi, Teasdale et al. 2017).

To start with the first item, related substances of phosphorothioate oligonucleotides arising from synthesis and manufacturing can be generally subdivided in three classes.
Shorter chain length (deletion sequences); (ii) sequences of longer chain length (addition sequences) and (iii) modified full length impurities. An overview of the general synthesis impurities are provided in Table 3.3.

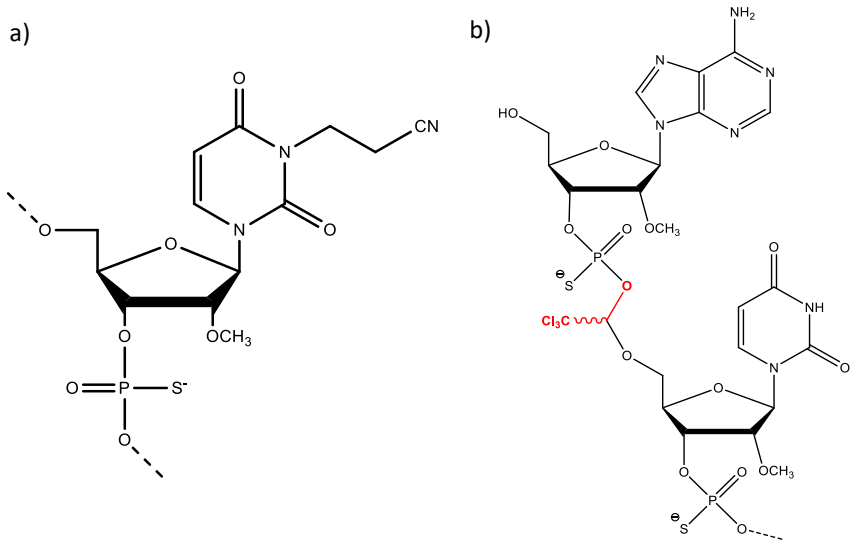


Figure 3.2: a) Structure of an internal N^3-(2-cyanoethyl)-2'-*O*-methyluridine residue. b) Structure of a modified internucleotide linkage caused by the presence of chloral in dichloroacetic acid and illustrated between a 2'-O-methyladenosine and a 2'-O-methyluridine.

Deletion sequences:

The most common impurities found in oligonucleotides include the n-1 deletion sequences (n-x) (Fearon, Stults et al. 1995, Temsamani, Kubert et al. 1995, Chen, Yan et al. 1999). Deletion sequences or also called shortmers typically arise from incomplete base coupling at the extending 5'-

end followed by a failed capping reaction. Any residual 5'-hydroxyl containing material is capped as an isobutyryl ester prior to the detritylation step and thus cannot participate in further chain elongation: n-x species are formed as a consequence. Incomplete capping can also lead to carryover of a deletion sequence that will be further chain extended and thus contributes to the so-called n-1 impurity, which is a mixture of small amounts of materials with different single internal deletions plus material arising from incomplete coupling in the final solid-phase synthesis cycle. Although thorough process development work leads to a robust scalable manufacturing process, nonetheless reactions do not all reach 100% and impurities generated by this non-perfect process accumulate throughout the synthesis. Fortunately, the use of anion-exchange purification enables removal of most of the shorter oligonucleotide impurities, whereas removal of n-2 and n-1 impurities is more challenging particularly for longer therapeutic oligonucleotides having a full phosphorothioate backbone. LC-MS analysis of process development batches as well as the nonclinical and clinical batches of DS and stability samples show, in many cases, measurable levels of n-x impurities down to n-20.

Modified full length impurities:

Capaldi et al. (Capaldi, Teasdale et al. 2017) listed several impurities from modification at a single internucleotide linkage, i.e. phosphate diester impurity in phosphorothioate diester oligonucleotides (Bergot and Egan 1992), trichloroacetaldehyde modified oligonucleotides (Gaus, Olsen et al. 2005), C-phosphonate oligonucleotides (Capaldi, Gaus et al. 2004), ethylene phosphodiester impurities (Rentel, Wang et al. 2005) and phosphorodithioate diester impurities in phosphorothioate diester oligonucleotides (Capaldi DC, 2014, EuroTides, Berlin, DE).

Modified full length impurities have the correct length but contains one or more modified nucleobases or phosphorothioate linkages.

Amongst others also several impurities with modified nucleobases were listed by Capaldi (Capaldi, Teasdale et al. 2017):

- Oligonucleotides containing a urea- or aminofuran residue in place of a pyrimidine nucleotide (Hill KW, 2007, DIA Conference, Washington, DC). Oligonucleotides containing a spiroiminodihydantoin (Fleming, Muller et al. 2012) (Hill KW, 2007, DIA Conference, Washington, DC) or isobutyryldiaminopurine in place of guanine (Rodriguez, Cedillo et al. 2014, Rodriguez, Cedillo et al. 2016), 5-amino-4-pyrimidinylimidazole in place of adenine (Lorenz S 2009, Rodriguez, Cedillo et al. 2016) and oxopropylimidazopyrimidinone in place of cytosine (Rentel, Wang et al. 2005).
- Addition of acrylonitrile on the N^3 of thymine (Capaldi, Gaus et al. 2003) or uridine (N+CNEt, characterized by a +53 Da peak in mass spectra) during deprotection, see Figure 3.2a above for uridine.
- Incomplete deprotection of the N^2-isobutyryl groups on the guanine residues (characterized by a +70 Da peak in mass spectra).
- N+isobutyryl and N+benzoyl denotes incompletely deprotected species that contain a residual N^2-isobutyrylguanosine and a residual N^6-benzoyladenosine respectively. N^2-isobutyrylguanosine can be subsequently conversed into an acetyl-modified diaminopurine moiety or an N^2-acetyl-2,6-diaminopurine impurity during the capping reaction (Gaus, Olsen et al. 2005, Rodriguez, Cedillo et al. 2014).
- The presence of trace amounts of trichloroacetaldehyde (chloral) in dichloroacetic acid used in the detritylation steps in the solid-phase synthesis can lead to a full-length oligonucleotide

impurity that is a family of species each with a single different modified internucleotidic linkage and observed as a +148 Da peak in the MS. The modified linkage is illustrated in Figure 3.2b.

- Among the other classes of impurities, several different types can occur at the base residues, such as deamination or depurination of adenine, cytosine, or guanine residues, which can be induced by thermal, basic, and acidic conditions (Capaldi, Teasdale et al. 2017, Roussis, Pearce et al. 2019) ; and iii) oxidation, primarily occurring at guanine residues (Fleming, Muller et al. 2012, Capaldi, Teasdale et al. 2017).
- A special category of modified full-length impurity is represented by a variant that arises by incomplete sulfurization or/and possible side reactions related to the reagent used (Xu, Musier-Forsyth et al. 1996), or partial oxidation during the solid-phase synthesis or by partial desulfurization during the deprotection step (Bergot and Egan 1992). This leads to a mono phosphodiester variant (mono PO) of phosphorothioate oligonucleotides, characterized by n-16 Da peak in the MS and comprises a mixture of n (n = number of nucleotide residues in oligo) individual species. This variant can be (partially) resolved from the full phosphorothioate species by analytical anion-exchange HPLC (IEX-HPLC). On IPRP-HPLC this species conveniently coelutes with the non-modified species and is classified as part of the active API. Phosphorus NMR spectroscopy gives an estimate of the level of the mono PO variant.

Addition sequences:

Addition sequences (n+x) or longmers are produced during the coupling step, whereby the incoming phosphoramidite species is added twice during a single coupling step. Addition sequences

are mostly restricted to n+1 and a small amount of n+2 (Krotz, Klopchin et al. 1997). Since these impurities elute just after the full-length product, they are, depending on their length, less or more difficult to remove by anion-exchange chromatography.

Degradation impurities:

As mentioned above, chemically modified oligonucleotides are usually stable at standard *in vitro* and *in vivo* conditions (El Zahar, Magdy et al. 2018). The most observed degradation impurities during shelf life studies, are mono PO oxidation products of n and deletion and addition sequences, that can already present after solid-phase synthesis (Bergot and Egan 1992). This was confirmed by stress testing with hydrogen peroxide, where oxidation of the phosphorothioate linkage PO analogs was determined to be the main degradation pathway. At higher concentrations of hydrogen peroxide also deletion sequences, depurination products, terminal phosphorothioates, ribose, ribophosphorothioates, and phosphoribophosphorothioates were observed (El Zahar, Magdy et al. 2018, Pourshahian).

Several forced degradation studies have been performed to determine the stability of oligonucleotides at different pH conditions (Calvitt, Levin et al. 2010, DC 2011, El Zahar, Magdy et al. 2018, Pourshahian). For four phosphorothioate oligonucleotides (1 deoxyribose, and 3 with MOE 2' ribose chemistry and methyl cytosine modifications) was determined that at acidic pH values significant degradation was observed. For pH 1.5 within the one hour all full-length products were completely degraded, whereas for pH 3.0 degradation took more than 48 h (El Zahar, Magdy et al. 2018). For pH 5.0, 7.0, 9.0, and 11.0, the full-length products were degraded by less than 20% within 48 h for all four oligonucleotides (El Zahar, Magdy et al. 2018). Acid treatment of DNA oligonucleotides resulted in loss of adenine and guanine followed

by the addition of water (Pourshahian). In contrast, acid hydrolysis of oligonucleotides containing a mixture of deoxy-, and 2′-MOE or 2′-OH and 2′-modified nucleotides (2′-OMe, 2′-F) at pH 2–3 at room temperature during several hours did not result in the cleavage of the inter-nucleotide linkages (Calvitt, Levin et al. 2010, DC 2011). This was confirmed by Pourshahian *et al*, who demonstrated that 2′-OH, 2′-MOE, 2′- OMe, and 2′-F modified residues made oligonucleotides resistant to acid treatment (Pourshahian).

El Zahar et al. observed that the sequence of oligonucleotides may have an impact on the rate of the degradation; both a higher pyrimidine content and the presence of sequences of AT, increased the rate of degradation (El Zahar, Magdy et al. 2018). Finally, Shishkina and Johnson reported that oligonucleotides are susceptible to degradation during evaporation to dryness (Shishkina and Johnson 2000).

Conclusions:

To properly analyze therapeutic oligonucleotides a combination of the techniques and strategies proposed here must be used. Adequate chromatographic resolution and MS sensitivity must be achieved in order to quantitate the impurities mentioned above. The constant tradeoff between chromatographic resolution and mass spectrometry sensitivity remains a big challenge in LC-MS analysis of oligonucleotides. Ion pair chromatography has been the most popular technique, but alternative approaches such as anion exchange, and HILIC methods have presented unique results for specific applications.

Here we describe several of the issues with mass spectrometry related to oligonucleotides. There is a dire need for increased sensitivity and resolution for routine analysis. Perhaps different mobile phase compositions can accomplish this without need for significant

instrumental improvements. Mobile phases can influence the charge state distribution, salt adduction and the major ionization pathway.

CHAPTER 4

MODELING CATIONIC ADDUCTION OF OLIGONUCLEOTIDES USING ELECTROSPRAY DESORPTION IONIZATION[3]

[3] J. Michael Sutton and Michael G Bartlett. 2020. Rapid Communications in Mass Spectrometry. 34. Reprinted with permission of the publisher.

Abstract

RATIONALE Cationic adduction causes poor sensitivity and increases spectral complexity during mass spectral analysis of oligonucleotides and alkylamines are used to reduce this adduction. It is unclear the effect of the physiochemical properties of the alkylamines on the reduction of the cationic adduction.

METHODS All samples were directly infused into a Waters (Milford, MA, USA) Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer in negative ion electrospray ionization mode through the native built-in fluidics system. The infusion flow rate was set to 50 μL/min. The TOFMS tuning parameters were as follows: capillary voltage −2.0 kV, cone voltage 25 V, extraction cone voltage 2 V, source temperature 125 °C, desolvation temperature 450 °C, cone gas 0 L/h, and desolvation gas (nitrogen) 1000 L/h.

RESULTS A quantitative model was created to predict the optimized alkylamine for MS analysis, while a qualitative model was generated to explain the most important physiochemical properties: proton affinity (13.83%), gas-phase basicity (11.79%), pKa (11.47%), boiling point (10.73%), MW (10.3%), Henry's Law Constant (9.56%), and partition coefficient (logP) (9.44%). The quantitative model was applied to RNA (microRNA) and a phosphorothioate and predicts the trend of cationic adduction.

CONCLUSIONS Two models are described to understand the physiochemical properties that contribute to the adduction and to provide users a quick mathematical tool to predict the best choice of alkylamine to lower cationic adduction and decrease spectral complexity while enhancing sensitivity.

Introduction

Bioanalysis has been advanced immensely with the invention of electrospray ionization mass spectrometry (ESI-MS) making the study of large biomolecules more routine (Fenn, Mann et al. 1989, Erb and Oberacher 2014). The study of the electrospray process, especially in biomolecules, has been an on-going investigation with many of the important tenets being described by Richard Cole in 2000 (Cole 2000). These tenets help to shape our understanding of the physiochemical properties of the electrospray solution and provide a rationale for increasing sensitivity and decreasing the complexity of spectra. Salt solutions can be useful in biological applications to study secondary structure of natively sprayed oligonucleotides (Ganem, Li et al. 1993, Light-Wahl, Springer et al. 1993, Kenderdine, Xia et al. 2018). While this provides useful structurally relevant biological information, cationic adduction caused by these salt solutions is generally undesirable due to the significant decrease in signal sensitivity and the increased spectral complexity.

To increase sensitivity of oligonucleotide analysis, charge state reduction has been one main focus to improve signal complexity and improve detection limits (Null, Nepomuceno et al. 2003), (Cheng, Bakhtiar et al. 1994), (Muddiman, Cheng et al. 1996). Reduction in the charge state leads to a reduction in the charge state envelope which pushes the signal to a smaller number of charge states and boosts their intensity. Adduction plays a similar role as a wide range of charge states where the signal can be spread out from the molecular peak over multiple cationic adducted peaks. There have been many attempts to address this from mobile phase additives to decrease the charge state and the extent of adduction, to on-line sample clean-up and desalting (Potier, Van Dorsselaer et al. 1994, Limbach, Crain et al. 1995, Gaus, Owens et al. 1997). Most of these techniques require extensive time and effort to reduce the cationic

adduction. Recently alkylamine ion-pair chromatography has largely replaced all other chromatographic methods for oligonucleotides. These alkylamines are commonly used during mass spectral analysis due to their ability to reduce the charge

Due to their superior performance over other methods, alkylamines have recently been employed in a modelling attempt to help explain their behavior (Basiri, Murph et al. 2017).It has been shown that even among similar ion-pairs there is a large amount of variability in the ESI response. There is debate about which alkylamine is the best choice: Chen and Bartlett have used N,N-diisopropylethylamine (DIEA) (Chen, Mason et al. 2013); Gong, et al. have used dibutylamine (DBA), dimethylbutylamine (DMBA), hexylamine (HA) and tripropylamine (TPA) (Gong and McCullagh 2011); Oberacher, et al. used N,N-dimethylcyclohexylamine (DMCHA)(Erb and Oberacher 2014); McGinnis and Bartlett used diisopropylamine (DIPA) (McGinnis, Grubb et al. 2013); Sharma, et al. used ,8- diazabicyclo[5.4.0]undec-7-ene (DBU (Sharma, Glick et al. 2012); Griffey, et al. and Bleicher, et al. used triethylamine (TEA) (Bleicher and Bayer 1994, Greig and Griffey 1995). An interesting observation by Basiri, et al. was that the ion-pair hydrophobicity should match the hydrophobicity of the additional mobile phase additives as well as the oligonucleotide itself (Basiri, Murph et al. 2017).

Combinations of offline and online methods have been used to address the adduction problem in oligonucleotide analysis including solid phase extraction cartridges (e.g. Oasis HLB, C18 ZipTip), chromatographic methods employing ion-exchange and hydrophobic techniques, molecular weight cutoff filters, dialysis and the addition of mobile phase modifiers like ion-pairs with fluorinated alcohols (Greig and Griffey 1995, Muddiman, Cheng et al. 1996, Apffel, Chakel et al. 1997, Gilar and Bouvier 2000, Ragas, Simmons et al. 2000, Gilar, Belenky et al. 2001, Jiang and Hofstadler 2003, Oberacher, Parson et al. 2004, Erb and Oberacher 2014).

Unfortunately, many of these techniques do not account for residual cations that can stem from glassware, plastic, mobile phase buffers and even the LC or MS system. It is therefore advantageous to use a method that is both quick and can address the reduction of residual cations in the mobile phase directly. The alkylamine ion-pairs provide a good solution to the problem due to their use in the separation phase of oligonucleotide analysis and their ability to suppress cationic adduction as suggested by Basiri & coworkers (Basiri, Murph et al. 2017).

The mechanisms of how larger biomolecules are ionized has been debated with two major competing theories, the charge residue model (CRM) and the ion evaporation model (IEM) (Kebarle and Peschke 2000, Nguyen and Fenn 2007, Hogan, Carroll et al. 2009). The CRM states that a charged droplet will transfer its charge to the macromolecule with the maximum charge before the molecule fissions out upon reaching the Rayleigh charge limit (Fernandez de la Mora 2000, Hogan, Carroll et al. 2009). These droplets will then break apart until ultimately there is only one analyte molecule per droplet. The IEM states that before the droplet can get too small the charge at the surface of the droplet causes the analyte to desorb (Iribarne and Thomson 1976, Fenn 1993). While the consensus is that small molecules are ionized primarily by IEM and biomolecules primarily by CRM, there is still no clear theory or model to fully explain the behavior of biomolecules in ESI-MS. This makes it difficult to predict how molecules will desorb and therefore difficult to determine to what extent this might affect adduction.

