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A high-throughput flow injection inductively coupled plasma mass spectrometry method for quantification of oligonucleotides

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ABSTRACT

Quantification of oligonucleotides is commonly performed by UV absorption or fluorescence based assays. However, chemical modifications may change the UV absorbance or fluorescence of oligonucleotides, making their accurate quantification difficult. Alternatively, atomic spectrometry can be used for direct quantification of phosphorus content in oligonucleotides, and their concentration can then be calculated using the phosphorus to DNA/RNA nucleotide mass ratio. Nevertheless, these methods require labor-intensive sample preparations, and thus have relatively low sample analysis throughput. In order to meet the need to quantify large libraries of synthetic structurally-modified oligonucleotides in drug discovery, a high-throughput inductively coupled plasma–mass spectrometry (ICP-MS) method has been developed in this study. Specifically, a high-performance liquid chromatography (HPLC) system is utilized in automated flow injection (FI) mode and coupled to an ICP-MS instrument to provide rapid and accurate quantification of phosphorus content in synthetic small interfering RNAs (siRNAs). Isobaric interferences at $^{31}P^{+}$ are avoided by monitoring the $^{31}P^{16}O^{+}$ molecular ion under optimized ICP-MS conditions. The use of a well-plate autosampler reduces sample injection cycle time and adds flexibility to the system by allowing direct analysis of aqueous samples in 96/384 well-plates without sample preparation. A throughput of 39 s per sample has been realized. The method requires minimal sample consumption (1–100 μL), which overcomes a major drawback of the conventional methods and makes it possible for practical use of oligonucleotide screening. Other advantages of this approach include excellent sensitivity (LOD = 136 ng L^{-1} as phosphorus), large linearity range and reduced solvent usage. The FI-ICP-MS method provides the capability of accurate quantitation of a large number of oligonucleotide samples with significantly improved sample analysis throughput over conventional methods.

1. Introduction

In the past decade, oligonucleotides as tool compounds for drug discovery or therapeutic medicines have gained significant interest in both academia and the pharmaceutical industry [1]. Oligonucleotides, including small interfering RNAs (siRNAs), microRNAs (miRNAs) and antisense oligonucleotides (ASO) are capable of interfering or blocking the normal translation of messenger RNA to its protein product [2,3]. This process has been utilized extensively to validate gene targets for supporting drug discovery, and has been of particular interest to the pharmaceutical industry due to its ability to interfere with targets that are considered undruggable by traditional small molecule therapies [4]. Recent advancements in RNA targeting and delivery technologies combined with evidence of clinical efficacy of oligonucleotide drugs have fueled rapid expansion of investment in developing oligonucleotides as therapeutic medicines [5–7]. Numerous clinical trials are currently ongoing for oligonucleotide drugs for a broad range of indications including viral infections, high cholesterol, cancer and muscular dystrophy [8–10].

Accurate quantification of oligonucleotides is essential for supporting their use in drug discovery and development. In the drug discovery stage, high sample throughput for quantification is also desired as oligonucleotides are often synthesized in a library format and in large numbers. High-throughput methods would allow rapid screening of more diverse sets of compounds, enabling discovery of candidate leads that would not be predicted by rational design.

Quantification of oligonucleotides has conventionally been performed by UV adsorption (OD 260 nm) [11] or fluorescence spectroscopy [11,12]. UV absorption is a rapid and simple technique, but suffers from low sensitivity and an insufficient selectivity due to the inaccuracies introduced by other UV absorbing functional groups or impurities [13]. Fluorescence spectroscopy provides highly sensitive detection; however, the presence of matrix constituents in the sample strongly affects the fluorescence intensity, making this method difficult to be applied as a definitive quantification method [14]. Another major disadvantage of these methods is that they require a

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well-characterized or quantified oligonucleotide reference material that often does not exist in the drug discovery or early development stages, particularly for structurally modified oligonucleotides.

A more recent development in oligonucleotide quantification is through direct determination of phosphorus, which is an effective method for accurate quantification of oligonucleotides due to the fixed stoichiometry of phosphorous from phosphodiester or phosphorothioate linkages in the oligonucleotide. The analysis of phosphorus can give a first, and more significantly, a precise indication of what the siRNA concentration will be in a certain sample. Atomic spectrometry is ideally suited for measurement of phosphorus, and the detection is theoretically independent from the molecular structure of the target analyte. Consequently, accurate quantification can be performed by using species-unspecific standards, e.g., inorganic phosphate salts, for all the oligonucleotides including those structurally modified [15].

Quantification of oligonucleotides using inductively coupled plasma optical emission spectrometry (ICP-OES) through direct determination of phosphorus content has been reported [16–19]. Although high accuracy has been achieved, the limit of detection for this method is not sufficient to cover samples with low (ng mL^{-1} level) phosphorus concentrations.

