Bio-Works

APPLICATION NOTE

AIEX as a sustainable option for oligonucleotide purifications to achieve high productivity – a comparative study between AIEX and IPC

Oligonucleotide (ON) therapy is a rapidly expanding field, driven by recent developments such as improved ON stability and delivery mechanisms, an increasing number of FDA-approved ON drugs based on both ASOs and siRNAs, as well as the recent mRNA vaccines. This increase in ON drug candidates leads to a high demand for efficient and sustainable manufacturing platforms, including synthesis, purification and formulation processes. Today, ion pair chromatography (IPC) is a dominant method for purifying and analyzing small quantities of ON drug candidates, offering a fast and high-resolution technique. This is however not a sustainable process, especially not at larger scales, due to the high amounts of organic solvents used and the high concentrations of toxic ion-pairing reagents, such as dibutylammonium acetate (DBuAA). Anion exchange chromatography (AIEX) is an alternative technique that is both sustainable and easy-to-use at all scales. While IPC separates based on hydrophobic properties, IEX separates based on charge. Since the ONs have a negative phosphate backbone, AIEX is suitable. Except for sustainability and scale-up reasons, it is important not only that the technique can provide high purities and yields, but also high productivity. To evaluate these characteristics, a comparative study was performed for ON purifications using both IPC and AIEX to investigate whether AIEX is an attractive alternative to IPC at production scale.

Introduction

Therapeutic oligonucleotides

Therapeutic ONs represent a promising next generation of drugs. These drugs offer the advantages of high therapeutic efficacy, low toxicity, and strong specificity, and can target more than 10,000 human proteins that were previously considered "undruggable" by small molecules or protein therapies. For example, antisense oligonucleotides (ASOs) are of special interest for treatment of approximately 8000 rare disorders. Moreover, the cost-effectiveness of designing, formulating, manufacturing, and purifying ON drugs at industrial scale is generally greater than for small molecules.

There is a diverse range of different ON drug candidates on the market today, such as ASOs (single stranded ONs), siRNAs (double stranded RNAs involved in the RNA interference pathway), splice-switching oligonucleotides (SSOs), and an RNA aptamer against a protein. Even if they seem diverse, they are all negatively charged molecules which is an advantage when it comes to purification processes.

Therapeutic ONs

- 20-50 nt DNA or RNA oligomers
- Single or double stranded
- May be modified to increase stability and/or delivery

Basics of AIEX and IPC

IPC is an HPLC technique, based on reversed phase chromatography (RPC) supplemented with so-called ion-pairing reagents, for separation of more polar and hydrophilic species that normally would not be retained on the highly hydrophobic RPC media. The retention of the highly polar ONs is poor under common RPC conditions but is significantly improved by addition of these ion-pairing reagents. Ion-pairing reagents are small molecules that contains one part that is hydrophobic and can interact with the hydrophobic ligands in the RPC media and another part that is charged with the opposite charge of the analytes for electrostatic interactions with the analytes. This means that the ions in the solution can be paired and be separated as ion pairs. Since oligonucleotides are negatively charged, the ion-pairing agent must be positively charged. IPC separations of ONs commonly use C18 as stationary phase and alkylamines, such as DBuAA, as ion-pairing reagents. The bound ONs are later eluted by a gradual increase in organic solvents (e.g. acetonitrile) to weaken the hydrophobic interactions. See Figure 1A for an illustration of the principle of IPC.

For AIEX, it is the charged analytes themselves that interact with ligands of opposite charge. For the negatively charged ONs, cationic ligands are needed, and the most frequently used AIEX is a strong ion exchanger derivatized with quaternary amines (Q resin). Since the net negative charge of the ON correlates with its length, the longest ON species will be the strongest retained ones on the resin, i.e. the ONs will be separated based on length. To elute the bound ONs, a linear salt gradient is commonly applied. See Figure 1B for an illustration of the AIEX separation technique. IEX is commonly used at bio-process scale, whereas IPC is more dominant at smaller scale.

Sustainability

Today, there is a strong focus on sustainability in all steps of pharmaceutical processes. Since IPC uses high concentrations of both organic solvents and toxic ion-pairing reagents, and is also conducted at elevated temperature which requires additional energy, there is a demand to switch from this method to a more sustainable method. IEX, a water-based method with high loading capacity and typically performed at ambient temperature, is an attractive alternative. However, comparative studies between the two methods regarding productivity and solvent consumption at preparative loadings are lacking.

Study setup

In this study, a DMT-off phosphodiester DNA of 20 nts (Qiagen) was used as a sample to mimic a typical ASO, with only preparative loadings applied. The ON feed was divided into two parts: one part was purified using AIEX in-house, while the other part was purified at an external company (IPC site) under their standard conditions for such a feed. After optimizing the processes with running conditions at each site, methods were set up to vary the sample load as well as the length of the elution gradient (i.e. the gradient slope). The AIEX eluents were collected in small fractions and sent for mass spectrometry (MS)