Alkylamine ion pairs play a variety of roles in the analysis of oligonucleotides including altering the properties of the ESI solution to increase ionization efficiency for MS. This improvement of the ESI signal has been shown to be variable and depend on the sequence of the oligonucleotide as well as the mobile phase properties. Many labs have tried to address these issues, but method development can still be time consuming and be dominated by trial and error.

Here we describe a series of experiments that have been used to model the effect of alkylamine physiochemical properties on the overall cationic adduction to address these challenges.

Materials and Methods

Chemicals and Reagents

The ion pairing agents dibutylamine (DBA), 1,8- diazabicyclo[5.4.0]undec-7-ene (DBU), N,N-diisopropylethylamine (DIEA), diisopropylamine (DIPA), N,N-dimethylbutylamine (DMBA), N,N-dimethylcyclohexylamine (DMCHA), N,N- dimethylhexylamine (DMHA), hexylamine (HA), N- methyldibutylamine (MDBA), octylamine (OA), propylamine (PA), tributylamine (TBA), triethylamine (TEA), tetramethylethylenediamine (TMEDA), tripropylamine (TPA), imidazole (IMI) and piperidine (PIP), as well as LC/MS grade methanol and water were purchased from Sigma- Aldrich Inc. (St. Louis, MO, USA). DNA Lobind microcentrifuge tubes were purchased from Eppendorf (Hauppauge, NY, USA). The following 24-mer ssDNA strands were purchased from Eurogentec (Seraing, Belgium): TTTTTTTTTTTTTTTTTTTTTTTT (100%T), CCCCCCCCCCCCCCCCCCCCCCCC (100%C), AAAAAAAAAAAAAAAAAAAAAAAA (100%A), ATTTCTTTGTTTATTTCTTTGTTT (75%T), ATTCTTGTTATTCTTGTTATTCTT (66%T), ATCTGTATCTGTATCTGTATCTGT (50%T), GTGTGTGTGTGTGTGTGTGTGTGT (50%G), TCGTACTAGTGGTCCTAATCGTAC (33%T), ATCGATCGATCGATCGATCGATCG (25%T), ACGACGACGTTTACGACGACGACG (13%T), CGGAGGAAACCTACGACGAG (4%T) and UGUAAACAUCCUACACUCUCAGC (miR-30c-5p). The following phosphorothioate modified RNA was obtained from Ionis Pharmaceuticals (Carlsbad, CA) TCCGTCATCGCTCCTCAGGG (Phosphorothioate DNA).

Working Solutions

The ion-pairing agents were used at a final concentration in aqueous solution of 15mM, except DBU which was 2.5mM. These were added to a newly made fresh solution of 50:50 methanol:water with the oligonucleotides at a concentration of 20 µg/mL. It has been found that oligonucleotides exhibit a large amount of non-specific binding unless DNA Lobind tubes (Hauppage, NY) are utilized (McGinnis, Cummings et al. 2013).

Instrumental Conditions

All samples were directly infused into a Waters (Milford, MA, USA) Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer in negative ion electrospray ionization mode via Intellistart, the native built-in fluidics system. The TOFMS tuning parameters were as follows: capillary voltage −2.0 kV, cone voltage 25 V, extraction cone voltage 2 V, source temperature 125 °C, desolvation temperature 450 °C, cone gas 0 L/h, and desolvation gas (nitrogen) 1000 L/h. The infusion flow rate was set to 50 µL/min and the data were collected in continuum full-scan MS mode with a 1 s scan time over the mass range from 500–3000 m/z. Measurements were performed in triplicate using the average of 50 scans and the sum of adduction peaks used to calculate the total adducted signal.

Databases and Computer Software

All MS operation, analysis and acquisition were performed on Waters (Milford, MA, USA) Masslynx 4.1 and Intellistart. Statistical analysis was performed using SAS JMP Pro 13 (Cary, NC, USA). Henry's law constants were computed using the HENRYWIN module of the US Environmental Protection Agency's EPI Suite ver. 4.11 (Washington, DC, USA). Proton affinity

and gas-phase basicity values were obtained from the NIST database (http://webbook.nist.gov/chemistry/). The remaining physiochemical properties were obtained from the CAS REGISTRY of the American Chemical Society (https://scifinder.cas.org/).

Results & Discussions

Alkylamines are routinely used as solution modifiers in oligonucleotide analysis due to their favorable properties for chromatography and also the enhancement of electrospray signals. Here we investigate the effect of alkylamine modifiers on the electrospray intensity of oligonucleotides and specifically their effect on cation adduct formation. We utilized 11 ssDNA strands of varying base compositions, Table 4.1, to account for the different nucleotides and 17 alkylamine modifiers, Table 4.2. The oligonucleotides were sprayed at a concentration of 20 μg/mL which provides robust and reproducible mass spectra. Previous studies in our lab showed that a 15mM concentration produces the best MS signal intensity (Basiri, Murph et al. 2017), except for DBU in which the optimal concentration of 2.5 mM produces the best MS signal intensity (Sharma, Glick et al. 2012). These parameters were chosen to ensure all spectra have adduction present so that this adduction can be modeled. The parameters were also chosen to reflect similar conditions used in standard LC/MS analysis with 2mm LC columns. In the experiments described the only variable is the alkylamine itself, all other conditions are held constant.

Table 4.1: Physiochemical properties of the 17 modifiers used in model of adduction

Ion - Pair	MW (g/mol)	Density (g/mL)	Boiling Point (°C)	pKa	Proton affinity (Kcal/mol)	Gas phase basicity (Kcal/mol)	Partition Coefficient ($logP_{oct/wat}$)	Water Solubility (g/L at pH=10)	Vapor Pressure (Pa)	Henry's Law Constant (H^{cp}, $\frac{mol}{m^3 Pa}$)
DBA	129.24	0.77	159	11.03	231.5	223.6	2.695	74	280	0.111
DBU	152.24	1.04	97	13.28	250.45	242.43	1.132	283	0.715	5.128
DIEA	129.24	0.74	127	10.5	237.6	230.3	2.354	87	1560	0.065
DIPA	101.19	0.72	83.9	11.05	232.3	224.3	1.364	235	10600	0.103
DMBA	101.19	0.72	95.9	10.02	231.6	224.2	1.647	51	6190	0.115
DMCHA	127.23	0.85	162	10.49	235.1	227.7	2.091	71	335	0.42
DMHA	129.24	0.74	147.1	9.99	231.5	223.5	2.666	13	771	0.065
HA	101.19	0.77	130	10.56	221.7	213.6	2.075	66	1180	0.368
MDBA	143.27	0.75	164.6	10.5	231.32	223.39	3.175	5.7	277	0.049
OA	129.24	0.78	179.6	10.65	221.86	213.77	3.094	17	129	0.012
PA	59.11	0.72	46.9	10.53	219.4	211.3	0.547	544	41500	0.667
TBA	185.35	0.78	216.5	10.89	238.6	231.3	4.704	0.67	12.5	0.062
TEA	101.19	0.73	89.7	10.65	234.7	227	1.647	155	7610	0.066
TMEDA	116.2	0.78	121	8.97	242.07	232	-0.037	688	2000	409.836
TPA	143.27	0.76	156	10.65	236.9	229.5	3.175	6.2	355	0.026
IMI	68.08	1.036	257	13.89	225.3	217.3	-0.099	57	32	2.625
PIP	85.15	0.8622	106	10.45	228.0	220.0	0.612	367	3773	2.217

DBA = dibutylamine DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene DIEA = *N,N*-diisopropylethylamine DIPA = diisopropylamine DMBA = *N,N*-dimethylbutylamine DMCHA = *N,N*-Dimethylcyclohexylamine DMHA = *N,N*-Dimethylhexylamine HA = hexylamine MDBA = *N*-methyldibutylamine OA = octylamine PA = propylamine TBA = tributylamine TEA = triethylamine TMEDA = tetramethylethylenediamine TPA = tripropylamine IMI = imidazole PIP = piperidine

Table 4.2: Oligonucleotides used in this study

Name	Backbone	Sequence (5' -> 3')	Length
100% A	ssDNA	AAAAAAAAAAAAAAAAAAAAAAAA	24-mer
100% C	ssDNA	CCCCCCCCCCCCCCCCCCCCCCCC	24-mer
100% T	ssDNA	TTTTTTTTTTTTTTTTTTTTTTTT	24-mer
75% T	ssDNA	ATTTCTTTGTTTATTTCTTTGTTT	24-mer
66% T	ssDNA	ATTCTTGTTATTCT TGTTATTCTT	24-mer
50% T	ssDNA	ATCTGTATCTGTATCTGTATCTGT	24-mer
50% G	ssDNA	GTGTGTGTGTGTGTGTGTGTGTGT	24-mer
33% T	ssDNA	TCGTACTAGTGGTCCTAATCGTAC	24-mer
25% T	ssDNA	ATCGATCGATCGATCGATCGATCG	24-mer
13% T	ssDNA	ACGACGACGTTTACGACGACGACG	24-mer
4% T		CGGAGGAAACCTACGACGAGGAAA	
miR-30C-2	RNA	UGUAAACAUCCUACACUCUCAGC	23-mer
Modified RNA	Phosphorothioate	TCCGTCATCGCTCCTCAGGG	20-mer

To account for the known ESI-MS signal intensity variability over the course of the several days under the same conditions, we utilized an Oligo/Ion-Pair (IP) normalization that has been previously characterized in our lab by Basiri (Basiri, Murph et al. 2017) and proposed by Leito & colleagues (Leito, Herodes et al. 2008, Oss, Kruve et al. 2010, Liigand, Kruve et al. 2015). This normalizes the oligonucleotide responses to one particular ion-pair, tetramethylethylenediamine (TMEDA). This simply becomes a ratio of the measured alkylamine, such as Octylamine (OA) divided by the normalizer TMEDA: OA/TMEDA. This allows us to easily compare results over the course of many days in the study due to a reduction in the MS signal intensity variability observed from ~20% down to 5%. In an attempt to understand how the alkylamines affect the ESI signal intensity, data was extracted from the most abundant charge

state and the adducted signal intensities were summed (eg: SA= [(M-)+1Na] +[(M-)+2Na] +….[(M-) +xNa]) . The molecular signal (M-) and the summed adduction signal (SA) were then normalized utilizing the TMEDA oligonucleotide normalization described above. To study the effect of sodium adduction on the overall quality of the mass spectrum in comparison to the molecular signal, the ratio of the M-/SA signal was calculated to provide a useful way to quickly visualize how much adduction is present in each spectrum and provide a useful numerical value that can be used to model the effect of each alkylamine on the mass spectra.

$$\frac{Normalized\ Molecular\ Peak\ Signal\ (M-)}{\Sigma Normalized\ Adduct\ Signal\ (SA)}$$

EQ 1: The normalization procedure used in the Partial Least Squares (PLS) and Bootstrap models. The Molecular signal is divided by the sum of all the adducted signals. The less adduction the higher the number for the model.

The result of utilizing 3 different common alkylamines during oligonucleotide analysis compared to no alkylamine (50:50 Water:Methanol) can be seen in Figure 4.1. There is a clear reduction in the sodium adduction in the spectra when the alkylamine choice is the only changed variable. It would be therefore be reasonable to conclude that this reduction in sodium adduction is directly related to the physiochemical properties of the added alkylamine. The spectra with no alkylamine added is so adducted with sodium that the main non-adducted peak is not present in the mass spectra. Signal intensity count was higher with octylamine (5x higher than TEA), then hexylamine (3x higher than TEA) and finally triethylamine. These alkylamines have different physiochemical properties and these can be examined to determine what properties are favorable to reduce the cationic adduction.

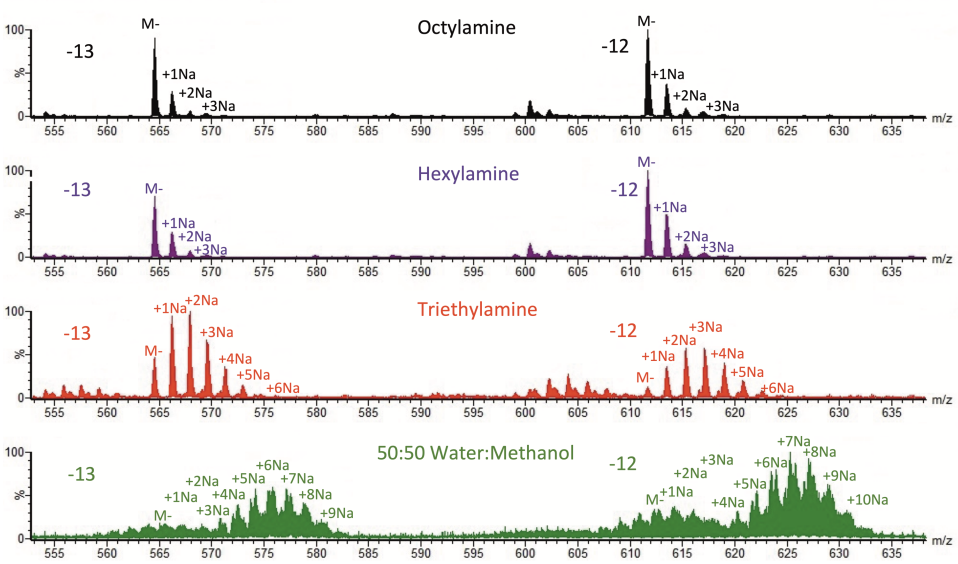


Figure 4.1: Representative chromatograms of 25% T ssDNA at m/z=-12 & -13 with 3 different alkylamines and a reference to the solvent system alone (50:50 MeOH:H_2O). The solvent system alone is highly cationically adducted and keeping concentration constant the alkylamines can reduce that cationic adduction.

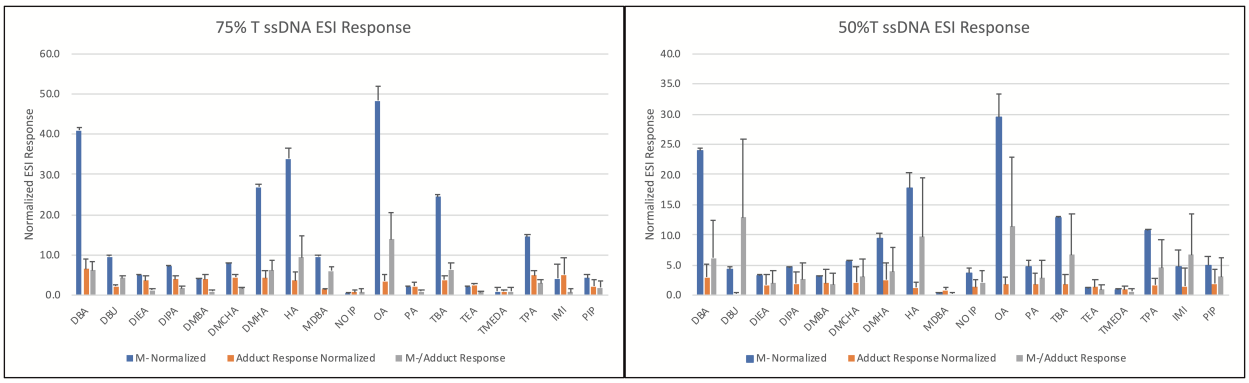


Figure 4.2: Comparison of the ESI response and cationic adduction of 75%T ssDNA and 50%T ssDNA. The overall intensity is higher for 75% T ssDNA due to the increased hydrophobicity but the trend among ion-pairs can be seen across the difference sequences used.

The alkylamine is not the only variable that can affect the signal intensity and it has been found that the base composition of the oligonucleotide strand also plays a significant role. Figure 4.2 shows the difference between 2 very similar ssDNA that differ only slightly in base composition. It has been suggested that the Thymine base (T) is more hydrophobic than the other bases, and this hydrophobicity is related to an increase in signal intensity (Huber, Oefner et al.

1992, Huber, Oefner et al. 1993, Gilar, Fountain et al. 2002, Null, Nepomuceno et al. 2003). Due to this observation we have utilized this base as a percentage for the naming convention for our model ssDNA's e.g. 25%T ssDNA has 25% of the bases as Thymine and the other 75% can be determined by looking at table 4.1. 75% T ssDNA has a higher ESI response than its 50% T ssDNA counterpart. This variability in the hydrophobicity of oligonucleotides contributes to the overall signal intensity and should be accounted for in any attempts at modelling the electrospray behavior of oligonucleotides. The base composition is also an important variable to investigate and evaluate its effects on the cationic adduction.

To qualitatively understand the effect of physiochemical properties on the cationic adduction we used a partitioning model. Partitioning is a statistical tool that attempts to classify data into groups by separating them in a decision-based manner. The partitioning divides the data into groups which differ in their adducted MS response. A random bootstrap forest partitioning model takes the dependent variable (mass spectral intensity of adduction) and uses the independent variables (physiochemical properties of alkylamines) to place the data into the groups and then averages the results of the many output partitioning trees. This is an advantageous approach because the averaging can overcome poor generalization and fit the data in a more accurate manner (Ho 1995). A bootstrap forest partitioning model was used to describe the physiochemical properties effect on the alkylamine adduction as seen in Figure 4.3. Many important variables for the overall electrospray response are highlighted from the model. Modeling the molecular peak/adducted peak signals, the model should provide a rationale for cationic adduction during the ESI process. Unsurprisingly, the top 7 parameters help to explain 77.12% of this phenomenon are all descriptors of the liquid-gas phase and contribute significantly to the electrospray ionization process.

