Optimisation of a Quality Control Method for Identity, Purity and Quantity for Oligonucleotide Therapeutics

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Introduction

Full length messenger ribonucleic acid (mRNA) based vaccines came into fame in the aftermath of the 2019 corona virus outbreak, which has rapidly developed into a global pandemic. The two mRNA vaccines developed by Moderna and BioNTech/Pfizer were the first mRNA therapeutics to be granted an Emergency Use Authorization by the EMA and US-FDA and proved to be a safe and efficacious treatments against COVID infection.

While full length mRNAs are a rather new class of therapeutic, shorter 18 to 40mer polynucleotide, so-called oligonucleotides, have been on the market for over two decades, with Fomivirsen being the first approved by the US-FDA in 1998. Since then (up to April 2021), a total of 13 oligonucleotide-based therapeutics have been approved by the US-FDA for several indication, most of them targeting the expression or translation of dysfunctional proteins.[1]

Natural RNAs however have a very limited therapeutic use as they have a very short half-life and their immunogenic properties limiting the maximum tolerated dose. These limitations are overcome by modifications of the oligonucleotides, some of which renders them resistant to nuclease degradation and reduce or inhibit immunogenicity.[2] Different types of modifications are used for this purpose e.g., modifications of the phosphate backbone, the ribose and more complex modifications like peptide nucleic acids and phosphorodiamidate morpholin. As most oligonucleotides approved to date only use on type of modification, we can expect to see different type of modifications used within one molecule to capitalise on the different physicochemical properties these modifications may lend to the molecule to modify half-life, affinity or immunogenicity.[1] What we can also expect to see are oligonucleotides conjugated to other molecules to achieve cell- or tissue-specific targeting.

Reliable analytical methods to display the quality (identity, purity and content) of an oligonucleotide is key throughout early development, preclinical and clinical phases and release testing. It is important to develop robust methods early on in a project, to have a history of critical quality attributes throughout product development, aids a data driven definition of meaningful acceptance criteria. The availability of platform methods with a good optimisation strategy enables the implementation of such methods early in new development programs without compromising time or costs.

In this application note we want to present an LC-MS based platform method for the determination of the identity, purity and quantity for oligonucleotides and which parameters are worth investigating to optimise the method in terms of chromatographic resolution of impurities, MS-parameter to minimise adduct formation to reduce the complexity of the mass spectra obtained.

Materials and Methods

Oligonucleotide standards were used to test the LC-MS system in terms of chromatographic and mass resolution as well as adduct formation and improving signal to noise; Oligo-T DNA by Waters MassPrep OST Standard (Waters cat.no.: 186004135-1), Agilent RNA resolution standard (Agilent cat.no.: 5190-3028). To optimise the parameters of the mass spectrometry system, in order to minimise adduct formation to monitor impurities and improve fragmentation to gain sequence information, a customised 40mer DNA oligonucleotide was designed (Thermo Fisher; Custom Standard DNA Oligos; ATG CCT TAA GCG ATG GAT TTT TTT CCA TCG CTT AAG GCA T). For chromatographic separation the Waters ACQUITY UPLC-Oligonucleotide BEH C18 (1.7 µm, 130 Å, 2.1 x 50 mm) (Water; cat.no. 186003949) and Waters ACQUITY Premier Oligonucleotide BEH C18 (1.7 µm, 1.2 x 50 mm) (Waters cat.no.: 186009484) were tested. The mobile phases were composed of LC-MS-grade water (Fisher Chemical W6-212; Fisher Scientific cat.no.: 10505904), as buffer we used Dibutylamine (DBA) (Merck KGaA cat.no.: 471232) and Hexafluoro-isopropanol (HFIP) (Merck KGaA cat. no.: 105228) was used as an ion pairing reagent. Analytical data was obtained from a Waters BioAccord System which includes ACQUITY I-class plus UPLC System (Binary Solvent Manager, Sample Manager FTN, ACQUITY Column Heater, TUV Detector) coupled to a Waters ACQUITY RDa Detector operated under the UNIFI Software (release version 1.9.9.3).

