TOSOH

Oligonucleotide purification and analysis

A chromatography overview

Tosoh Bioscience is your expert partner for the analysis and purification of biomolecules in the laboratory and in manufacturing.

Our chromatography columns and media are produced according to Japanese values and principles. This guarantees the highest possible quality and reliability over many years.

Sharing our expertise with individual customers is our top priority. This is appreciated particularly by users in the pharmaceutical and biotech industries and in academia.

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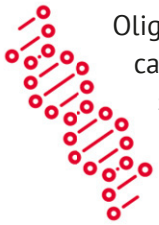
What to expect from this whitepaper

Oligonucleotide-based therapeutics have been investigated over the last decades and their promise as a new drug modality is now being realized. There has been tremendous development since the first antisense approach in living cells in 1978. The growing interest in oligonucleotides is driven by the high potential of oligonucleotides to be used in the treatments of a variety of medical conditions, the growing number of FDA approved oligonucleotide drugs, and an increased focus on personalized medicine and on the development of therapies for rare diseases. Two major types of oligonucleotide drugs are currently being developed as therapeutic platforms for the reduction of target gene expression: short interfering RNA (siRNA) and antisense oligonucleotides (ASOs). The potential of aptamer RNAs that modulate protein functions is another popular area of research.

The increased demand for oligonucleotides requires a cost-effective and easy scale-up from research amounts to commercial needs. Chromatographic methods are part of the typical purification schemes of therapeutic oligonucleotides. (U)HPLC methods are also applied for purity control and characterization of synthetic oligonucleotides. This whitepaper will give you an **overview of the current methods** that are available for purifying and characterizing oligonucleotides. **You will get to know the advantages and disadvantages of the various chromatography methods available and receive useful practical tips for simplifying your processes and reducing costs.**

The whitepaper will help you to select the appropriate column materials and chromatography media and introduce you to viable approaches to specific applications.

1. The purification of therapeutic oligonucleotides



Oligonucleotides (ONs) are short (typically 20-25 bases) synthetic single strand or double strand DNA or RNA molecules that have a wide range of applications in genetic testing, research, forensics, and therapy. In nature, oligonucleotides are usually found as small RNA molecules acting in the regulation of gene expression (e.g. microRNA).

Oligonucleotides are synthesized chemically on solid supports through repeated cycles adding the desired nucleotides to the enlarging molecule. As a consequence, the typical purification scheme is different and less complex than the purification of a large recombinant biomolecule - e.g. a monoclonal antibody - out of a cell culture feedstock.

Oligonucleotides are synthesized stepwise including the repeated use of protection groups. Upon synthesis, an appropriate deprotection method is selected. Subsequent purification involving liquid chromatography ensures the purity required for the intended use.

The removal of N-1 (shortmer) and N+1 (longmer) impurities is the most important purification task to be achieved in the chromatography step. These impurities emerge through the faulty addition of two similar nucleotides in one cycle or a failed coupling of the last nucleotide.

Chromatography can also separate ONs carrying protection groups from deprotected oligonucleotides and it can even facilitate the cleavage and removal of protection groups.

Different chromatographic techniques are used to implement successful purification strategies:

1. Ion exchange chromatography (IEC)

2. Hydrophobic interaction chromatography (HIC)

The selection of the chromatographic mode depends on the intended use.

Modified Oligonucleotides

Oligonucleotides for therapeutic use are often modified to enhance stability, improve base pairing or improve pharmacokinetics. Locked nucleic acids (LNAs) are modified RNA nucleotides in which the ribose moiety is modified with an extra bridge connecting the 2‘ oxygen and 4‘ carbon. The bridge „locks“ the ribose and enhances base stacking and backbone pre-organization. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide and hybridize with DNA or RNA according to Watson-Crick base-pairing rules.

Phosphorothioate (PTOs) are the most widely used nuclease resistant oligos for antisense applications. In PTO oligos, a non-bridging oxygen is replaced by a sulfur atom. Phosphorothioates can be introduced to an oligo at the 5‘- or 3‘-end in order to inhibit exonuclease degradation or internally to inhibit endonucleases. Both PTOs and LNA-containing oligonucleotides can be purified and analyzed using the same methods employed for standard oligonucleotides.

1.1 Removal of N-1 and N+1 impurities by anion exchange chromatography

Ion exchange chromatography is based on the competitive interaction of charged ions or molecules. The target molecules compete with salt ions for the charged positions on an ion exchange matrix. For the separation of nucleic acids, anion exchange chromatography is one of the most popular chromatographic modes.