Term	Number of Splits	SS	Portion
Proton Affinity	1170	738.248816	0.1383
Gas Phase Basicity	1058	629.423228	0.1179
pKa	1276	612.282273	0.1147
Boiling Point	1218	572.803945	0.1073
MW	1116	549.857794	0.1030
Henry's Law Constant	1066	510.598199	0.0956
Partition Coefficient	1134	503.857707	0.0944
Density	1142	409.044276	0.0766
Vapor Pressure	1088	399.879905	0.0749
Water Solubility	947	230.369865	0.0431
Content G	1169	56.6817466	0.0106
Content T	1042	52.1069738	0.0098
Content C	879	46.0663642	0.0086
Content A	893	27.9888004	0.0052

Figure 4.3: Bootstrap model highlighting important physiochemical parameters of cationic adduction. The important top 7 parameters account for 77.12% of the signal response.

Re-examining figure 4.1, we can see that this qualitative model can help explain the factors that lead to lower amounts of adduction. The model in figure 4.3 suggests that 7 parameters can explain 77.12% of this phenomenon and they are as follows: proton affinity (13.83%), gas-phase basicity (11.79%), pKa (11.47%), boiling point (10.73%), MW (10.3%), Henry's Law Constant (9.56%), and partition coefficient (logP) (9.44%). Water solubility, partition coefficient and Henry's law constant all play a major role in the ion evaporation mechanism that has been successfully applied to oligonucleotide ionization (Chen and Bartlett 2013, McGinnis, Grubb et al. 2013, Basiri, Murph et al. 2017) This was also partially explained by the model proposed by Chen et al., which indicates alkylamines with a lower Henry's law constant evaporate from electrospray droplets and concentrate the oligonucleotide at the surface of the droplet (Chen and Bartlett). This higher surface concentration would increase the charge on the analyte at the edge of the droplet, ultimately increasing the electrospray signal and this

also appears to reduce the cationic adduction (Null, Nepomuceno et al. 2003). Proton affinity and gas phase basicity follow the trend of OA>HA>TEA where the lower values for each indicates reduced cationic adduction which increases the signal intensity on the molecular peak. pKa and MW do not seem to follow any particular trend. Boiling point and partition coefficient follow a trend where the higher value indicates reduced cationic adduction which increases the signal intensity on the molecular peak. These trends can be visualized in Table 4.2.

The variables that are involved in cationic adduction with the highest weight according to our bootstrap model are proton affinity and gas phase basicity. The removal of sodium cations from solution can be discussed by 2 previously described mechanisms by Muddiman, et al. (Muddiman, Cheng et al. 1996). The first is that the alkylamines can essentially sequester the sodium cations by binding to the sodium cations in solution thus eliminating them from binding to the phosphodiester bond of the oligonucleotide. This relies on the acid-base properties of the phosphodiester bond (pKa <2) vs alkylamine (pKa ~10). This does not account for the differences seen between ion pairs with similar pKa values since the amount of sodium cation binding still varies drastically between these species. The second mechanism involves the alkylamine hydrogen bonding to the backbone and leaving as a neutral base in the gas phase. This relies on the relative basicity of the alkylamine and is supported by the model. It is reasonable to believe that this mechanism accounts for a greater portion of the sodium cation adduction reduction in the samples since the sodium adduction varies drastically with different alkylamines. During the electrospray process, dissociation will occur as the droplet evaporates and eventually the ssDNA will leave as an ionized species and the ion pair will leave as a neutral as proposed by Muddiman, et al. The proton affinity of the phosphodiester group was estimated by Lum and Grabowski (Lum and Grabowski 1992) to be 315 Kcal/mol and the proton affinity

for sodium hydroxide is 256.2 Kcal/mol. It can be expected that sodium would bind to the phosphodiester backbone but the alkylamine proton affinities are 219-250Kcal/mol. Thus, it can be proposed that the phosphodiester bond preferentially binds to the ion-pair over the sodium in solution. This helps to explain why the alkylamines that reduce the cationic adduction most significantly have the lowest proton affinities.

Another variable at play here is the ability of OA and HA to reduce the charge states over TEA. In general, the data for OA and HA shows the most abundant peak in the spectrum 1 charge state lower than for TEA. This is most likely due to the slight globular compaction that OA and HA impart on the oligonucleotide as discussed by Basiri, et al(Basiri, van Hattum et al. 2017). This reduction in charge state would mean that there are less negative charges on the phosphodiester backbone and in turn less binding sites for the cationic adduction.

The proton affinities of the alkylamines help explain part of the adduction phenomenon but the hydrophobicity also plays a large role. In the strands that contain a large number of thymine bases, the sensitivity is greater than strands with lower thymine composition (Gilar 2001, Gilar, Fountain et al. 2002, Null, Nepomuceno et al. 2003). Thymine is one of the most hydrophobic bases and this increased hydrophobicity plays an important role in the droplet during desorption. In a droplet the most hydrophobic species will cluster at the surface while the more hydrophilic components will reside in the center. Oligonucleotides contain a series of hydrophilic phosphates along their backbone but also contain a series of hydrophobic bases. An assumption can then be made that the hydrophilic backbone will be more likely to stay in the center of the droplet with the sodium cations and various anions. Alkylamines also contain a hydrophilic portion, amine group, and a hydrophobic portion, usually a carbon chain or even ring. When an alkylamine is added to the droplet the phosphate backbone can interact with the

hydrophilic portion of the alkylamine and the alkylamine can pull all or part of the oligonucleotide to the surface of the droplet, which would aid in the desorption of the oligonucleotide. We suggest that this mechanism most follows the ion evaporation model (IEM) of electrospray desorption. In this model, the alkylamine essentially increases the hydrophobicity of the oligonucleotide and assists with transport to the droplet surface. Therefore, the oligonucleotide would be desorbed before the droplet has undergone all of the fission events that would lead to a small droplet with only one analyte present. This mechanism of desorption would help explain why the cationic adduction is reduced as well. The oligonucleotide is pulled to the surface by the alkylamine and this doesn't allow the cations to electrostatically bind to the phosphodiester backbone of the oligonucleotide.

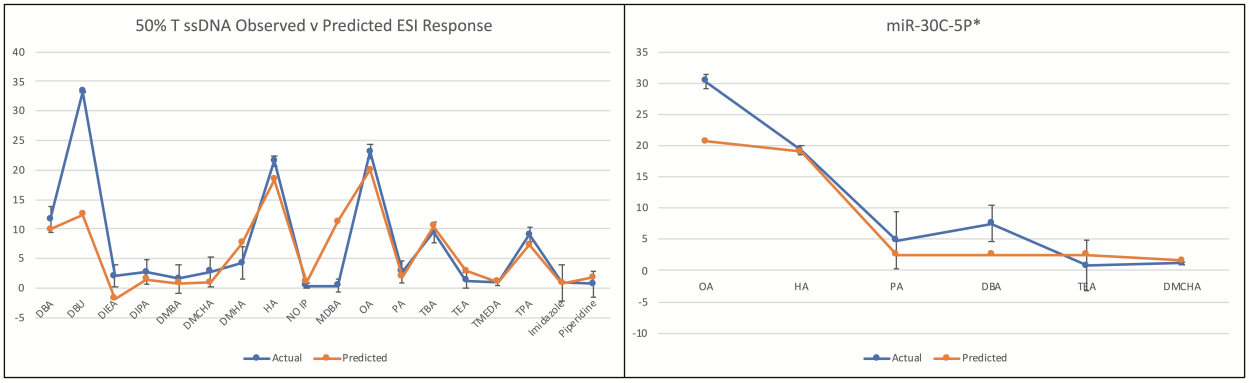


Figure 4.4: Using the PLS model to get a predicted (orange) vs actual (blue) signal intensity utilizing 2 different oligonucleotide sequences and sugar chemistries: 50%T ssDNA compared with a biologically relevant microRNA, miR-30c-5p*.

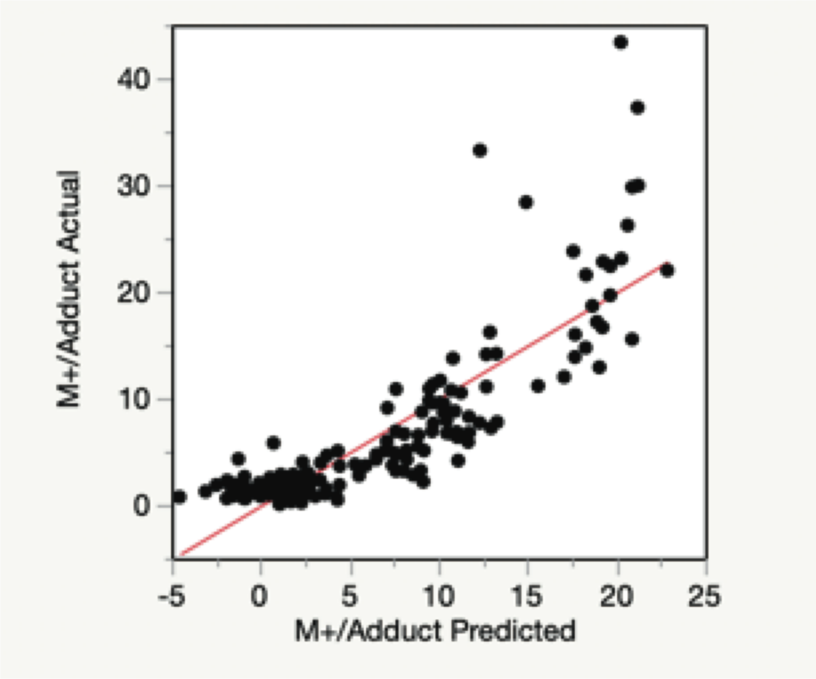


Figure 4.5: The plot of the Partial Least Squares (PLS) regression model used to obtain Equation 2 of the M- Normalized/Adduct Normalized data.

Practical application of this model by mathematical modeling was required to make this a useful tool. We chose a partial least squares model to determine the cationic adduction mathematically. The PLS model uses linear regression to model predicted variables against observed variables. This type of model is particularly useful when the number of predictors (physiochemical properties) vastly outweighs the observation (cationic adduction). A PLS model was performed as seen in Figure 4.5. Some of these data points lie above the line with an M+/Adduct Actual higher than 25 but the M+/Adduct Predicted shows that these should fall within 15-22.5. These outliers are the ssDNA containing octylamine which decreased the adduction significantly when compared to the other alkylamines used. Our model utilized 17 different alkylamines and it resulted in a large amount of variance among the alkylamines. 2 of the alkylamines reduce the cationic adduction more significantly than the other 15 which resulted

in these outliers. This alkylamine concentrates the signal in the main peak and is not spread across many sodium adducts unlike many of its competitor alkylamines. This skews the PLS regression line towards the alkylamines that contain signal across many sodium adducts. In general, since this only accounts for 5.88% of the data the model can still be used to accurately predict the adduction of many different alkylamines with the only downside being that it underpredicts the M+/Adduct signal for some alkylamines that reduce the cationic adduction more significantly than the others. This allows us to generate equation 2, which can be used to predict unknown alkylamines sodium adduction. This is useful so that method development time for this experiment is cut down significantly and if there is a small amount of sample the best data set can be generated without sample waste. This equation has been put into a user-friendly format in supplemental sheet 1. Users can quickly input their desired oligonucleotide sequence and choose between alkylamines that will result in less cationic adducted spectra.

$$\begin{aligned} &Normalized\ Adduct\ Signal\ Intensity \\ &= -0.1863MW + 92.9058Density - 0.1904Boiling\ Point + 2.986pKa \\ &+ 7.3193Proton\ Affinity - 7.9235Gas\ Phase\ Basicity \\ &+ 14.1397Partition\ Coefficient - 0.0233Water\ Solubility \\ &- 0.0001Vapor\ Pressure + 0.0702Henry's\ Law\ Constant \\ &- 2.0227Content\ A - 0.5545Content\ T + 0.6488Content\ C \\ &+ 7.0867Content\ G + 0.2411 \end{aligned}$$

EQ 2: The resulting mathematical descriptor equation obtained from the Partial Least Squares (PLS) model. This is useful in determining the 'predicted' signal intensity for an oligo of any composition with any alkylamine.

The model was then applied to the ssDNA that consists of 50% T-base. As can be seen in the trend, the prediction fits the actual data quite well. The biggest outlier is DIEA and the negative predicted signal intensity means it is predicted to have a larger cumulative adduction signal than the molecular peak. This can be explained by the rather high gas phase basicity of DIEA and its predicted inability to selectively bind the phosphodiester bond over a sodium cation. In practice we can see that DIEA actually suppresses the cationic adduction quite well. In fact, DIEA has similar properties to DIPA only differing by a methyl group and they perform very similarly in practice. Octylamine (OA), Propylamine (PA), Hexylamine (HA) and Dibutylamine (DBA) were the top 4 cationic adduction reducing ion-pairs in the model. These were selectively chosen to test the utility of the model against an RNA strand, Triethylamine (TEA) and Dimethylcyclohexylamine (DMCHA) were included for comparison. We chose a 23-mer microRNA, miR-30c-5p, to represent a biologically relevant molecule to test our model. The biggest difference between an RNA strand and a DNA strand is the 2'-OH (RNA) vs 2'-H (DNA) and the bases uracil (RNA) vs thymine (DNA). The 2'-OH modification for RNA would make the RNA sugar slightly more hydrophilic than the DNA and the addition of the uracil makes the base also makes it slightly more hydrophilic. These two changes would in theory suggest that the RNA would behave slightly differently than the DNA in the electrospray desorption process. In Figure 4.4, it can be seen that the model can accurately predict the trend for ssRNA even though the model was built with only data from ssDNA. In fact, this agrees with data recently obtained in our lab regarding analysis of microRNA's from biological systems using a non-traditional ion-pair for improved sensitivity and lower adduction during mass spectral analysis (Basiri, Sutton et al. 2019).

Next, we were curious if the model would hold up against a slightly different olignoculetide modification a phosphorothioate backbone. Phosphorothioates contain a substitution in the phosphodiester backbone replacing one of the oxygen molecules with a sulfur instead. Sulfur is larger than oxygen, should be more hydrophobic, has a larger valence shell which can expand to hold more electrons and is much less electronegative than oxygen. If you substitute 23 oxygens for 23 sulfurs in a 24-mer oligonucleotide, the strand should be much more hydrophobic than a DNA and behave fundamentally different due to the differing properties of oxygen and sulfur. Figure 4.6 shows how well our model from equation 2 predicts the cationic adduction (orange line) vs the actual data (blue line). We took the 4 alkylamines that reduce the cationic adduction the most Octylamine (OA), Propylamine (PA), Hexylamine (HA) and Dibutylamine (DBA) and compared them with Triethylamine (TEA) and Dimethylcyclohexylamine (DMCHA). The bar graph in figure 4.6 shows the normalized ESI response for the experimental data. Our model shows a similar trend in the predicted vs actual data plot and this is a little surprising given that the altered chemistry of the phosphorothioate would desorb differently than a ssDNA. We would suggest that the model would predict a lower signal intensity than actually observed. The model slightly under predicts 4/6 alkylamines tested but this is due to using the top 4 cationic reducing alkylamines and 2 average alkylamines for comparison. The more important observation is that the model still provides a general trend for the cationic adduction. It is unclear how the model would perform with even more highly modified oligonucleotides and we would suggest exercising caution utilizing the model beyond what is shown here.

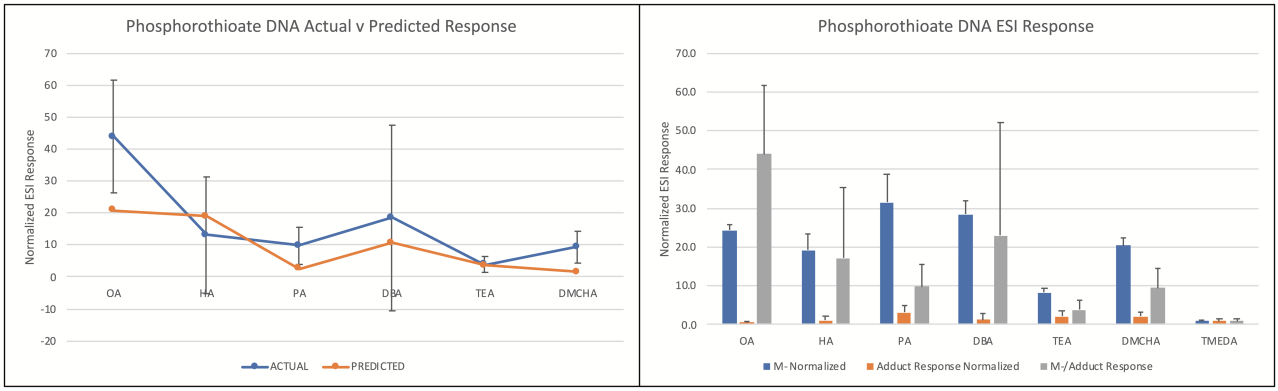


Figure 4.6: Showing the phosphorothioate DNA response using the. PLS model observed (blue) v predicted (orange) and the overall normalized signal intensity from the 5 highest performing alkylamines. This figure demonstrates the usefulness of the model and how it can predict ssDNA, RNA and even phosphorothioate modified oligonucleotides.