Inductively coupled plasma–mass spectrometry (ICP-MS) offers higher sensitivity and provides a wide linear dynamic range (up to eight orders of magnitude). However, the quantitative determination of phosphorus – as a mono-isotope element at mass 31 u – by a quadrupole-based ICP-MS is hindered by its high first ionization potential (~10.5 eV) and severe spectral interference derived from the sample matrix and laboratory environment. Isobaric interferences such as $^{15}N^{16}O^{+}$ (30.995 u), $^{14}N^{16}O^{1}H^{+}$ (31.00583 u), $^{12}C^{1}H_{3}^{16}O^{+}$ (31.01839 u), all with a nominal mass to charge ratio of 31, overlap with the $^{31}P^{+}$ signal (30.97376 u) significantly [20]. This problem can be resolved by the use of a double-focusing sector field ICP-MS [20,21] that has an enhanced mass resolution (m/Δm) greater than 1500; sufficient to separate the interferences from the P signal. The main drawback of this technique is the high cost of such high resolution ICP-MS instruments. A recent development in phosphorus determination by a quadrupole-based ICP-MS is to determine the oxide ion $^{31}P^{16}O^{+}$ (47 u), thus avoiding the isobaric interferences at 31 u, as long as any other spectral interference at mass 47 u (e.g., $^{47}Ti^{+}$) is negligible. This is normally realized with a reaction/collision cell using oxygen as a reaction gas [17,22,23]. However, under optimized conditions the oxide ion can be formed without the use of oxygen as reaction gas [24].

Both ICP-OES and ICP-MS methods require labor intensive sample preparation steps for direct phosphorous quantitation in oligonucleotides, which limits the sample analysis throughput. In addition, both approaches require relatively large amount of samples (milliliter range), restricting their use for the routine quantification of oligonucleotides in the drug discovery stage where the sample availability is often limited. This has become a limiting factor for practical use of the technique. While there is a substantial need for a high-throughput analytical method for accurate quantitation of synthetic structurally-modified oligonucleotides using small amounts of sample, particularly in the drug discovery stage, no such methods have been reported in the literature, to the best of our knowledge.

In this study, we report the development of a high-throughput microanalytical procedure for quantification of low volume siRNA samples (down to a volume of 1 μL). In our previous work, a high-performance liquid chromatography (HPLC) system was utilized in automated flow injection (FI) mode and coupled to a quadrupole ICP-MS for rapid assessment of residual metal content in pharmaceutical samples [25]. The flow injection analysis system has been modified to provide direct, fast and accurate quantification of phosphorus content in synthetic siRNAs.

2. Experimental

2.1. Instrumentation

A FI-ICP-MS method has been developed in our laboratory for fast screening of metal concentrations in organic solvents [25]. For this application, the configuration was simplified by removing the membrane desolvation unit since only aqueous solutions were used. An Agilent 7500cs ICP-MS (Agilent Technologies, Wilmington, DE, USA), which was equipped with an Octopole Reaction System (ORS), was used for the determination of phosphorus. An Agilent 1100 HPLC binary pump system with a vacuum degasser was used for the introduction of a continuous carrier stream, and a thermostated HPLC regular autosampler or a well-plate autosampler was used for sample injection. No HPLC column was used and the outlet was connected directly to the nebulizer. Deionized water with a flow rate of 1.0 or 2.0 mL min^{-1} was used as a carrier stream. The injection volume for each sample ranges from 1 to 100 μL, with larger volumes used for samples with lower phosphorus concentrations. "Chromatographic" data analysis was made by using the Agilent Plasma Chromatographic software.

The analysis is performed under optimized conditions for the oxide ion ($^{31}P^{16}O^{+}$) in order to avoid isobaric interferences. The initial instrument tuning was performed with a tuning solution of 10 μg L^{-1} of ^{7}Li, ^{89}Y, ^{140}Ce and ^{205}Tl in 2% nitric acid. Then it was optimized to give the maximum oxide ion formation ratio ($^{140}CeO^{+}/^{140}Ce^{+}$) without the introduction of oxygen as a reaction gas. Further optimization was performed by maximizing the signal at *m/z* 47 ($^{31}P^{16}O^{+}$) using a solution of 500 μg L^{-1} phosphorus (as PO_4^{3-}) standard solution in deionized water. The instrument settings were checked daily and optimized when necessary.

The ChemStation and Plasma Chromatographic software integrated with the Agilent HPLC and ICP-MS makes the FI-ICP-MS analysis a fully automated process, and this configuration is well suited for an easy and robust setup of high-throughput phosphorus analysis and for standardization.

For purpose of comparison, a Thermo Scientific iCAP 6500 ICP-OES spectrometer (Thermo Scientific, Pittsburgh, PA, USA) was also used for sample analysis using a conventional procedure [26]. Operating conditions of all the instrumentation are summarized in Table 1.

Table 1
Operating parameters for the instruments used in the experiments.

Agilent 7500cs ICP-MS conditions for FI-ICP-MS	
RF power	1050 W
Plasma gas flow	14.9 L min^{-1}
Auxiliary gas flow	1.15 L min^{-1}
Spray chamber temperature	1 °C
Sampling depth	4.0 mm
Peristaltic pump speed	0.50 rps
Isotope monitored	47 (PO^{+})
Acquisition mode	Time resolved analysis
Integration time	0.50 s
Agilent 1100 HPLC conditions	
Mobile phase	Deionized H_2O
Flow rate	1.0 to 2.0 mL min^{-1}
Injection volume	1–100 μL
No HPLC column was used	
Thermo scientific ICP-OES conditions for P analysis with conventional sample introduction	
Power	1350 W
Coolant gas flow	12 L min^{-1}
Auxiliary gas flow	0.50 L min^{-1}
Nebulizer gas flow	0.75 L min^{-1}
Plasma view	Axial
Emission lines	177.495 nm, 178.284 nm, 185.942 nm
Sample introduction	Concentric nebulizer with a cyclonic chamber
Number of repeats	3