Setting up a Platform Method

As already pointed out, it is important to have a robust quality control method early on in development program to have a solid data set documenting product quality. In order to "afford" such a method early on in a development program, it is important to invest some initial work to identify a solid method setup, which can be readily adapted to suit the needs of other parallel or future oligonucleotide projects.

Therefore, we started with identifying a chromatographic setup able to resolve oligonucleotides in a suitable mass range for most anti-sense oligonucleotides (18 to 30 mer). Therefore, we selected the Waters Oligo-T standard which featured thymidine oligomers from 15 to 35 in 5mer intervals to test different chromatographic conditions. Looking at suggestions for oligonucleotides provided by the vendors of chromatography columns and other work groups, we investigated the combination of 15 mM DBA as a buffer and 25 mM HFIP as an ion pairing reagent in water for eluent A and in methanol for eluent B. We frequently observed droplets in the aqueous eluent due to the limited miscibility of HFIP in water. In an attempt to eliminate droplet formation, the HFIP was reduced to 15 mM, but this caused a broadening the chromatographic peaks of the of the thymidine oligomers. The addition of 5% (v/v) methanol to eluent A with 25 mM HFIP stabilised the solution and eliminated droplet formation with no adverse effect on the chromatographic resolution (Figure 1). This setup is compatible with mass spectrometric detection as

both, the ion pairing reagent and the buffer, evaporate and are removed in the first stages of the vacuum system.

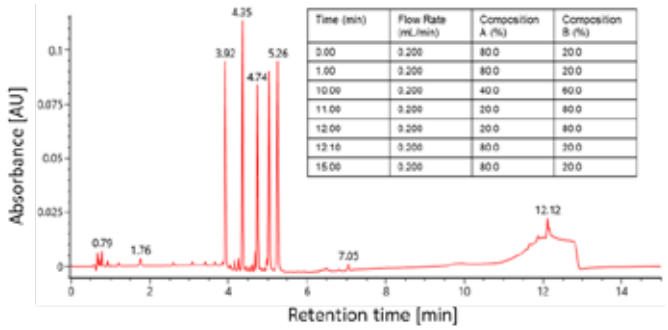

Figure 1: Standard chromatographic conditions for the separation of 15 to 35 mer oligonucleotides. Eluent A: 95% (v/v) water, 5% (v/v) ethanol, 25mM HFIP, 15mM DBA. Eluent B: methanol 25mM HFIP, 15mM DBA. Column: ACQUITY Premier Oligonucleotide, BEH C18 column, 1.7µM; 1.2 x 50 mm. Column temperature: 60°C

The MS-System was setup according to the recommendation of the vendor for oligonucleotide analysis (polarity: negative; mass range: 400-5000 m/z; cone voltage: -30V). The resulting mass spectra were densely populated with signals. In case of the 25 oligomer, we could observe overlapping ion series belonging to DBA and sodium adducts. The DBA and sodium adduct ion series of the 25 oligomer make up the largest ion population, resulting in deconvolutet mass spectra in which the signal representing the free 25 oligomer is barely visible over the noise. The strong adduct formation and the densely populated mass spectra hinder the identification of low-level impurities which may not be resolved by chromatography and may interfere with the spectral deconvolution, leading to inaccurate mass calculations. Therefore, different cone voltages (voltage offset applied between spray needle and MS-orifice) were tested to evaluate ionisation conditions capable of stripping adducts but keeping covalent bonds of the molecules intact. The optimal voltage is dependent on the different parameters e.g., the mass and charge state of the oligonucleotide or the nature of the adduct. In our case a cone voltage of -80V was sufficient to remove the DBA adducts (Figure 2).