Conclusions

In this study we have described a set of experiments that were designed to determine the effect of the physiochemical properties of alkylamines and their effect on cationic adduction and reducing spectral complexity. With investigations into the physiochemical properties of alkylamines that contribute to the cationic adduction, we are able to leverage these properties to address these problems. The major factors highlighted in this study, proton affinity and gas phase basicity, support the previously suggested mechanisms for cationic adduction and reducing spectral complexity proposed by Muddiman, et. al (Muddiman, Cheng et al. 1996) and explains 77.12% of the adduction that can be seen. We propose a mechanism in which the oligonucleotides may undergo IEM desorption. The use of a complex set of test oligonucleotides gives us confidence that the model can be applied to diverse sets of oligonucleotides including RNA and slightly modified RNA/DNAs that could represent some therapeutic oligonucleotides.

Many previous studies have shown techniques to reduce spectral complexity and cationic adduction. A set of comprehensive experiments have been described that highlight the

importance of alkylamine physiochemical properties in ESI analysis to reduce these problems. Method development involving oligonucleotides can be extensive and time consuming. Utilizing our PLS model (Supplemental Sheet 1) users will be able to quickly choose an alkylamine that will give them the lowest amount of cationic adduction. TEA has long been the gold standard for oligonucleotide analysis but for cationic adduction TEA is not the best choice. A mixture of offline and online sample preparation methods has been previously described but they fail to address cations that can stem from the mobile phase, LC or MS instrument and glass/plastic used during analysis. Our study shows that utilizing our model can quickly provide mass spectrometry users with clean, robust mass spectra with little to no cationic adduction and decreased spectral complexity. Being able to quickly analyze an oligonucleotide with reduced cationic adduction allows users to easily get a molecular ion peak which can be used to confirm the molecular weight of their oligonucleotide. This method allows for the quick analysis of a sample without complicated desalting procedures like: combinations of offline and online including solid phase extraction cartridges (e.g. Oasis HLB, C18 ZipTip), chromatographic methods employing ion-exchange and hydrophobic techniques, molecular weight cutoff filters and dialysis (Greig and Griffey 1995, Muddiman, Cheng et al. 1996, Apffel, Chakel et al. 1997, Gilar and Bouvier 2000, Ragas, Simmons et al. 2000, Gilar, Belenky et al. 2001, Jiang and Hofstadler 2003, Oberacher, Parson et al. 2004, Erb and Oberacher 2014). This procedure is a one-step analysis method that can be useful to a broad range of applications.

Here we have shown that octylamine and hexylamine reduce the spectra complexity by the greatest amount and also reduce the cationic adduction by the greatest amount in ssDNA and ssRNA. For a single stranded phosphorothioate, we have shown that OA, PA and DBA are all acceptable choices. Many current studies target the analysis of RNA and modified DNAs. Here

we describe a model using mixed base ssDNA that we have shown can be applied to biological applications (microRNA) and even therapeutic applications (phosphorothioate modified). Our quantitative model provides us with the rationale for predicting and choosing alkylamines based on their physiochemical properties to reduce cationic adduction and the qualitative model allows users to quickly choose the best alkylamine to provide the least cationic adducted spectra and can be used during method development.

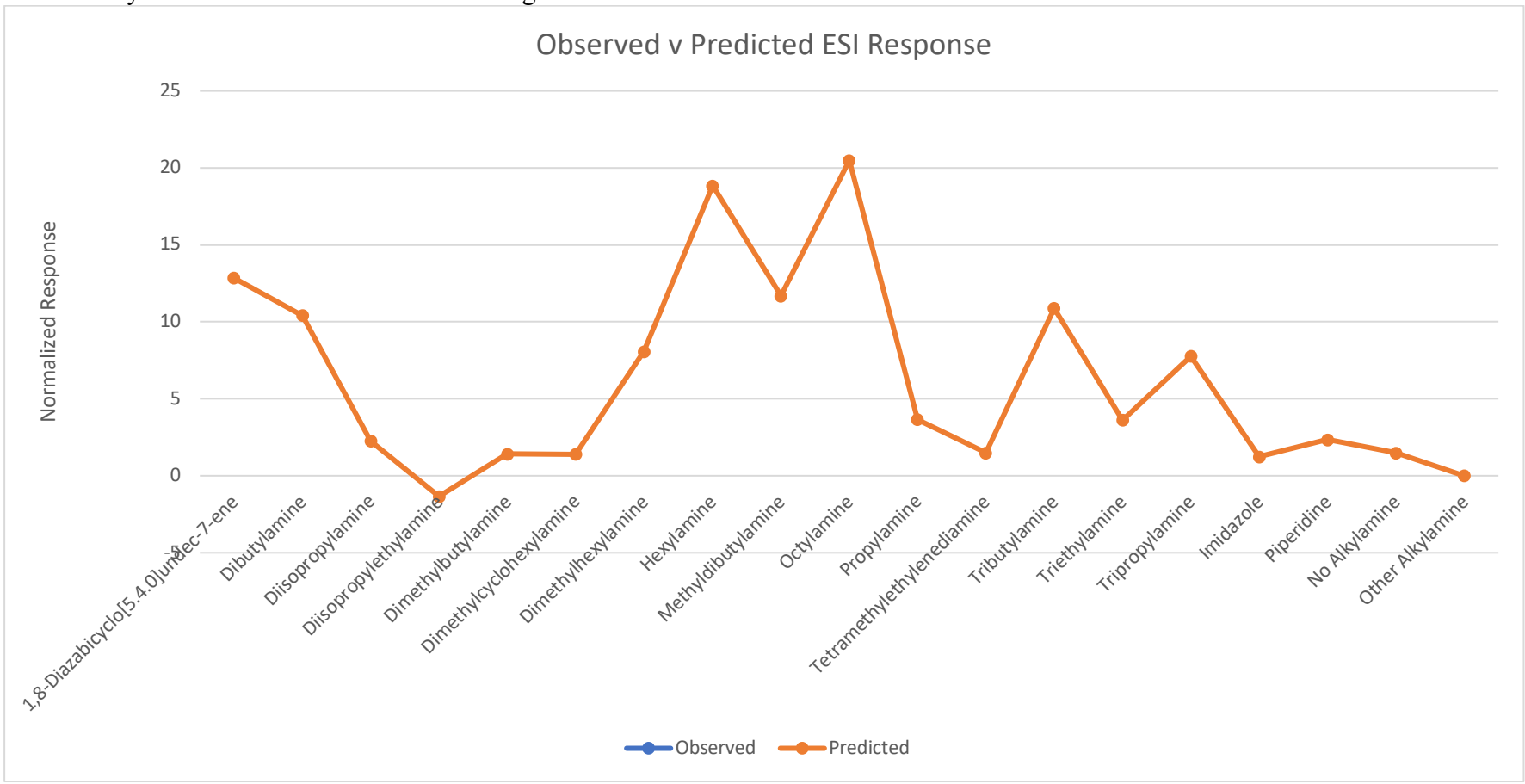
IP agent	Observed	MW	Density	Boiling Point	pKa	Proton affini	Gas phase ba	Partition Coe	Water Solub	Vapor Pressu	Henry's Law	Content A	Content T	Content C	Content G	Predicted
1,8-Diazabicyclo[5.4.0]undec-7-ene		152.24	1.04	97	13.28	250.45	242.43	1.132	283	0.715	5.128	0.25	0.25	0.25	0.25	12.8469235
Dibutylamine		129.24	0.77	159	11.03	231.5	223.6	2.695	74	280	0.111	0.25	0.25	0.25	0.25	10.4171671
Diisopropylamine		101.19	0.72	83.9	11.05	232.3	224.3	1.364	235	10600	0.103	0.25	0.25	0.25	0.25	2.2616777
Diisopropylethylamine		129.24	0.74	127	10.5	237.6	230.3	2.354	87	1560	0.065	0.25	0.25	0.25	0.25	-1.3347751
Dimethylbutylamine		101.19	0.72	95.9	10.02	231.6	224.2	1.647	51	6190	0.115	0.25	0.25	0.25	0.25	1.4113117
Dimethylcyclohexylamine		127.23	0.85	162	10.49	235.1	227.7	2.091	71	335	0.42	0.25	0.25	0.25	0.25	1.3934616
Dimethylhexylamine		129.24	0.74	147.1	9.99	231.5	223.5	2.666	13	771	0.065	0.25	0.25	0.25	0.25	8.0713841
Hexylamine		101.19	0.77	130	10.56	221.7	213.6	2.075	66	1180	0.368	0.25	0.25	0.25	0.25	18.8405648
Methyldibutylamine		143.27	0.75	164.6	10.5	231.32	223.39	3.175	5.7	277	0.049	0.25	0.25	0.25	0.25	11.6759159
Octylamine		129.24	0.78	179.6	10.65	221.86	213.77	3.094	17	129	0.012	0.25	0.25	0.25	0.25	20.4869246
Propylamine		59.11	0.72	46.9	10.53	219.4	211.3	0.547	544	41500	0.667	0.25	0.25	0.25	0.25	3.6500369
Tetramethylethylenediamine		116.2	0.78	121	8.97	242.07	232	-0.037	688	2000	409.836	0.25	0.25	0.25	0.25	1.4914774
Tributylamine		185.35	0.78	216.5	10.89	238.6	231.3	4.704	0.67	12.5	0.062	0.25	0.25	0.25	0.25	10.8861576
Triethylamine		101.19	0.73	89.7	10.65	234.7	227	1.647	155	7610	0.066	0.25	0.25	0.25	0.25	3.6101238
Tripropylamine		143.27	0.76	156	10.65	236.9	229.5	3.175	6.2	355	0.026	0.25	0.25	0.25	0.25	7.7648886
Imidazole		68.08	1.036	257	13.89	225.3	217.3	-0.099	57	32	2.625	0.25	0.25	0.25	0.25	1.2352273
Piperidine		85.15	0.8622	106	10.45	228.0	220.0	0.612	367	3773	2.217	0.25	0.25	0.25	0.25	2.35644474
No Alkylamine		0	0	0	0	0.0	0.0	0	0	0	0	0.25	0.25	0.25	0.25	1.492425
Other Alkylamine		***	***	***	***	***	***	***	***	***	***	***	***	***	***	#VALUE!

Input values into tiles marked *** with the appropriate physiochemical data to get a prediction of the adduction for an alkylamine not listed here.

For the oligonucleotide strand input the composition as a percentage, not to exceed 1 when A, T, C & G are added up. Substitute T for U if predicting for RNA. 25 % of each base is used as an example.

Supplemental Table 4.1: The equation modeled into an excel spreadsheet for rapid modelling of the cationic adduction, see supplemental figure 4.1 for corresponding graph.

Supplemental Figure 4.1: The model is graphed automatically in the provided excel document. Users may input observed data to test the accuracy of the model with their desired oligonucleotides.



CHAPTER 5

OLIGONUCLEOTIDE ANION ADDUCT FORMATION USING NEGATIVE ION ELECTROSPRAY ION MOBILITY MASS SPECTROMETRY[4]

[4] J Michael Sutton, Noha M El Zahar and Michael G Bartlett. 2020. Submitted to Journal of the American Society for Mass Spectrometry.

Abstract

Improving the mobile phase of electrospray oligonucleotides has been a major focus in the field of oligonucleotides. These improved mobile phases should reduce the charge state envelope of oligonucleotides coupled with electrospray ionization which is key to reducing spectral complexity and increasing sensitivity. Traditional mobile phase compositions with fluorinated alcohol and alkylamine, like hexafluoroisopropanol (HFIP) and triethylamine (TEA), have a large amount of cationic adduction and many charge states. Utilizing different fluorinated alcohol and alkylamine combinations, like nonafluoro-tert-butyl alcohol (NFTB) and octylamine (OA), can selectively reduce the charge states analyzed. Other classes of biomolecules have been analyzed with anionic salts to stabilize complexes, increase the molecular peak detection, and even provide unique structural information about these molecules; however, there have been no studies using anionic salts with oligonucleotides. Our experiments systematically study the stability and binding of ammonium anionic salt. We show that anions selectively bind low charge states of these oligonucleotides. Ion-mobility measurements are made to determine the collision cross section (CCS) of these oligonucleotides with anion adduction. We utilize both a nucleic acid exact hard sphere simulation (EHSS) calibration and a protein calibration. We are able to show that NFTB/OA is a good choice for the study of oligonucleotides with reduced charge states for the binding of anionic salts and the determination of CCS using ion-mobility.

Keywords: Oligonucleotide, Ion-mobility mass spectrometry, Anion adduction, Electrospray ionization

1. Introduction

With the invention of electrospray ionization by Fenn et al. there has been great interest in analyzing biomolecules with mass spectrometry (Fenn, Mann et al. 1989). One of the greatest advantages of mass spectrometry is the ability to gain structural information from molecules. There has been significant work done to determine secondary structural characteristics of biomolecules like proteins, carbohydrates, lipids and oligonucleotides (RNA/DNA) (Clemmer and Jarrold 1997, Bohrer, Merenbloom et al. 2008, Bleiholder and Bowers 2017). A significant amount of biomolecule secondary structural information comes from x-ray crystallography or NMR spectroscopy. Electrospray ionization mass spectrometry and ion mobility spectrometry can provide lower resolution measurements. This is ideal because mass spectrometry is a faster technique when compared to x-ray crystallography sample preparation times and does not require eliminating all free protons from solutions like NMR. Mass spectrometry also requires significantly less sample which is advantageous for studying structural elements of biomolecules with limited sample amounts. Studying oligonucleotides in the gas phase presents a unique set of challenges, like the ability of biomolecules to maintain solution state characteristics during the transition to the gas phase. Gabelica, et al. propose that despite molecular dynamic simulations suggesting otherwise, oligonucleotides can maintain their solution structure at the time scale of mass spectrometry experiments. (Baker, Bernstein et al. 2006, Arcella, Portella et al. 2012). This holds true for many different oligonucleotide systems that have been successfully studied using mass spectrometry (Hoaglund, Liu et al. 1997, Parkinson, Lee et al. 2002, Guo, Bruist et al. 2005, Mazzitelli, Wang et al. 2007, Smargiasso, Rosu et al. 2008, Marchand and Gabelica 2014, Lippens, Ranganathan et al. 2016, Scalabrin, Palumbo et al. 2017).

Charge state reduction has been a common theme to reduce spectral complexity and to increase detection limits (Cheng, Bakhtiar et al. 1994, Muddiman, Cheng et al. 1996, Null, Nepomuceno et al. 2003). Apffel, et al. proposed utilizing a fluorinated alcohol (HFIP) with an alkylamine to improve detection and oligonucleotide analysis, drifting away from using ammonium acetate salts to an organic-based mobile phase (Apffel, Chakel et al. 1997). The field quickly began attempting to find the perfect combination of these mobile phase additives for their analysis (Greig and Griffey 1995, Sharma, Glick et al. 2012, Chen and Bartlett 2013, McGinnis, Grubb et al. 2013, Erb and Oberacher 2014, Gong and McCullagh 2014, Basiri, Murph et al. 2017, Basiri, van Hattum et al. 2017). Recently, Basiri et al. proposed that NFTB & OA promotes a lower charge state distribution envelope and that these lower charge states are hairpins of the oligonucleotides studied (Basiri, van Hattum et al. 2017). Oligonucleotides have a highly acidic backbone and are generally analyzed in negative ion mode with electrospray. Cations can highly adduct to the phosphate backbone and cause poor sensitivity by causing the signal intensity to be spread across many charges and adduct states (Sutton and Bartlett 2019). However, anionic adducts have been shown to provide stability and can be used in mass spectrometry experiments to provide unique data (Cole and Zhu 1999, Cai and Cole 2002, Cai, Concha et al. 2002). Fabrik et al. leveraged phosphate anions to study structural features of free oligosaccharides (Fabrik, Čmelík et al. 2012). Collision-induced dissociation of oligosaccharides in the presence of fluoride, acetate and chloride was found to increase the molecular peak of neutral oligosaccharides during analysis (Jiang and Cole 2005). In another study utilizing sulfate, iodide, nitrate, dihydrogen phosphate and trifluoroacetate, Cole and coworkers were able to create a model to leverage the use of anion attachment for the study of peptides (Liu and Cole 2011). In all of these studies, gas phase basicity (GPB) plays the most important role in

determining the binding of anions to the analyte of interest. This is supported by Grandori, et al. whom look at conformational effects of proteins in electrospray and suggest that GPB and solvent accessible area play the most important roles in determining an analytes secondary structure (Li, Santambrogio et al. 2016).

Here we investigate the ability of nonafluoro-tert-butyl alcohol combined with octylamine as a novel solvent system to facilitate the study of lower charge state oligonucleotides with ion-mobility. A selection of anions was used to determine the utility of these additives to improve stability of lower charge states or to alter the size of adducted oligonucleotides during negative ion electrospray ion mobility of oligonucleotides. We look at a 20-mer phosphorothioate and a 32-mer RNA to determine the effectiveness of promoting secondary structure. Calibration profiles of collision cross section CCS (Ω, $Å^2$) for the anion adducts were compared utilizing two distinct calibration methods, one utilizing a protein calibration (Ruotolo, Benesch et al. 2008) and another using a modular set of nucleic acids(Lippens, Ranganathan et al. 2016).