Anion exchange chromatography can achieve purities over 96%.

Every nucleotide typically adds one negative charge of the phosphate group backbone to the overall charge of an oligonucleotide. Therefore, ONs of different length can be easily separated by anion exchange chromatography (AIEX).[1]

Quaternary ammonium groups (Q) or the weak alkaline diethylaminoethyl (DEAE) group are typical

Load

Buffer A low salt concentration

Elution

Buffer B high salt concentration

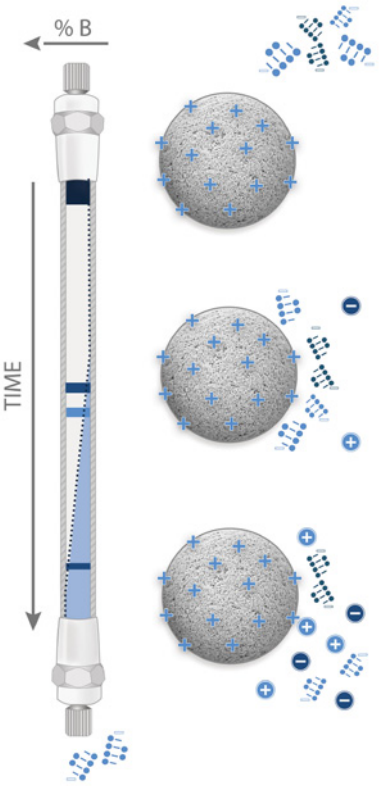


Fig. 1: Ion exchange chromatography.

anion exchange ligands. The ion exchange phase is equilibrated with a binding buffer of low ionic strength that enables the binding of the target molecule to the charged groups of the stationary phase. After an optional washing step, the bound target is typically eluted by increasing the ionic strength of the mobile phase through a salt gradient.

The high performance anion exchange medium TSKgel SuperQ-5PW (20) has an impressive track record in the purification of oligonucleotides for various therapeutic approaches.[1,2] Through its relatively small particle size of 20 µm it ensures high resolution while providing a high loading capacity at the same time. The higher purity and capacity achieved with TSKgel SuperQ-5PW compared to other commercially available AIEX media pays off in better process economics at large scale manufacturing.

One example is the purification of P=O, P=S and P=O/P=S mixed backbone oligonucleotides through AIEX mentioned in patent EP 2 540 734 A2 filed by Alnylum Pharmaceuticals Inc., the manufacturer of the first approved siRNA therapeutic.

Industry proven purification

Anion exchange chromatography with TSKgel SuperQ-5PW is one of the most cited approaches to purify oligonucleotides for therapeutic use. To ensure the best productivity and efficiency of manufacturing while putting patient safety first key players in the field rely on TSKgel® SuperQ-5PW (20) for the purification of siRNA-based biopharmaceuticals. This high-resolution anion exchange resin offers high purity at high loading, accounting for more efficient production processes. As published in 2011 by a team of scientists of former Roche Kulmbach GmbH, now Axolabs, on the manufacturing strategies for siRNA, TSKgel SuperQ-5PW delivered the best separation between single stranded (ss) and double stranded (ds) oligonucleotides.[2]

1.2 A useful tool for trityl-on oligonucleotides: Hydrophobic interaction chromatography

In most production schemes all protection groups used during synthesis are cleaved by acidic and/or alkaline treatment. But the ON can also be submitted to chromatographic purification with the terminal 5′-DMT (dimethoxytrityl) group attached (DMT-on; trityl-on). The trityl-group increases the hydrophobicity of the ON and accordingly, reversed phase and hydrophobic interaction chromatography are very effective in separating the DMT-protected oligonucleotide from charged impurities

Hydrophobic interaction chromatography (HIC) is a useful tool to separate molecules based on their hydrophobicity, while maintaining their native configuration and biological activity. Nonpolar surface regions of a sample are basically exposed at high salt concentrations and bind to the hydrophobic ligands of the matrix (e.g. alkyl groups).

The greater the hydrophobicity of the molecule, the less salt is required for the binding to the HIC medium. Stationary phases for HIC typically use ethyl, butyl, hexyl, phenyl or polypropylene glycol groups as the functional groups. Elution is usually performed over a decreasing salt gradient. With decreasing ionic strength, the grade of hydrophilic regions of the analytes increases and they elute.