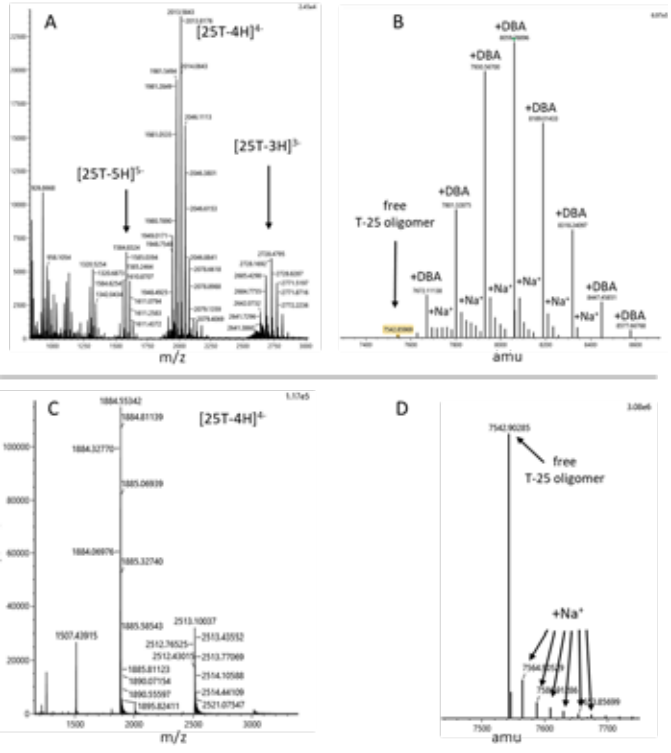

Figure 2: Mass spectra and the transformed masses obtained with different cone voltages to remove adducts from the 25 thymidine oligomer (25T). The mass spectrum A) was obtained with a standard cone voltage of -60V. The transformed masses in B) clearly show clusters of DBA and sodium (Na+) adducts. The Mass spectrum C) was obtained with a cone voltage of -80V which removed all DBA and most of the sodium adducts as can be seen in the transformed masses in D).

Optimisation of the Chromatographic System for RNA Oligonucleotides

An RNA-based oligonucleotide mix (Agilent RNA resolution standard), which is in a similar mass range (14 to 21 mer) than the previously tested thymidine oligomer mix, was subjected to the chromatographic setup described above. Only two peaks out of the four expected peaks could be observed in the chromatogram. With slight changes to gradient program, it was possible to achieve full baseline resolution of all oligomer species, even of the 20 and 21 mer oligonucleotide (Figure 3). This indicates that the chromatographic setup, the combination of column and eluent system, can be utilised as a platform setup from which molecule-/project specific methods can be readily adapted.

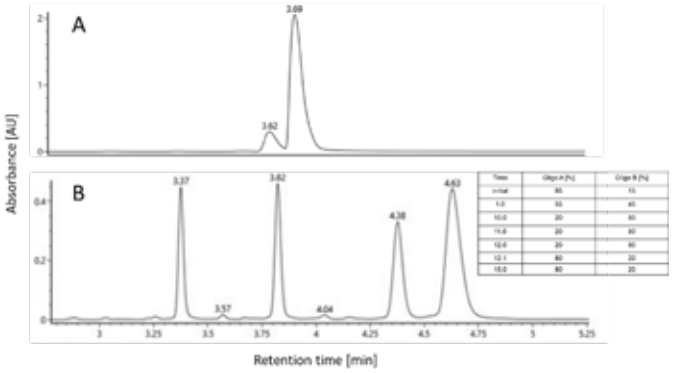

Figure 3: Chromatograms of the Agilent resolution standard, a mix of four RNA oligonuclotides (14, 17 20, 21 mer). Chromatogram A was obtained with the standard gradient program. Chromatigram B was obtained with an optimized gradient while all other parameters remained unaltered.