2. Experimental & Methods

2.1 Chemical & reagents:

LC-MS grade omni-solv water, acetonitrile and methanol were obtained from Millipore Sigma (St. Louis, MO). Ammonium acetate, ammonium sulfate, ammonium perchlorate, ammonium phosphate, ammonium oxalate, ammonium nitrate, ammonium iodide, ammonium bromide, octylamine, triethylamine, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), nonafluoro-tert-butyl alcohol (NFTB), equine myoglobin (horse heart) (cat #:M1882), equine cytochrome c (horse heart) (cat#:C-2506) and bovine ubiquitin (red blood cells) (cat#:U6253) from Sigma Aldrich

(St. Louis, MO). ssDNA calibrants: 14B (GTTAAGTCGTATTA), 24B (TATCAGAAGAAGGTAACGAGTAGG), 32B (TTAAACAGTATCAGAAGAAGGTAACGAGTAGG) (Lippens, Ranganathan et al. 2016) and HpB, 32-mer aminoglycoside binding RNA (GGCGAUACCAAGCCGAAAGGCUUGGUAUCGCC), were obtained from Eurogentec, NA (Fremont, CA). Oligo 1, 20-mer phosphorothioate (T*C*C*G*T*C*A*T*C*G*C*T*C*C*T*C*A*G*G*G), where * indicates the phosphorothioate (oxygen to sulfur substitution), was obtained from Ionis Pharmaceuticals (Carlsbad, CA).

2.2 Preparation of working and stock solutions

1mg/mL stock solutions of oligonucleotides were prepared in LC-MS grade water and stored at -80ºC. Anionic salt solutions were freshly made at 1mg/mL. Final concentrations of working solution in 50:50 methanol:water with 15mM ion pair and 25mM fluorinated alcohol, except for NFTB, which was used at 2mM are: 10 µM oligonucleotide and 75 µM anionic salt (Basiri, van Hattum et al. 2017, Sutton and Bartlett 2019). All surfaces and equipment were cleaned with 1% sodium dodecyl sulfate followed by 70% ethanol prior to analysis to prevent degradation of oligonucleotide samples. Eppendorf DNA Lobind tubes (Hauppauge, NY) were used to minimize any non-specific loss of the oligonucleotides to plastic surfaces.

2.3 Instrumental conditions & software

The built-in fluidics system of the Waters Synapt G2 HDMS (Milford, MA) with Masslynx v4.2 was used for the data collection and analysis of the samples. All samples were infused with the built-in direct infusion pump system at 50uL/min and data collected in Mobility-TOF mode with

1s scan times in continuum full-scan mode. Mass spectra were acquired over the mass range 400-3000 *m/z*. Data was acquired and analyzed in triplicate by combining the average of 50 scans for each replicate. TOF-MS conditions were as follows: capillary voltage 3.0kV, sampling cone 25V, extraction cone 2.0V, source temperature 125°C, desolvation temperature 450°C, cone gas 0L/h and desolvation gas 1000L/h. Ion-Mobility specific parameters as follows: IMS gas (N_2) 100mL/min, trap gas (Ar) 2mL/min, wave velocity 450m/s and wave height 18V.

The procedure of Ruotolo et al. was used for calibration using proteins (Ruotolo, Benesch et al. 2008). Collision cross sections (Ω) were determined from the procedure determining corrected drift times (t''_D) vs known CCS (Ω, $Å^2$) values of equine myoglobin, equine cytochrome c and bovine ubiquitin. The known CCS (Ω) values were obtained from table 2 in Ruotolo et al. (Ruotolo, Benesch et al. 2008). Protein calibrants included: equine myoglobin: charge (z)= -13 (Ω=3136 $Å^2$), (z)= -14 (Ω=3243 $Å^2$), (z)= -15 (Ω=3230 $Å^2$), (z)= -16 (Ω=3313 $Å^2$), z=-17 (Ω=3384 $Å^2$), z=-18 (Ω=3489 $Å^2$), z=-19 (Ω=3570 $Å^2$), z=-20 (Ω=3682 $Å^2$), z=-21 (Ω=3792 $Å^2$); equine cytochrome C: z=-10 (Ω=2226 $Å^2$); and bovine ubiquitin: z=-9 (Ω=2303 $Å^2$). The resulting calibration curve (R^2>0.99) was verified by plotting charge states from the calibrants not utilized in the calibration curve. Additionally, a nucleic acid calibration following the procedure by Lippens, et al. was performed and resulted in a calibration curve (R^2>0.98) (Lippens, Ranganathan et al. 2016). Nucleic acid calibrants included 14B: z=-4 (Ω=743 $Å^2$); 24B: z=-4 (Ω=992 $Å^2$), z=-5 (Ω=1000 $Å^2$); and 32B: z=-5 (Ω=1217 $Å^2$), z=-6 (Ω=1367 $Å^2$). The resulting calibration curves were used to determine the CCS (Ω) values of all oligonucleotides present.

Graphs were processed with Microsoft Office Excel v16.36 for Mac. pH measurements were recorded using a symphony SB80PC pH meter from VWR (Radnor, PA). The instrument was

calibrated following the procedure listed in the manual prior to use with Fisher Scientific pH standards of pH 4.0 (catalog #: SB101), 7.0 (catalog #: SB107) and 10.0 (catalog #: SB115). Gas phase ion data was obtained through the NIST webbook (Mallard).

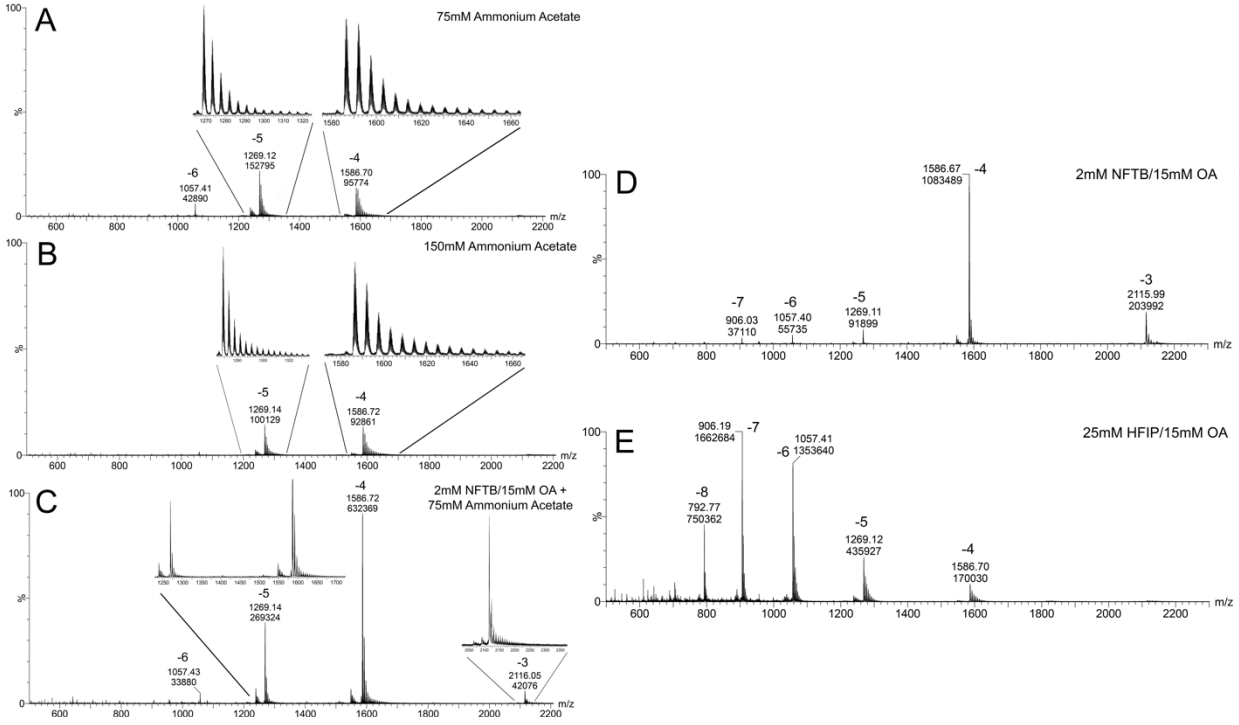
3. Results & Discussion

3.1 Charge Reduction Effect of NFTB v HFIP v Ammonium Acetate

Commonly, ammonium acetate is used to mimic biological conditions for native electrospray (Guo, Bruist et al. 2005, Scalabrin, Siu et al. 2014). For oligo 1, $[M - 4H]^{4-}$ and $[M - 5H]^{5-}$ are the most abundant charge states for 75mM ammonium acetate and 150mM ammonium acetate, figure 1. Ammonium acetate solution has a significant amount of Na^{+} adduction as seen in the inserts (figure 1A & 1B). There is a slight charge state distribution shift and significant signal intensity increase when the solvent changes from 100% water to 50/50 water/methanol spiked with 2mM NFTB/15mM OA. 50/50 water/methanol as a bulk solution has a lower boiling point than 100% water and this contributes to the increased ionization efficiency (figure 1C). It has been shown that nonafluoro-tert-butyl alcohol (NFTB) when used in combination with larger hydrophobic ion-pairing alkylamines shifts the electrospray charge state distribution of oligonucleotides. This shift occurs in the presence of alkylamines like octylamine (OA), hexylamine (HA) and dibutylamine (DBA) (Basiri, van Hattum et al. 2017). Octylamine was chosen as the representative alkylamine due to its ability to provide the greatest mass spectral intensity (Basiri, van Hattum et al. 2017) and the ability of OA to significantly reduce sodium adduction (Sutton and Bartlett 2019). This shift in the charge state distribution has been attributed to the oligonucleotides adopting a compact globular or perhaps a folded hairpin structure, similar to previous studies (Porrini, Rosu et al. 2017). When ammonium acetate is not

present in 50/50 water/methanol spiked with 2mM NFTB/15mM OA there is a clear shift away from the $[M - 5H]^{5-}$ charge state to the $[M - 4H]^{4-}$ and $[M - 3H]^{3-}$, in addition to decreased Na^{+} adduction, figure 1D. HFIP/TEA has been used to study the secondary structure formation of G-quadruplexes (Scalabrin, Palumbo et al. 2017); however, when compared to NFTB/OA there is a shift towards higher charge states, figure 1E. The fluoroalcohol hexaisopropanol does not shift the charge state distribution, and therefore is thought to correspond to a more linear or non-hairpin folded structure. Interestingly, the control sample of ammonium acetate (pH=7, pKa acetate=4.57, pKa ammonium=9.25) closely matched the distribution of the NFTB/OA solution and based on matching pKa values this seems reasonable; however, the ammonium acetate control has a much lower pH than the NFTB/OA solution and more closely matches the pH of the HFIP/OA solution. This suggests the pKa of the mobile phase additive is more important to the ultimate charge state distribution than the sample pH.

Figure 5.1: A: 10 µM Oligo 1 in 100% water with 75mM ammonium acetate buffer **B:** 10 µM Oligo 1 in 100% water with 150mM ammonium acetate buffer **C:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA + 75mM ammonium acetate **D:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA **E:** 10 µM Oligo 1 in 50/50 water/methanol 25mM HFIP/15mM OA. Y axis - % relative abundance X axis - m/z.



In previous experiments by Cech and Enke (Cech and Enke 2001), they reported that trifluoroethanol (TFE) promoted higher charge states than HFIP. This is likely due to the pKa of TFE (pKa= 11.49) being higher than that of HFIP (pKa=7.97). The resulting solution of TFE is significantly more basic, which promotes higher protonation of the phosphate backbone of the oligonucleotides. Basiri, et al. postulated that this same phenomenon happens with HFIP (pKa=7.97) and NFTB (pKa=4.91) (Basiri, van Hattum et al. 2017). Muddiman, et al. also noted that in more acidic samples with acetic acid there was a lower charge state envelope. This lower charge state envelope was attributed to the ability of the basic modifier to be charged and thus bind to the phosphate backbone and subsequently leave as a neutral (Muddiman, Cheng et al. 1996).

This explanation does not take into account the concentrations of HFIP v NFTB, OA and the solvent composition. For a solution containing 25mM HFIP, 15mM OA in 50/50 water/methanol, the pH is 9.90 ±0.06 and for a solution containing 2mM NFTB, 15mM OA in 50/50 water/methanol the pH is 10.78±0.01. This is quite surprising since NFTB has a lower pKa and we would generally expect a lower pH at similar concentrations. Due to the suppressive properties of NFTB it is used at a much lower concentration than HFIP (2mM vs 25mM) and therefore the pH is higher for the HFIP solution. The caveat is that this pH measurement is of bulk solution; therefore, it would follow that when droplets are formed during ESI the pH of the droplets containing NFTB will be lower than that of HFIP.

It would reason that as the organic (methanol, BP=64 °C) and mobile phase additives evaporate during the electrospray process, eventually the solvent composition will be primarily water. It has been shown that macroscopically, water-alcohol mixtures are miscible, but microscopically, there are clusters of the alcohol and clusters of water (Wakisaka and Ohki 2005). Several studies have shown that this phenomenon occurs during ESI with ethanol-water & methanol-water (Matsumoto, Nishi et al. 1995, Nishi, Takahashi et al. 1995, Takamuku, Yamaguchia et al. 2000). HFIP-water also has this microheterogeneity and it has been proposed that the CF_3 groups turn inwards towards each other and the -OH groups interact with the water environment around it through hydrogen bonding (Yoshida, Yamaguchi et al. 2003). These microenvironments contain clusters of hydrophobic molecules and other clusters of hydrophilic molecules. The boiling points of NFTB, HFIP, and OA are 45°C, 59°C, and 179°C, respectively. NFTB's boiling point is 45°C, HFIP's boiling point is 59°C and OA's boiling point is 179°C. As the droplet is evaporated you would lose molecules in the order: NFTB>HFIP>Methanol>OA>Water based on boiling point alone, but the fluorinated alcohols

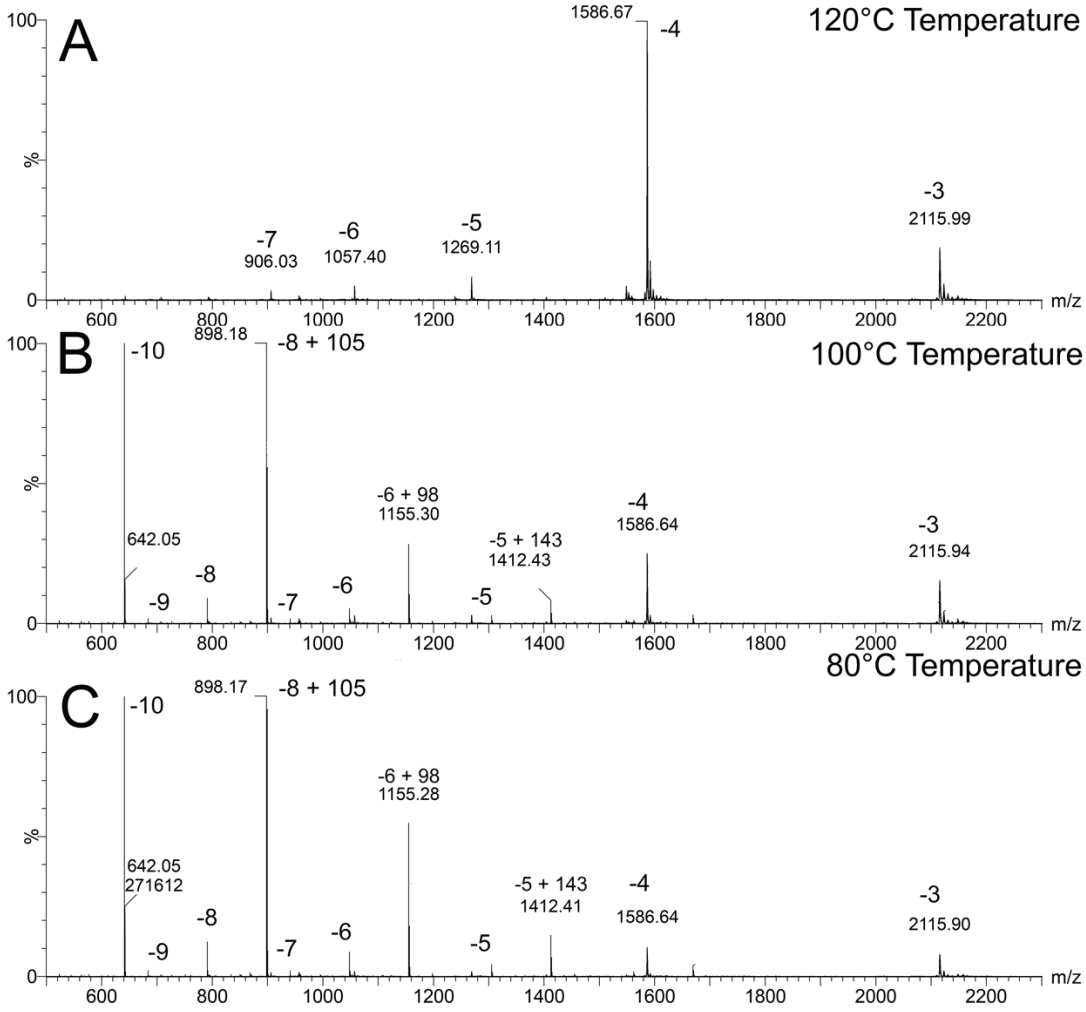
prefer a more hydrophilic microenvironments and the alkylamine prefers more hydrophobic microenvironments. The alkylamine forms a gradient in the droplet according to its hydrophobicity. As the droplet evaporates and loses the organic, the alkylamine concentrates on the droplet's surface (Chen, Mason et al. 2013). It can be proposed that you may lose solvent in the order Methanol>OA>NFTB>HFIP>Water, since the OA preferentially sits near the hydrophobic clusters. Hydrophobic clusters containing methanol evaporate faster which results in a predominately aqueous droplet containing oligonucleotide, the acidic modifier and a lower concentration of the alkylamine.