HIC can combine purification and deprotection of oligonucleotides.

The DMT protection group adds a highly hydrophobic part to the full-length ON relative to all failure sequences. The DMT-on target binds to the HIC resin while failure sequences missing the 5′-DMT group will not bind and elute in the flow-through. The bound full-length ON can then be eluted in a very high purity and the 5′-DMT group can be easily removed. Trityl-on purification is particularly effective for the purification of long oligonucleotides (40 to 150 nucleotides). Most TOYOPEARL HIC media can be used for this approach.[3]

An additional advantage of Trityl-on purification through HIC is that the bound full length ON can be cleaved from the DMT protection group on-column after the unprotected impurities were washed off the column. A shift to acidic pH cleaves the ON and leaves the DMT group bound to the resin. The DMT can be removed and the column can be regenerated by cleaning-in-place (CIP). Through this approach a purity of >99% at a recovery of 99% of was achieved using TOYOPEARL Phenyl-650M and on-column cleavage at about pH 4.[3]

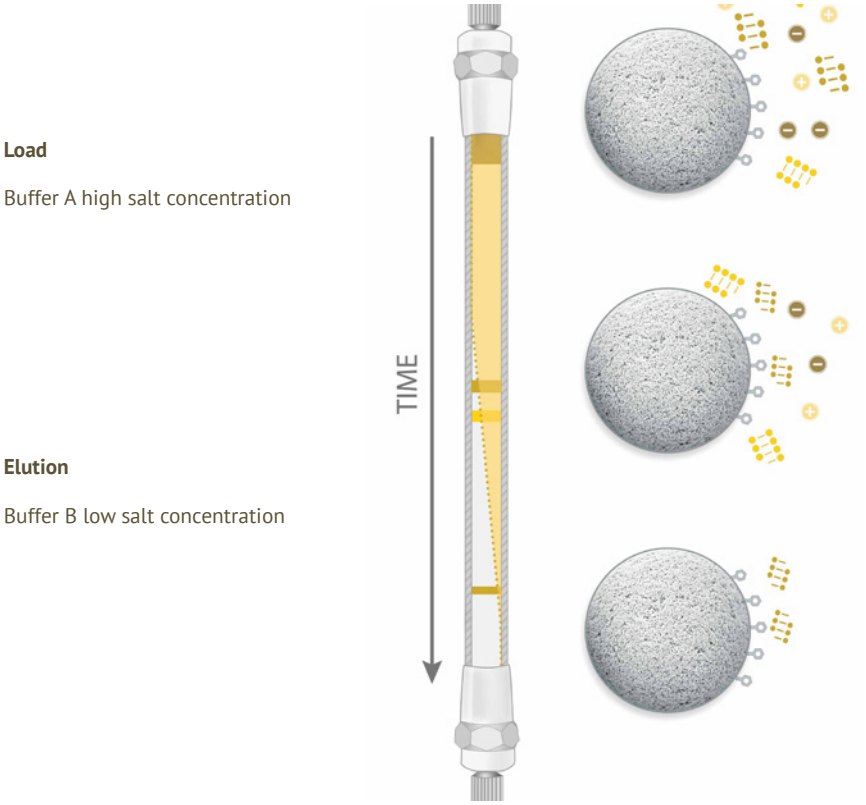


Fig. 2: Hydrophobic interaction chromatography (HIC)

HIC for Plasmid Purification

The recent developments in gene therapy and DNA vaccine have fostered the development of efficient plasmid DNA (pDNA) purification processes. Hydrophobic interaction chromatography is a popular tool for purification of supercoiled plasmids for gene therapy.[4]

2. Analytical chromatography to characterize oligonucleotides

In principle, the same modes that are used for purification of oligonucleotides are also applied for characterization and quality control analysis. In order to achieve short analysis times while retaining high resolution, non-porous particles are often used for ON analysis by HPLC or UHPLC.