Setup of the Mass Spectrometer to Obtain Sequence Information

Mass spectrometers are not only powerful tools to monitor compounds that cannot be resolved chromatographically (impurities under the main peak) or investigating the identity of unknown impurities, but especially fragmentation data of oligonucleotides can be used to confirm the sequence (identity). Depending on the mass spectrometer (mass separation technology) applied, the maximum length of an oligonucleotide, from which reliable sequence information can be derived, is about 40 nucleotides in length. Oligonucleotides can be readily sequenced by collision induced fragmentation in a mass spectrometer. The fragmentation mainly occurs at the phospho-diester bonds between the nucleotides and the fragmentation is more or less random, giving rise to ions that resulting from cleavages at each phosphate-diester. However, fragmentation occurs at four possible sites in each phosphate-diester link (see Figure 4), whereas the charge from cleavage may reside at the 5´ or the 3´ end, giving rise to 8 possible fragment ions describing one part of the sequence. When choosing

suitable fragmentation settings, it is important to make sure, that there are sufficient fragments representing the full oligonucleotide sequence, while on the other hand not to induce too much fragmentation of the nucleobases which do not hold sequence information. Even though the oligonucleotides fragment in distinct units, it is still time-consuming for a skilled mass spectrometrist to manually piece together the full sequence, due the many fragments generated. There a very useful software tools available to support the sequence building process, however they rely on good quality fragmentation spectra and in doubt on skilled humans.

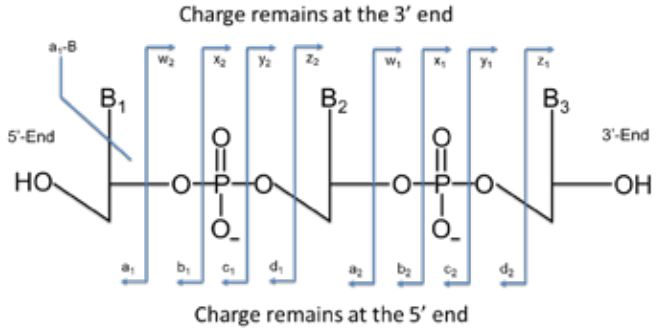

Figure 4: Fragmentation scheme of nucleotides. In a_n to d_n the charge resides at the 5´-End of fragment ion; in w_n to z_n at the 3´-End. The fragments resulting from nucleobase cleavage are termed a_n-B or w_n-B depending on where the charge resides.

In this example an unmodified 40mer DNA oligonucleotide was used to optimise the fragmentation. This represents, what experts perceive, as the maximum sequence length that can be readily sequenced by mass spectrometry. The fragmentation energy (fragmentation cone voltage) was subsequently increased to achieve maximum backbone (phosphate-diester) fragmentation, without inducing nucleobase sidechain fragmentation. The fragment spectrum gained only allowed the Spectrum Tools software (Waters) to determine 62% of the DNA sequence.

Conclusion

Reversed phase chromatography using a mass spectrometry compliant buffer system (DBA at 15mM) and ion pairing reagent (HFIP at 25mM) is a robust system to resolve oligonucleotides in a 14 to 40 mer length. The addition of 5% (v/v) to the aqueous eluent stabilised the poorly miscible HFIP without interfering with the chromatographic properties. An LC-method optimized for a DNA oligonucleotide mix was readily optimised to resolve a RNA Oligonucleotide mix by only slightly changing the gradient program. Since the parameter settings of MS-systems is highly dependent on the system used, recommendation of general settings cannot be given. But commonly ionisation or ion source settings should be identified that induce sufficient energy to strip off buffer and salt adducts and reduce the complexity of the mass spectra to enable monitoring and identification of low level impurities. The identity of an oligonucleotide is conferred from this nucleotide sequence. Most MS-systems should be able to produce quality fragmentation spectra to enable sequence analysis up to a 40mer oligonucleotide. Here it is important to find fragmentation energy settings that effectively fragments the phosphor-diester backbone by not inducing nucleobase fragmentation.

LC-MS is a robust readily adaptable quality control method for oligonucleotides. It can be implemented early on in projects to collect QC-data on the product throughout the development program and may be validated later on for release and stability testing.

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