HFIP is ~10x more concentrated in these solutions than NFTB but the pKa suggests that the droplets of NFTB should be a lower pH when in the electrospray plume. In fact, when compared to ammonium acetate solutions for other biomolecular ESI studies, ammonium acetate undergoes an acidification during ESI due to the oxidation of water during electrospray (Van Berkel and Kertesz 2007, Konermann 2017). There is a pH shift from 7 down to the pKa of acetate (4.75). Functionally, the fluorinated alcohols and the acetate serve the same acidifying purpose and therefore the final pH of the solutions being sprayed are closer to the pKa of the respective fluorinated alcohol. OA will still bind to the phosphate backbone of the oligonucleotide over other cations, like sodium, due to a more preferential proton affinity (Sutton and Bartlett 2019). Therefore, the pH of the final electrospray droplet is slightly higher than the pKa of the fluorinated alcohol. The final pH is ~8 for HFIP and the final pH is ~5 for NFTB which closely matches the phenomenon for acetate. This holds up experimentally in figure 1 when you compare charge state envelopes between ammonium acetate solutions with solutions containing NFTB/OA and HFIP/OA. The more acidic environment of NFTB/OA leads to a

lower charge state distribution, while the more basic environment of HFIP/OA leads to a higher charge state distribution.

The source temperature dictates the temperature/energy that is imparted on a droplet that has almost completely undergone desolvation fission. We would expect solvent loss in this order: Methanol>OA>NFTB>Water, as discussed previously. Higher temperature would suggest that methanol and OA would desolvate quickly, resulting in a lower pH and charge state distribution that is shifted lower. As the temperature is lowered, more OA would be present in the droplets bringing the pH up and ultimately resulting in a higher charge state distribution. Softer ionization transfer conditions were tested to demonstrate effects of desolvation on the charge state envelope of an oligonucleotide in the presence of the NFTB/OA buffer solvent system. At lower source temperature conditions, 80°C and 100°C, there is a significant shift of the charge state to higher charges compared to 120°C, Figure 2. This supports the idea that these ESI droplet microenvironments are greatly affected by the mobile phase additives present, and that by adjusting the microenvironments the entire mass spectra can be affected. Gabelica, et al. suggest that lower charge states are the result of charge residue pathway (CRM) ionization and that higher charge states are the result of chain ejection model (CEM) ionization(Khristenko, Amato et al. 2019). The CEM pathway is likely the reason the 80°C and 100°C samples have higher charge states than the 120°C samples which are likely undergoing CRM.

Figure 5.2: 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA. Source temperature conditions varied as follows: A - 120°C, B - 100°C and C - 80°C. Y axis - % relative abundance X axis - m/z.



3.2 Anionic Salt Adduction

Previously, anionic salt adduction has been reported for other biomolecules like proteins and saccharides, and these adducts have been shown to improve stability and provide unique MS/MS spectra for structural analysis (Cole and Harrata 1993, Cole and Zhu 1999, Cai, Concha et al. 2002, Jiang and Cole 2005, Fabrik, Čmelík et al. 2012). Anionic salt adduction has not been

extensively studied for single stranded oligonucleotides. Using DNA in solution as a model, the ion atmosphere in solution helps form the dsDNA complex; and in the absence of this ion atmosphere it is estimated to require 60kcal/mol of thermal energy to overcome the opposing charges for a dsDNA complex to form (Lipfert, Doniach et al. 2014). Likewise, a similar scenario is true for ssDNA/ssRNA complexes that form hairpins, secondary structure or crumpled globular forms where the presence of counterions significantly reduces the energy required to form these complexes. Like-polarity ion adduction is somewhat unusual due to the energy required to overcome the electrostatic repulsion. Oligonucleotides are a particularly net negatively charged class of molecules when analyzed in negative ion-mode electrospray. There is also a tendency for metal cationic adduction, primarily sodium and potassium, to dominate the adduction on the phosphate backbone, and it is therefore imperative that studies of anionic adduction minimize the non-specific cationic adduction that can occur by using ammonium salt solutions. It has been shown that since ammonium salts are fairly volatile it leaves the oligonucleotide with a net neutral charge, since the ammonium can dissociate to NH_3 and H^+ (Cech and Enke 2001). We systematically explore the stability and adductive effects of various ammonium-anionic salts, table 1, in the negative ion-mode for a phosphorothioate oligonucleotide, oligo 1.

Due to the ability of NFTB/OA to only promote lower charge states, we focus our analysis on these charge states, figure 3. The most abundant charge states for this oligonucleotide are the $[M - 3H]^{3-}$ and $[M - 4H]^{4-}$ charge states. Data was normalized to the signal intensity of a solution of 2mM NFTB/15mM OA in 50/50 water/methanol with 10 µM oligo 1; all salt solutions contained 2mM NFTB/15mM OA in 50/50 water/methanol with 10 µM oligo 1 with 75 µM ammonium salt. None of the salt solutions exhibited a higher signal intensity than the control

solution for either the $[M - 4H]^{4-}$ or the $[M - 3H]^{3-}$ charge states. For example, chloride, bromide and phosphate had the highest signal for the $[M - 4H]^{4-}$ charge state and these signals were 72%, 71% and 66% compared to the control signal, respectively. Additionally, iodide, chloride and nitrate had the highest signals for the $[M - 3H]^{3-}$ charge state with 83%, 69% and 65% compared to the control signal, respectively. This is not unusual since the ammonium salt solutions contain a large number of additional ions that compete for ionization and alter the physiochemical properties of the solution as compared to control. In fact, when the $[M - 3H]^{3-}$ & $[M - 4H]^{4-}$ are summed, chloride, bromide, iodide, nitrate, formate and acetate result in the highest signal intensity. Upon inspection of these spectra, there are no salt specific anionic adducts present in the spectra; however, there are still Na^+ and K^+ cationic adducts. Phosphate, perchlorate, arsenate and sulfate have the lowest signal intensity, and all contain at least 1 anionic adduct, figure 4.

The adducts form more readily on the $[M - 3H]^{3-}$ charge state as compared to the $[M - 4H]^{4-}$ charge state. In almost all of the spectra, the $[M - 4H]^{4-}$ base peak was more abundant than the $[M - 3H]^{3-}$ base peak. However, this was not the case for sulfate, perchlorate, nitrate and iodide, figure 4 and supplemental figure 1. For sulfate and perchlorate, this can be partially explained by the ability of the respective salts to more readily adduct to the $[M - 3H]^{3-}$ peak but not the $[M - 4H]^{4-}$ peak. This suggests that the anionic adduction of sulfate and perchlorate either stabilizes the oligonucleotide in solution which promotes the $[M - 3H]^{3-}$ charge state or that the sulfate and perchlorate play a role during the ionization that promotes fewer charges. Phosphate adducts to the $[M - 3H]^{3-}$ charge state as well, but only as a single adduct; meanwhile, sulfate and perchlorate have multiple adducted anionic species on the $[M - 3H]^{3-}$ peak. It is unclear why nitrate and iodide have increased signal intensity of the $[M - 3H]^{3-}$ over the $[M - 4H]^{4-}$ charge state. Work with N-linked glycans spiked with ammonium nitrate has shown that $[M + NO_3]^-$

formed stable complexes with the neutral glycan even in the presence of increasing RF-1 voltage that would normally destabilize other anionic complexes in negative ion mode (Harvey 2005). Additionally, as more sialyation was present, there were more sites for ionization. This lead to multiply charged glycans on which nitrate did not form stable adducts. Here, the nitrate may have stabilizing properties in the oligonucleotide solution by providing a positive charge to create a net neutral charge environment; however, when the oligonucleotides undergo ionization and become multiply charged, the nitrate is unable to form a stable adduct. This stabilization of the nitrate adduct could promote the binding of nitrate to the lower charge state $[M - 3H]^{3-}$ preferentially over higher charge states like $[M - 4H]^{4-}$.

Interestingly, the sulfate peak has a nominal mass difference of 97 or 98 daltons compared to the base peak. This suggests that the sulfate is adducted as an HSO_4^- or H_2SO_4 adduct, possibly in the forms: --H+--[SO_4] or --H+--[SO_4]--H+--. Cole & coworkers propose that the stability of anionic adducts in negative electrospray is governed by several phenomena such as the solution phase affinity for the anion, the gas phase basicity and the ability of the anion to multiply hydrogen bond to the analyte (Cai and Cole 2002). In fact, sulfate, chlorate, phosphate and arsenate all have some extent of hydrogen bonding as evidenced in figure 4. This observation suggests that these anions can form adducts through hydrogen bonding with the phosphorothioate backbone or the bases. Smith, et al. found that binding of metal cations to a 12-mer oligonucleotide was site specific and dependent on the sequence and position of thymine within the sequence (Wu, Cheng et al. 1996). For example, they found that a uranyl, UO_2^{2+}, bound selectively to the 7^{th} and 8^{th} phosphodiester groups, Na^+ was found to be delocalized across the strand. Mg^{2+} was found delocalized across the strand but not to the same degree as Na^+. It was also found that the metal ions studied selectively bound the lone central thymine

instead of the terminal 2 thymine caps of the strand. This rationale can be used to hypothesize why the anions bind the $[M - 3H]^{3-}$ over the $[M - 4H]^{4-}$ charge state. The $[M - 3H]^{3-}$ may be structured in a way that allows the anions to bind to a base while the $[M - 4H]^{4-}$ does not share the same structure as $[M - 3H]^{3-}$.

While many of the anions used in this study have known gas phase basicity (GPB, kcal/mol) values, a few do not and "bracketing" methods can be used to estimate relative GPB values, table 1. It can be hypothesized that the gas phase basicity of perchlorate, which is currently not known, appears to be close to that of sulfate due to their similar behavior in this study. The ability of perchlorate and sulfate to create dipole moments can explain why they adduct more strongly than the other anions. Arsenate and phosphate also appear to have similar gas phase basicity (phosphate is known but arsenate is unknown) since they have a weaker anionic interaction in the form of adduction compared to sulfate and perchlorate. This would not be surprising, since these anions are isoelectronic and arsenate can replace phosphate in many chemical and biological reactions (Tawfik and Viola 2011).

Figure 5.3: Normalized intensity of 75 µM ammonium-anion salts in 50/50 Water/Methanol containing 2mM NFTB/15mM OA spiked with 10 µM oligo 1 oligonucleotide in the negative ion mode. Ammonium-anion salts were normalized to the intensity of a 50/50 Water/Methanol solution containing 2mM NFTB/15mM OA spiked with 10 µM oligo 1. Data shown is the average (n=3) of 50 scans.

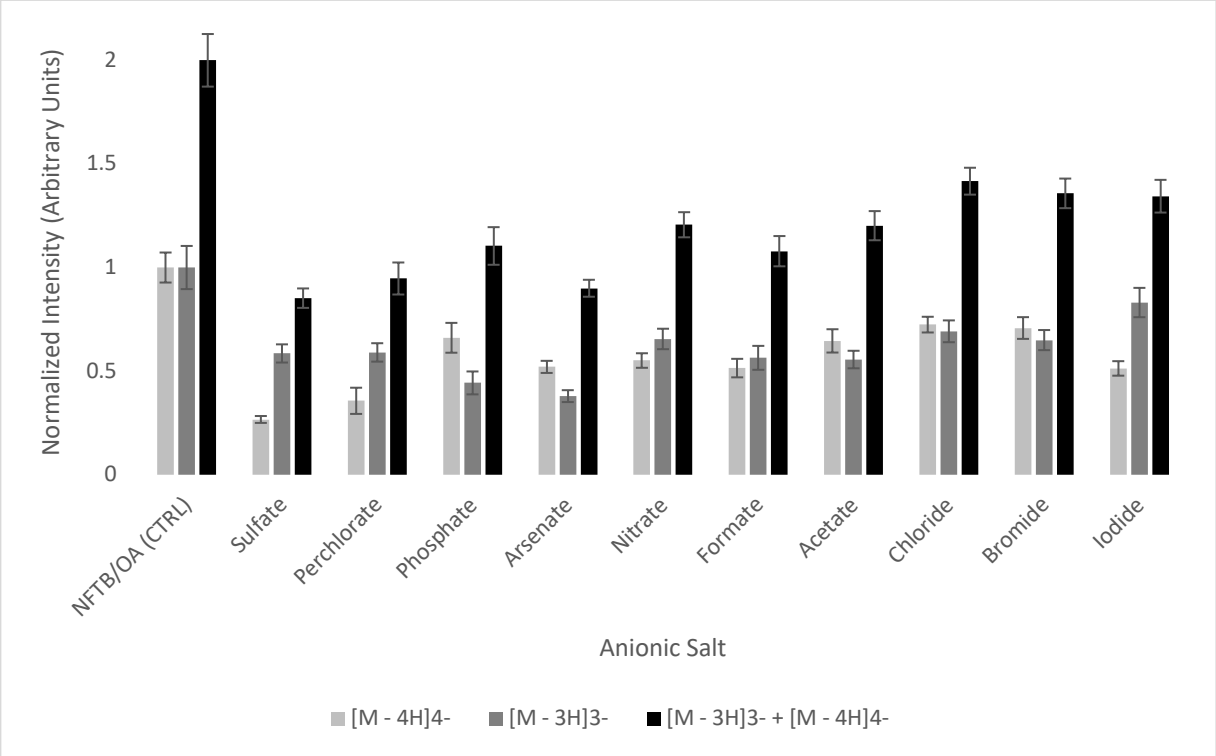


Figure 5.4: Mass spectra of oligo 1 with various anionic salts, Y axis - % relative abundance X axis - m/z.**A:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA (control) **B:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA + 75 µM NH_4AsO_4 **C:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA + 75 µM NH_4PO_4 **D:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA + 75 µM NH_4ClO_4 **E:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA + 75 µM NH_4SO_4

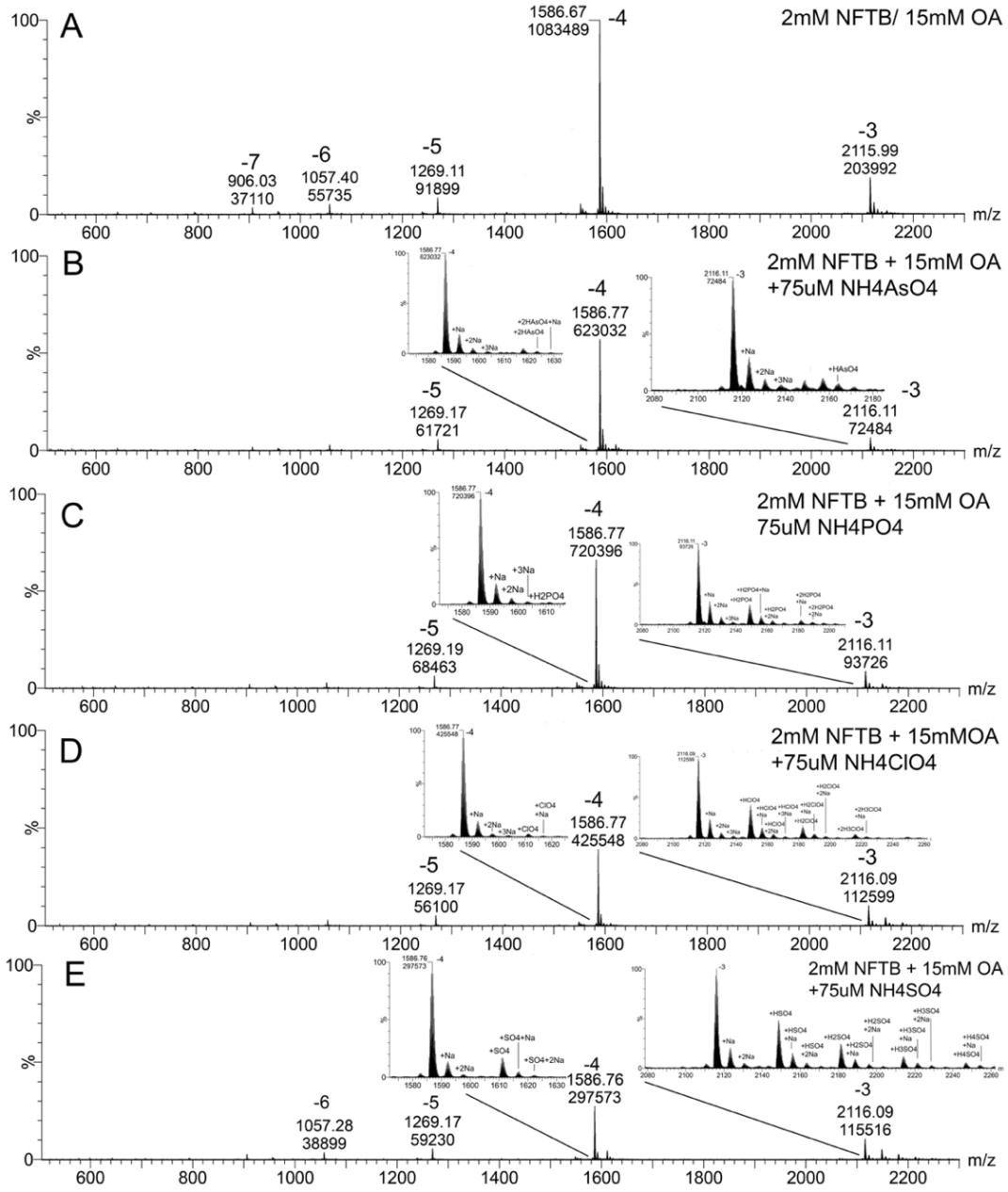
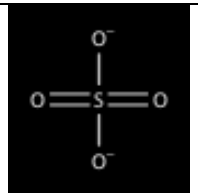
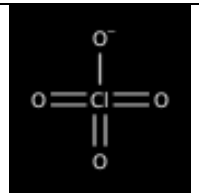
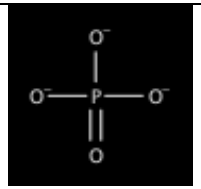
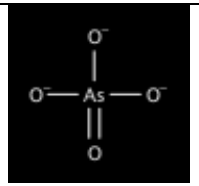
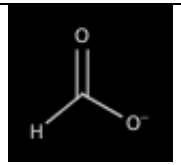
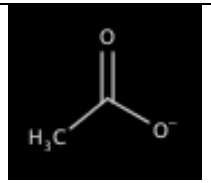
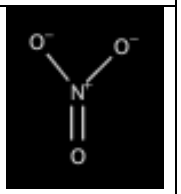


Table 5.1: Structures were drawn using MarvinSketch: MarvinSketch was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin 17.27.0, ChemAxon (https://www.chemaxon.com). Gas Phase Basicity values obtained from NIST webbook (Mallard). Atomic Radii obtained from various sources:(Jenkins and Thakur 1979)* ChemAxon calculations†. (Chloride, Bromide, and Iodide ions in supplemental table 5.1)

	Sulfate	**Perhlorate**	**Phosphate**	**Arsenate**	**Formate**	**Acetate**	**Nitrate**
Salt							
Anion GPB (kcal/mol)	302.34 ± 2.39	N/A	322.90 ± 5.02	N/A	N/A	N/A	317.81 ± 0.20
Atomic Radii (Å)	2.58*	2.40*	2.38*	2.87†	2.45†	2.85†	1.79*

3.3 Effect of Anionic Adduction on Size of Oligonucleotides Measured in Gas Phase

Oligonucleotides are known to keep their structure from the solution to the gas phase and can retain secondary structure on relatively short time scales (Gabelica 2014). It is also known that the main charge state for electrospray can be estimated from the solvent accessible area of a molecule (Rueda, Kalko et al. 2003, Rueda, Luque et al. 2005, Arcella, Portella et al. 2012). This implies that the lower charge states in the gas phase correspond to compacted or possibly folded states in solution. In fact, higher charge states of oligonucleotides are thought to be in an elongated form similar to proteins (Felitsyn, Kitova et al. 2002, Arcella, Portella et al. 2012).