A more detailed characterization of the molecular structure can be achieved when hyphenating (U)HPLC with mass sensitive detection such as multi-angle light scattering or mass spectrometry. We will focus on three examples for the use of analytical (U)HPLC for ON analysis. A comprehensive review on the characterization of therapeutic oligonucleotides was recently published by Alexandre Goyon and co-workers from Genentech.[5]

2.1 Impurity profiling by AIEX – a routine analysis

The purity of a synthetic oligonucleotide can easily be checked by ion exchange chromatography. Due to the negative charge of the ON, anion exchange is again the method of choice. TSKgel (U)HPLC columns based on non-porous particles are available with the same surface and ligand chemistries as the TOYOPEARL and TSKgel purification media. A non-porous base matrix ensures fast kinetics and high resolution of the separation. TSKgel DNA-STAT or DNA-NPR columns have been successfully applied to reliably quantify the amounts of N-1 and N+1 impurities. Figure 3 shows a purity check of a 20mer ON within 20 minutes analysis time.

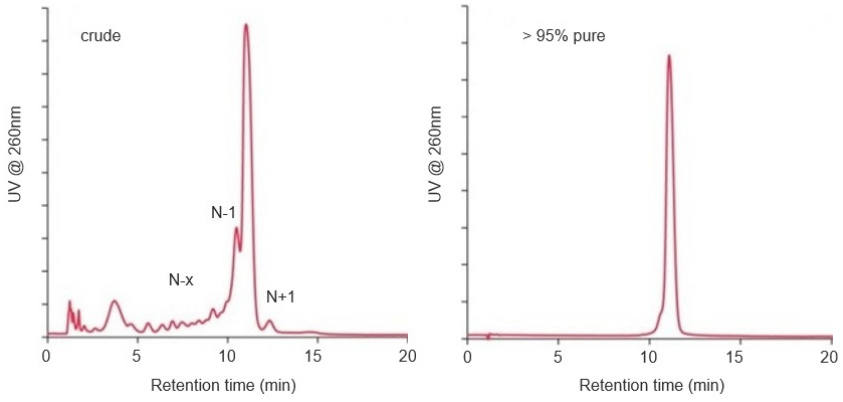


Fig. 3: AIEX analysis of crude and purified 20mer oligonucleotide on TSKgel DNA-STAT

Plasmid Analysis by AIEX

Analytical anion exchange chromatography is also very popular to check the purity of plasmid DNA. Pharmaceutical grade plasmids are used for various application such as gene therapy or vaccination. Urthaler et al.[4] describe the use of TSKgel DNA-NPR to analyze the amounts of supercoiled or covalently closed circular (ccc), open circular (oc), and linear forms of plasmids that are obtained by an improved downstream. Today, this 5 minutes method is widely used in the industry.

„The availability of identical ligand chemistries from UHPLC columns over prepacked method development columns to industrial process resins simplifies method development for purity control and manufacturing."

2.2 SEC-MALS – for deeper insights

The purity check of therapeutic oligonucleotides is typically done by AIEX or capillary gel electrophoresis. Size exclusion chromatography (SEC) can also be applied as orthogonal method or for hyphenation with light scattering detection.

SEC separates by hydrodynamic volume, ideally without interactions between sample and stationary phase. Best separation of biomolecules is achieved when using silica-based columns. UHPLC columns have now also become available for high resolution, yet robust and reproducible SEC-analyses. The TSKgel UP-SW series is going to become a new standard for this.

SEC over a bank of two 12.5 nm pore size TSKgel UP-SW2000 UHPLC columns can discriminate oligonucleotides differing by one base in length. Multi-angle light scattering detection on a Tosoh LenS3™ MALS detector can be used to get a more detailed picture of the purity of the oligonucleotide.[6]