The size of an oligonucleotide should theoretically increase marginally with anions adducted, especially if the adducts are present on the phosphorothioate backbone. Collision cross sections (CCS, Ω, $Å^2$) were determined by ion mobility mass spectrometry employing 2 orthogonal calibration techniques, table 5.1. A standard protein calibration utilized equine myoglobin (m/z = -13 thru m/z = -21), equine cytochrome C (m/z = -10) and bovine ubiquitin (m/z=-9) and was found to have excellent linearity R^2>0.99. An additional calibration curve utilized a nucleic acid calibration consisting of: 14B: z=-4 (Ω=743 $Å^2$); 24B: z=-4 (Ω=992 $Å^2$), z=-5 (Ω=1000 $Å^2$); and 32B: z=-5 (Ω=1217 $Å^2$), z=-6 (Ω=1367 $Å^2$) (Lippens, Ranganathan et al. 2016) and was found to have excellent linearity R^2>0.98. Lippens et al., obtain the theoretical CCS values for the nucleic acid ladder by molecular dynamics simulations using exact hard sphere scattering (EHSS) or projections superposition approximation (PSA). The authors found that the PSA and EHSS performed similarly and that PSA generally had the most consistent results. EHSS had better performance when the topology of the analyte was matched to the calibrant. For the data set acquired, there was no noticeable differences between the PSA and

EHSS calibration curves, therefore since our calibrants closely matched the topology of our samples we chose to display the CCS values obtained with EHSS.

In general, the nucleic acid calibration should provide a more accurate representation of the topology of other nucleic acids. In general, the calibration curves are reasonably consistent for the lower charge states studied (-3 to -5). The 2mM NFTB / 15mM OA solution predicted: Ω_{EHSS}= 906 Å^2 v $\Omega_{Protein}$= 940 Å^2 for m/z = -3, Ω_{EHSS}= 1078 Å^2 v $\Omega_{Protein}$= 1029 Å^2 for m/z = -4, and Ω_{EHSS}= 1246 Å^2 v $\Omega_{Protein}$= 1117 Å^2 for m/z = -5. In general, these CCS values are in general agreement of the size of the nucleic acids at these low charge states. It is expected that the lower charge states correspond to a smaller structure because the oligonucleotide can either be folded or compacted by the formation of non-native hydrogen bonds among the backbone and the bases. Interestingly, for the higher charge states: Ω_{EHSS}= 2848 Å^2 v $\Omega_{Protein}$= 1956 Å^2 for m/z = -13 and Ω_{EHSS}= 2863 Å^2 v $\Omega_{Protein}$= 1964 Å^2 for m/z = -14, are not in agreement.

A common approach for ion-mobility calibration is to measure a few calibrants with known CCS values and then to extrapolate to the unknown samples being analyzed(Ruotolo, Benesch et al. 2008, Lippens, Ranganathan et al. 2016, Sun, Vahidi et al. 2016). In this experiment, the protein calibrants used were at higher charge states and generally corresponded to a larger size which required extrapolation to the lower charge states (m/z = -3, -4 & -5) of the oligonucleotide analytes. Conversely, the nucleic acid calibrants used were at higher charge states and generally corresponded to a smaller size which required extrapolation to the higher charge states (m/z = -13 & 14) of the oligonucleotide analytes. In general, for the topology to closely match the predicted CCS values, the calibrants should match in size and charge. In this experiment, the nucleic acid calibration contained lower charges & sizes than the protein calibration, but the relative size of these ions significantly impacts the degree of accuracy for the

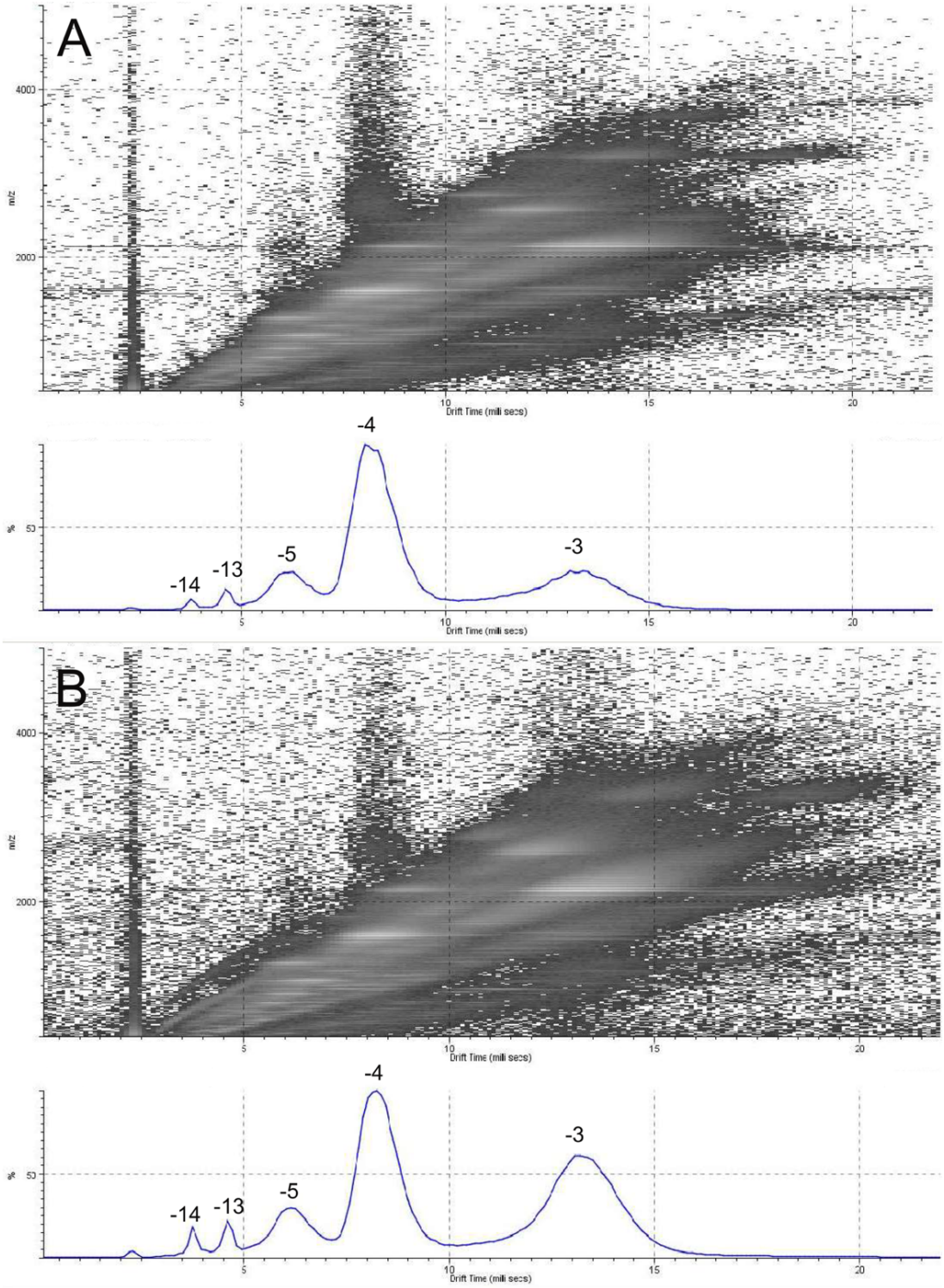
CCS measurements. The R^2 for the protein vs nucleic acid calibration is nearly identical (0.99 vs 0.99, respectively); however, an effective CCS calibrator must have a known CCS value and must be sufficiently "rigid" to minimize conformational errors in the measurement (Lippens, Ranganathan et al. 2016). The rigidity of nucleic acid sequences are likely in closer agreement than that of a nucleic acid and a protein, which gives us a greater degree of confidence in the nucleic acid calibration. An important observation across both calibrations is that they suggest smaller topology at lower charge states and larger topology at higher charge states.

The expected result of adduction would be to see an increase in the size of the ions measured when compared to non-adducted molecules. The average drift time of the non-adducted peaks does not shift, as expected; however, the drift time does not increase to any significant degree despite a large amount of sulfate binding on the $[M - 3H]^{3-}$ charge state as seen in figure 5. The $[M - 3H]^{3-}$ charge state has an increased ion intensity (figure 5B) compared to the control (figure 5A). The increased signal for the $+SO_4$ can be seen in the upper portion of figure 5B. As the m/z ratio increases ($+SO_4$ adduction) there is not a significant increase in drift time and therefore no significant increase in size. This is likely due to the large nature of the oligonucleotides at this charge (Ω_{EHSS}= 906 Å^2 v $\Omega_{Protein}$= 940 Å^2 for m/z = -3) compared to the relatively small nature of sulfate: +1 SO_4 (2.6Å), +2 SO_4 (5.2Å), +3 SO_4 (7.8Å) and +4 SO_4 (10.4Å).

Table 5.2: Collision Cross Sections (Ω) of oligo 1 using a nucleic acid calibration with exact hard sphere scattering (EHSS) simulation CCS calibrants (Lippens, Ranganathan et al. 2016) or using proteins with known measured CCS values (Ruotolo, Benesch et al. 2008)

Charge	CCS EHSS N2 (Ω, Å2)	CCS Protein Calibrants (Ω,	Charge	CCS EHSS N2 (Ω, Å2)	CCS Protein Calibrants (Ω,
NFTB/OA			**Chlorate**		
-3	905.86 ± 1.39	939.35 ± 0.73	**-3**	906.46 ± 2.39	939.66 ± 1.25
-4	1077.59 ± 1.34	1029.26 ± 0.70	**-4**	1079.13 ± 1.32	1030.06 ± 0.69
-5	1245.94 ± 2.46	1117.40 ± 1.29	**-5**	1245.94 ±2.46	1117.40 ± 1.29
-13	2847.73 ± 20.31	1956.02 ± 0.63	**-13**	2847.73 ± 20.31	1956.02 ± 10.63
-14	2863.04 ± 30.90	1964.04 ± 16.18	**-14**	2863.04 ± 30.90	1964.04 ± 16.18
Sulfate			**Nitrate**		
-3	906.77 ± 1.05	939.83 ± 0.55	**-3**	905.56 ± 0.92	939.19 ± 0.48
-4	1082.89 ± 2.23	1032.03 ± 1.17	**-4**	1077.59 ± 1.34	1029.26 ± 0.70
-5	1245.91 ± 4.87	1117.38 ± 2.55	**-5**	1248.75 ± 2.41	1118.87 ± 1.26
-13	2870.27 ± 25.66	1967.82 ± 13.43	**-13**	2858.73 ± 34.08	1961.78 ± 17.84
-14	2897.59 ±1 4.47	1982.12 ± 7.58	**-14**	2871.40 ± 40.14	1968.41 ± 21.02
Phosphate			**Acetate**		
-3	907.97 ± 1.37	940.45 ± 0.72	**-3**	905.25 ± 1.90	939.03 ± 1.00
-4	1078.35 ± 2.30	1029.66 ± 1.21	**-4**	1075.23 ± 3.61	1028.02 ± 1.89
-5	1247.33 ± 4.22	1118.12 ± 2.21	**-5**	1248.75 ± 2.41	1118.87 ± 1.26
-13	2847.73 ± 20.31	1956.02 ±1 0.63	**-13**	2852.56 ± 43.74	1958.55 ± 22.90
-14	2854.32 ± 26.76	1959.47 ± 14.01	**-14**	2913.32 ± 36.68	1990.36 ± 19.20
Arsenate			**Formate**		
-3	905.56 ± 0.92	939.19 ±0.48	**-3**	907.08 ± 0.52	939.98 ± 0.27
-4	1079.13 ±1.32	1030.06 ± 0.69	**-4**	1078.35 ± 2.30	1029.66 ± 1.21
-5	1248.75 ± 2.41	1118.87 ± 1.26	**-5**	1245.94 ± 2.46	1117.40 ± 1.29
-13	2853.31 ± 26.50	1958.94 ± 13.87	**-13**	2865.04 ± 9.67	1965.08 ± 5.06
-14	2845.20 ± 30.89	1954.94 ± 16.18	**-14**	2910.91 ± 75.82	1989.10 ± 39.70

Figure 5.5: Ion-mobility drift times vs m/z ratio (top) and drift time vs relative ion intensity (bottom) for both: **A:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA **B:** 10 µM Oligo 1 in 50/50 water/methanol 2mM NFTB/15mM OA + 75 µM NH_4SO_4



3.4 Selective Sulfate Adduction of HpB RNA Aptamer

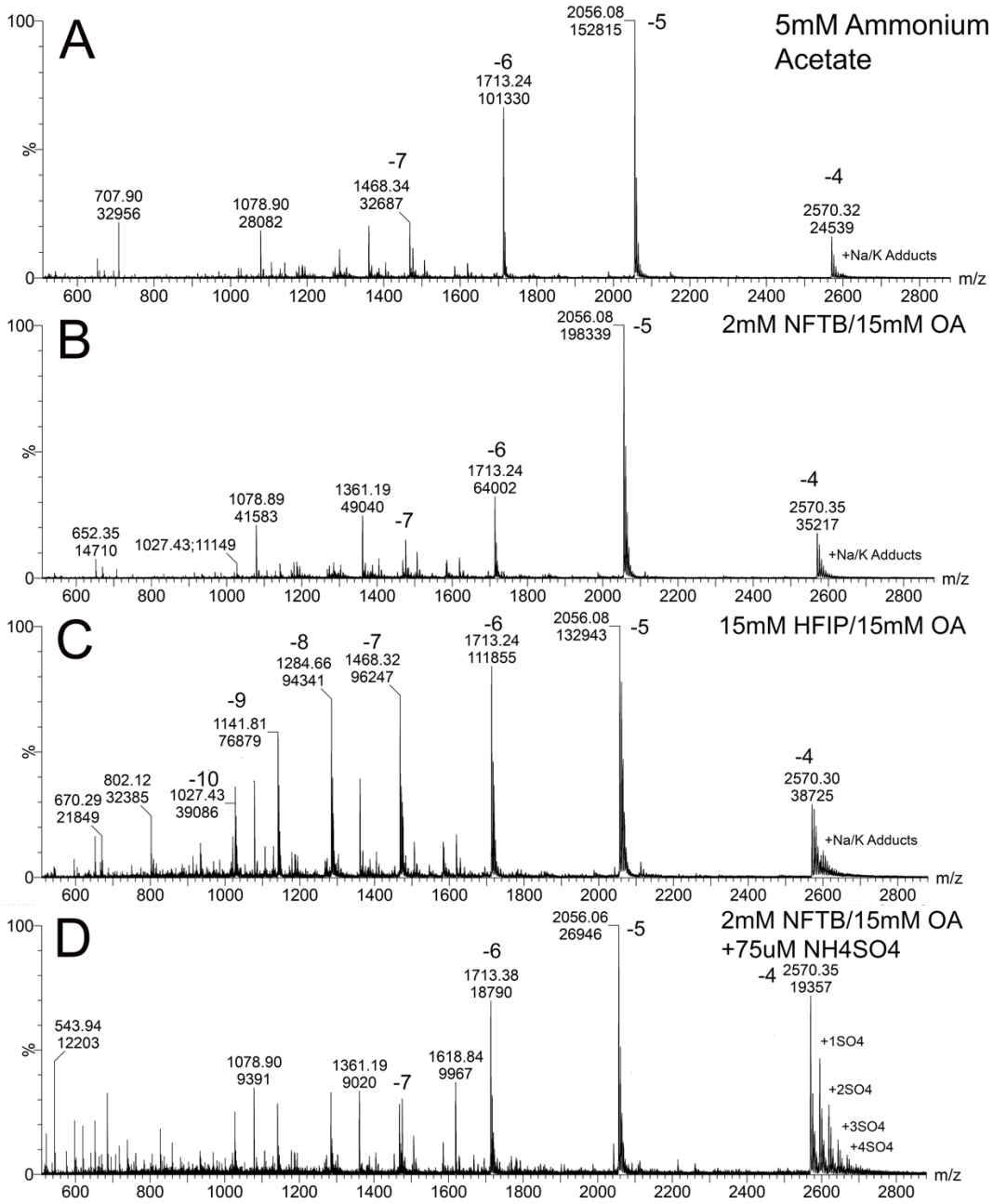
Mobile phase additives play a large role in the electrospray charge state distribution of oligonucleotides (Greig and Griffey 1995, Chen and Bartlett 2013, Erb and Oberacher 2014, Basiri, Murph et al. 2017). Basiri, et al. showed that the NFTB & OA mobile phase when used with a 33-mer phosphorothioate closely resembles the collision cross section of a similarly folded G-quadruplex by Baker et al. (Baker, Bernstein et al. 2006, Basiri, van Hattum et al. 2017). They propose that when the oligo is folded the collision cross section is half of the higher charge state linear forms. Similarly small collision cross sections were calculated for an RNA aptamer HpB at the $[M - 4H]^{4-}$ charge state (Ω_{EHSS} =1148.84±1.30Å^2 , $\Omega_{Protein}$ =1097.20±1.35Å^2) compared with the $[M - 18H]^{18-}$ charge state (Ω_{EHSS} =2418.68±12.01 Å^2, $\Omega_{Protein}$ =2608.39±20.55 Å^2). The $[M - 18H]^{18-}$ charge state is nearly 2.2 times larger than the $[M - 4H]^{4-}$ charge state, suggesting that the $[M - 18H]^{18-}$ charge state is a more linear structure for HpB and that the $[M - 4H]^{4-}$ charge state is likely in a compacted state, supplemental table 2. We propose that the -5 and -6 charge states have a larger solvent accessible area and are therefore less compacted in solution which corresponds to the most abundant charge state, which can be determined from the solvent accessible area (Rueda, Kalko et al. 2003, Rueda, Luque et al. 2005, Arcella, Portella et al. 2012).

HpB exhibits similar adduction behaviors to oligo 1 in the fact that sulfate appears to selectively adduct only lower charge states, figure 6. For example, the $[M - 4H]^{4-}$ charge state exhibits 4 sulfate adducts, similarly to the $[M - 3H]^{3-}$ charge state which also exhibits 4 sulfate adducts. Unlike oligo 1, HpB does not increase in size with the sulfate adducts on the $[M - 4H]^{4-}$

charge state, ammonium acetate (control) (Ω_{EHSS} =1148.40±0.76Å^2 , $\Omega_{Protein}$ =1096.30±0.78Å^2) vs sulfate (Ω_{EHSS} =1147.97±0.76Å^2 , $\Omega_{Protein}$ =1096.30±0.78Å^2).

Site-specific binding of metal ions has been shown for a 12-mer d(TTGGCCCTCCTT) where the binding was found to occur only on the central thymine but not on terminal thymines for Mg^{2+} and also UO_2^{2+} (Wu, Cheng et al. 1996). Oligo 1 is a 20-mer with 1-terminal thymine but 4 internal thymines, (T*C*C*G*T*C*A*T*C*G*C*T*C*C*T*C*A*G*G*G). Interestingly, when oligo 1 is infused with a solution of ammonium sulfate, there are a maximum of 4 sulfates found to be adducted to the $[M - 3H]^{3-}$ charge state which could correspond to the 4 internal thymine bases, figure 4E. While HpB is an RNA, the methyl loss on uracil vs thymine may not be critical for sulfate binding and HpB has 5 internal uracil bases, (GGCGAUACCAAGCCGAAAGGCUUGGUAUCGCC). When HpB is infused with a solution of ammonium sulfate, we can see in the spectra on the $[M - 4H]^{4-}$ peak there are 4 sulfate adducts of the 5 possible uracil binding sites. This can perhaps suggest that the sulfate is binding selectively to the thymine/uracil base of these oligonucleotides, similarly to the Mg^{2+} and UO_2^{2+} ions.

Figure 5.6: Mass spectra of a 32-mer aminoglycoside binding RNA aptamer, Y axis - % relative abundance X axis - m/z. **A:** 10uM HpB sprayed with 5mM Ammonium Acetate in 100% water (control), 5mM was optimized to obtain sufficiently high signal intensity. **B:** 10uM HpB sprayed with 2mM NFTB/15mM OA in 50/50 water/methanol. **C:** 10uM HpB sprayed with 25mM HFIP/15mM OA in 50/50 water/methanol. **D:** 10uM HpB sprayed with 2mM NFTB/15mM OA in 50/50 water/methanol + 75uM NH_4SO_4.



4. Conclusions

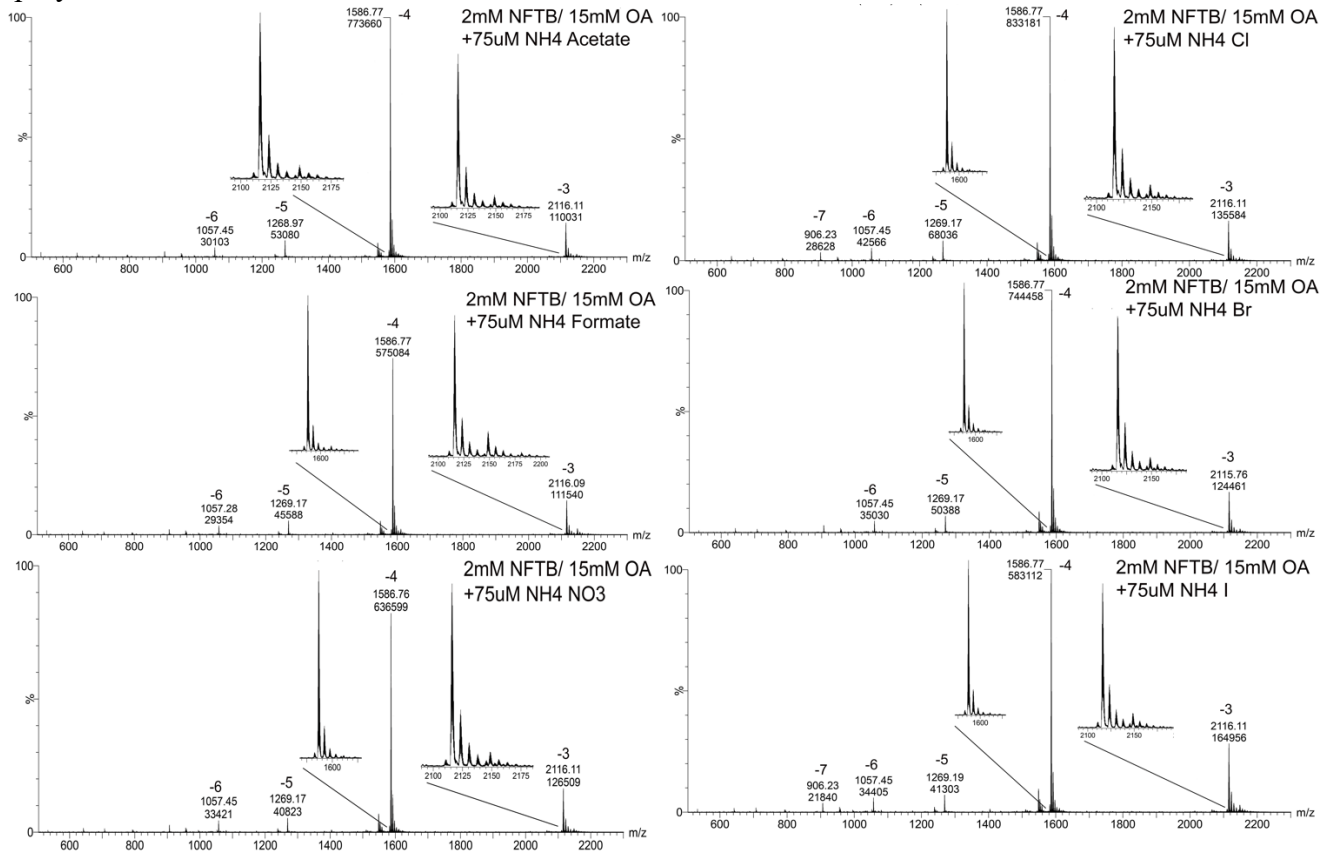
In this study we introduced the ability of a novel mobile phase composition to selectively reduce the charge state of oligonucleotides and for anions to selectively adduct to these reduced charge states. Anionic adduction was systematically studied for its ability to bind and its ability to stabilize lower charge state oligonucleotides. Anionic adduction has been studied for other molecules in the past, but this is the first report of anionic adduction for oligonucleotides. Hydrogen bonds and electrostatic interactions drive the anionic adduction. This is likely dictated by the matching proton affinities of the polyanionic backbone and the anionic salt in solution. This complex likely forms in solution and then, when the molecule is desolvated, the salt is not lost as a neutral like alkylamines (Sutton and Bartlett 2019).

This suggests the anions bind in a mechanism that is unique to the structure of these lower charge state oligonucleotides. In an attempt to elucidate the binding structure, ion-mobility was employed to measure the topology (CCS) of these anionic complexes and their ultimate effect on the topology of the oligonucleotides. Comparison between a protein and a nucleic acid calibration profile provided confidence in the relative size of each charge state analyzed. A trend was found that as the charge state decreases, the size of the oligonucleotide decreases, and this is a key feature for studying folding of oligonucleotides. These reduced sizes are likely due to the crumpled mismatched hydrogen binding of backbone and base which allows for anion binding of openly available protons. We have shown NFTB/OA is a good mobile phase choice to selectively study lower charge states of oligonucleotides with electrospray mass spectrometry and ion-mobility determinations.

Supplementary Table 5.1: Structures were drawn using MarvinSketch: MarvinSketch was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin 17.27.0, ChemAxon (https://www.chemaxon.com). Gas Phase Basicity values obtained from NIST webbook (Mallard). Atomic Radii obtained from a database of ionic radii (Shannon 1976).

	Chloride	**Bromide**	**Iodide**
Salt			
Anion GPB (kcal/mol)	N/A	318.21 ± 1.1*	309.20 ± 0.20*
Atomic Radii (Å)	1.81	1.96	2.2

Supplemental Figure 5.1: Mass Spectra of additional anionic salts sprayed with 10uM Oligo 1 sprayed with 2mM NFTB/15mM OA in 50/50 water/methanol with 75uM Ammonium Salt



Supplementary Table 5.2: Collision Cross Sections calculated for HpB using the EHSS nucleic acid calibration and the protein calibration. 10uM HpB sprayed with 5mM Ammonium Acetate in 100% water (control), 5mM was optimized to obtain sufficiently high signal intensity. 10uM HpB sprayed with 2mM NFTB/15mM OA in 50/50 water/methanol. 10uM HpB sprayed with 25mM HFIP/15mM OA in 50/50 water/methanol. 10uM HpB sprayed with 2mM NFTB/15mM OA in 50/50 water/methanol + 75uM NH_4SO_4.

Charge	CCS EHSS N2 (Ω, Å2)	CCS Protein Calibrants (Ω, Å2)
Ammonium Acetate		
-4	1148.84 ± 1.30	1097.20 ± 1.35
-5	1316.32 ± 1.57	1178.19 ± 1.56
-6	1476.90 ± 4.61	1254.82 ± 4.46
NFTB/OA		
-4	1147.97 ± 0.76	1096.30 ± 0.78
-5	1316.32 ± 1.57	1178.19 ± 1.56
-6	1478.39 ± 6.96	1256.27 ± 6.74
HFIP/OA		
-4	1147 ± 2.02	1095.38 ± 2.09
-5	1317.08 ± 2.69	1179.08 ± 2.69
-6	1481.46 ± 4.52	1259.24 ± 4.38
NFTB/OA + Ammonium Sulfate		
-4	1148.40 ± 0.76	1096.30 ± 0.78
-5	1319.00 ± 1.54	1180.87 ± 1.54
-6	1479.94 ± 5.21	1257.77 ± 5.05

CHAPTER 6

CONCLUSIONS

The experiments and results presented here provide a set of new ideas and provide the framework for future studies. The investigations regarding mobile phase additive optimization provides valuable insight into the electrospray ionization process. The analysis of oligonucleotides suffers significantly from sensitivity issues for both bioanalysis and impurity studies of therapeutic and biological oligonucleotide constructs.

Mass spectrometry as a tool for oligonucleotide analysis is critical to gather non-biased critical data for the understanding of oligonucleotide metabolism and degradation. Hybridization techniques out-perform mass spectrometry on a quantitative level due to their significantly better sensitivity, but hybridization cannot compete with mass spectrometry in regard to selectivity. In this aspect, it is important to choose the analysis technique best suited for the data required for a specific project. Routine use of mass spectrometry to quantitate oligonucleotides over using hybridization requires significant method development of mass spectrometry methods to compete with sensitivity. Data is presented here to enhance the sensitivity and improve the selectivity of oligonucleotides by controlling the amount of counterion adduction present during the mass spectral analysis.

Perhaps the easiest way to improve any analysis with mass spectrometry is by changing the sample, since the alternative is purchasing a new expensive instrument. Changing the sample can be as simple as increasing the concentration of the sample and as complex as optimizing the mobile phase additives. Oligonucleotides have a highly polyanionic backbone and when

analyzed with mass spectrometry in the negative ion mode they gain many negative charges in a non-homogenous manner. This results in the original concentration of oligonucleotides being spread out across many different charge states. In addition to this, these negative charges attract positively charged cations that may not be eliminated from the sample or are present during ionization. Optimization of the mobile phase additives are essential to reducing the number of charge states and preventing cationic adduction. Oligonucleotides are commonly analyzed with alkylamine ion-pairs combined with fluorinated alcohols. Alkylamines only differ in their physiochemical properties and chapter 4 provides both a qualitative model and quantitative model to study these physiochemical properties, their ability to reduce or promote cationic adduction and the deleterious effects on the mass spectral intensity and complexity. It was shown that despite using a ssDNA test set this analysis can be used for phosphorothioate and mircoRNA molecules successfully.

Ion-mobility mass spectrometry is a powerful tool that can characterize a molecules size in the gas phase. In recent years, this has been used to study the effects of gas-phase oligonucleotides successfully, in particular G-quadruplexes. Our lab has discovered that nonafluorotertbutyl alcohol (NFTB) when combined with long chain alkylamines, like octylamine (OA), can selectively reduce the charge state of oligonucleotides in the gas phase. In chapter 5 studied the effect of anionic salts on the ability to promote or stabilize these low charge state species and found that certain anions selectively adduct the polyanions in the gas phase. In particular, sulfate preferentially binds the lowest charge state present. Ultimately, we show that anionic salts can bind to these oligonucleotides and that perhaps this can provide mass spectrometry users with unique and useful information.

In conclusion, the studies herein evaluate mobile phase additives and their ultimate ability to electrostatically adduct to oligonucleotides. We provide a method to reduce cationic adduction in electrospray and provide physiochemical properties that are the major contributors to this phenomenon. Lastly, we show anionic salts selectively adduct certain charge states in solution and that this provides users with useful information. These studies will help the field of oligonucleotide mass spectrometry better understand the effect of mobile phase additives on electrostatic interactions that occur during electrospray desorption.

Perhaps the most limiting factor in utilizing mass spectrometry for all RNA biomarker studies and all therapeutic oligonucleotide analysis is the poor sensitivity of LC-MS. Fundamental studies like the ones presented here provide insight into desorption mechanisms and the effects of mobile phase optimization on the improvement of oligonucleotide analysis.

Despite the promising progress made in these studies there are some fundamental questions that can still be answered. This is the first-time anion adduction has been reported for an oligonucleotide. Constructs should be designed in a way to study the binding location of the anionic salts. Carefully designed constructs could reveal the precise binding location of these anions to the oligonucleotides. Orthogonal techniques could provide helpful insight into the effects of the anionic adducted oligonucleotide binding. It would be worth pursuing if the anionic binding could provide novel insights for MS/MS analysis of these oligonucleotides. In addition, utilizing heated UV to look at folded vs non-folded constructs and their ability to bind the oligonucleotides could provide a deeper understanding of the anionic binding effects.

There is still research that shows there is a small population of oligonucleotides that contain post-translational modifications in specific disease states. These studies require a control set of samples and a treated set of samples to analyze these micro-populations. Utilizing LC-MS

for these studies would be valuable since they can provide a higher degree of selectivity and survey these post-translational modification profiles. It is perhaps most important that efforts for these studies include mobile phase optimization to improve sensitivity and provide insight into these micro-populations. With current technologies these studies cannot be accomplished with current LC-MS technologies. There is promise in utilizing anionic adduction combined with MS/MS to obtain novel fragmentation and qualitatively analyze these micro-populations. Once these populations have been identified quantitation utilizing the most appropriate techniques could provide deeper insight into molecular processes of these disease states and potentially provide new insights into biomarker identification.

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