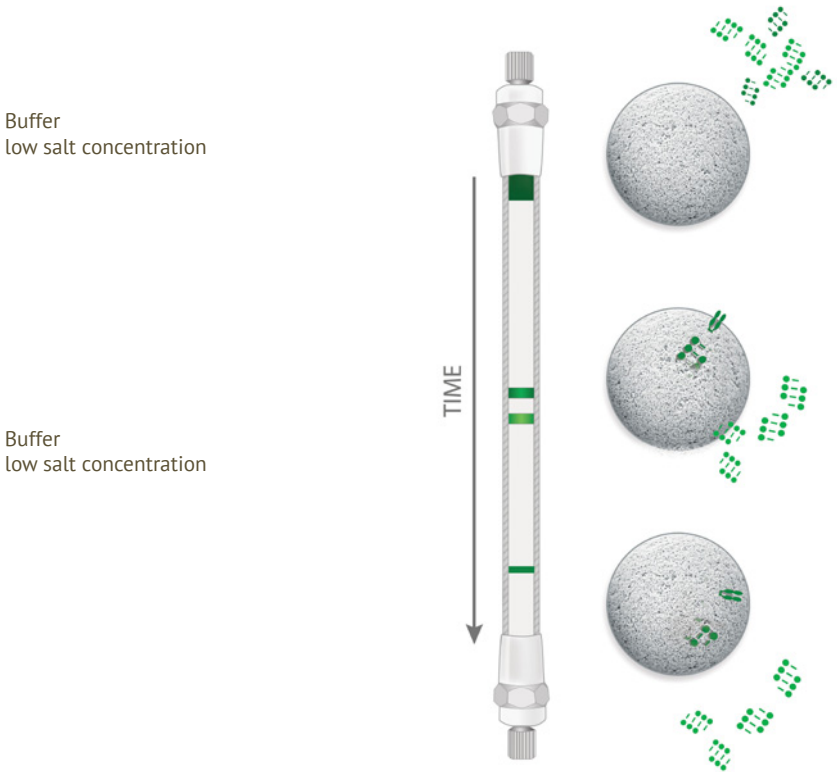


Fig. 4: Size exclusion chromatography (SEC)

SEC-MALS for Biopolymers

The new LenS3 MALS detector provides unmatched sensitivity for the analysis of low concentrations of biomolecules. While the green laser increases the light scattering signal intensity, the revolutionary flow chamber material and design reduce noise. The result is a higher signal-to-noise-ratio and thus higher sensitivity.

2.3 Characterization of oligonucleotides by reversed phase, HILIC, and LC-MS

Reversed phase chromatography relies only on hydrophobicity for retention and separation. Because of the ionic nature of oligonucleotides ion-pair reagents are needed to achieve adsorption on reversed phase (U)HPLC columns. Suitable counter-ions or ion-pair reagents such as long-chain alkyl amines like triethylammonium acetate (TEAA) are typically added in low concentration to the mobile phase to help with the retention and separation of negatively charged nucleic acid molecules.

Ion-pair reversed phase liquid chromatography (IP-RPLC) on TSKgel OligoDNA-RP can also be used to check the purity of the full-length oligonucleotide after the on-column cleavage approach on HIC resins described in chapter 1.2.[3]

When coupling reversed phase separation with mass spectrometric (MS) detection, the ion-pair reagent in the mobile phase needs to be changed to a volatile one to achieve good ionization and high response.

Several authors suggest a mixture of 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) with low concentrations of triethylamine (TEA) or hexylamine (HA) as ion-pair reagents for IP-RP-MS. As HFIP is insoluble in acetonitrile, methanol must be used as organic mobile phase. [5]

Hydrophilic interaction liquid chromatography (HILIC) is a popular technique for the analysis of polar molecules and it has the advantage to be MS-compatible. In addition, HILIC can provide higher MS intensity compared to RPLC due to the higher amount of volatile organic solvent in the mobile phase.

The TSKgel Amide-80 series of UHPLC and HPLC columns, that are well-known for glycan analysis, can also be used for oligonucleotide analysis.[5]

3. Summary

Therapeutic oligonucleotides can accurately be directed against their ribonucleic acid (RNA) target and represent a promising approach in previously untreated diseases. As these new drugs emerge, new demands arise for their purification and characterization.

This sets a broad range of requirements for chromatographic media and HPLC columns. Due to the stringent regulation of the biopharmaceutical industry, chromatography resins and columns need to be available in consistently high quality for production processes and (QA) analytics over a long period of time. At the same time, the development of chromatography media and tools for downstream processes and analytics must keep pace with the ever more rapid development of new biopharmaceuticals.

With its TSKgel and TOYOPEARL product lines, Tosoh Bioscience offers columns and chromatography resins for the analysis, isolation, and purification of biomolecules, covering all the usual applications from ion exchange and size exclusion chromatography to HIC, HILIC and reversed phase chromatography.

For a seamless scale-up of analytical methods to production scale, there are the TSKgel HPLC columns, which are manufactured using similar chemistries as the high resolution TSKgel resins and TOYOPEARL process resins for manufacturing and possess the same chromatographic selectivity.

In addition, an extensive portfolio of analytical (U)HPLC columns is available for more specific applications in biopharmaceuticals and small molecules. This portfolio is constantly being enhanced by new developments that are made to reflect advances in pharmaceutical development and production and to anticipate customer requirements.

Supplementing its products, Tosoh Bioscience offers a range of services such as chromatography workshops and support in packing columns for processes and in method development for specific projects.

Literature

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