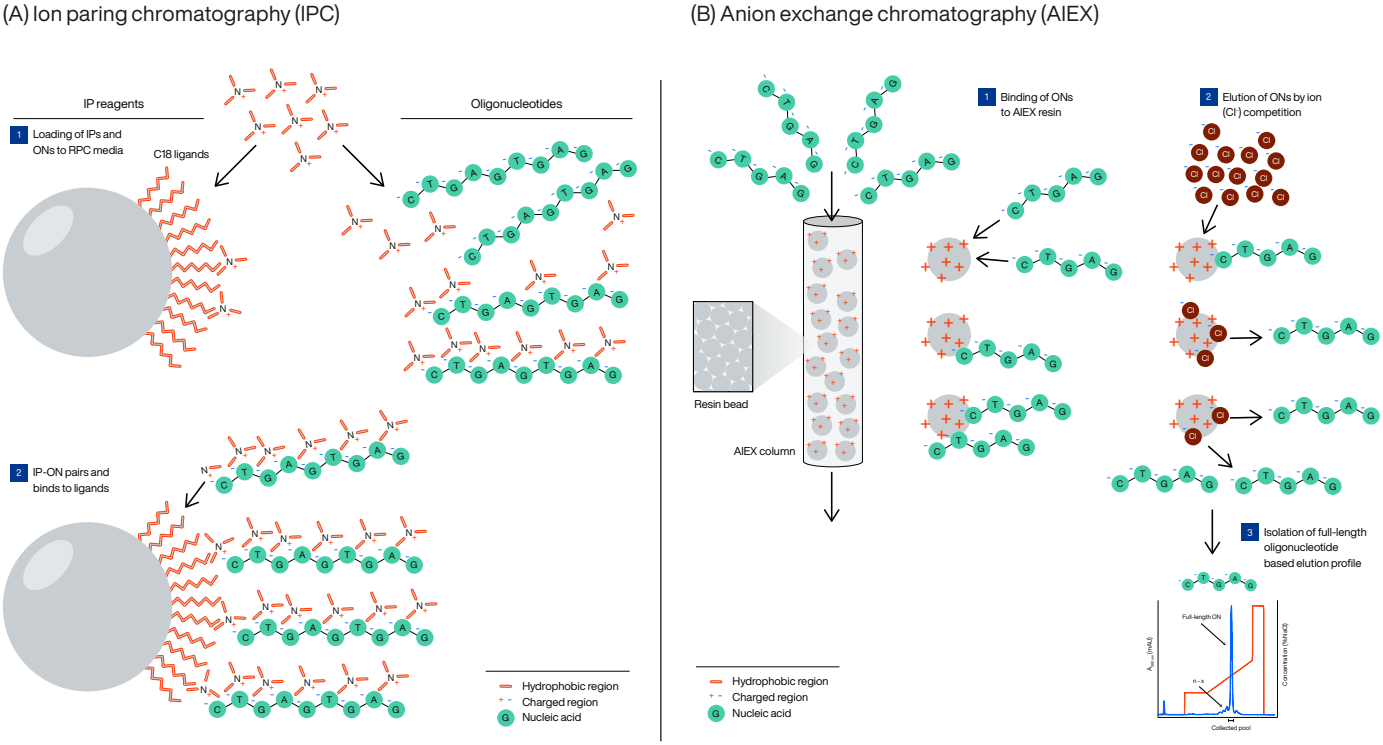

Figure 1. Illustrations of principles of oligonucleotide separations by (A) ion pair chromatography and (B) anion exchange chromatography.

analysis to determine purity and yield, while the IPC purifications were analyzed in-line by MS during the runs. Based on this data, productivity and solvent consumption were calculated, also considering the cycle time.

In Table 1, the running conditions for both methods are specified. The AIEX resin, WorkBeads™ 40Q, is a quaternary amine derivatized agarose-based resin with a high ligand density and a relatively small average pore size and a narrow pore-size distribution. The IPC column contains a high-resolution C18 media with a smaller bead size.

Table 1. Running conditions applied in the study

	AIEX	IPC
Resin	WorkBeads 40Q (45 µm)	XBridge C18 (5 µm)
Max capacity	~ 48 mg ON/mL resin (DBC)	~ 2.5 mg ON/mL media
Column	5 × 25 mm	2.1 × 50 mm
Temperature	Ambient	50°C
Binding buffer	20 mM tris, pH 8	20% MeCN, 60 mM DBuAA
Elution buffer	20 mM tris, 1 M NaCl, pH 8	50% MeCN, 60 mM DBuAA
Elution gradient*	20-80% (10-40 CV)	24.5-39.5% (2-10 min)
Sample load*	19-38 mg/mL resin	1.1-1.8 mg/mL resin
Flow rate	0.25 mL/min (75 cm/h)	0.5 mL/min

* Elution gradient and sample load were variables in this study

Variables in this study

Sample load

- 40% of maximum resin capacity
- 60% of maximum resin capacity
- 80% of maximum resin capacity

Gradient slope

- Steep
- Mid
- Shallow

To calculate the purity and yield plots, the UV responses were converted to concentrations based on the mass spectrometry analyses values. Productivity was calculated as mg product obtained per minute per mL resin, and solvent consumption was calculated as consumed solvent in mL per mg product obtained.

Steps included in total cycling time

- Sample injection
- Wash of unbound species
- Gradient elution
- Wash with 100% elution buffer
- Re-equilibration of column

Results

In Figure 2, the AIEX purification at 40% load using the mid gradient (20 CV) can be seen where both the UV trace and the individual ON species are visualized including full-length product (FLP) and n-1 to n-6. By holding at 20% elution buffer, early eluting impurities are removed before start of the linear gradient. The hold is designed to minimize the cycle time in order to increase productivity. The green solid line represents the FLP in each fraction (dots). As expected, the n-1 (the dark blue impurity trace) is the most difficult one to remove due to close resemblance to the FLP. Longmers (n+x) were insignificant in this feed. The peak shape of the FLP (yield shown as concentration) has an anti-Langmuirian shape (fronting) which will have an impact on the purity-yield relation.

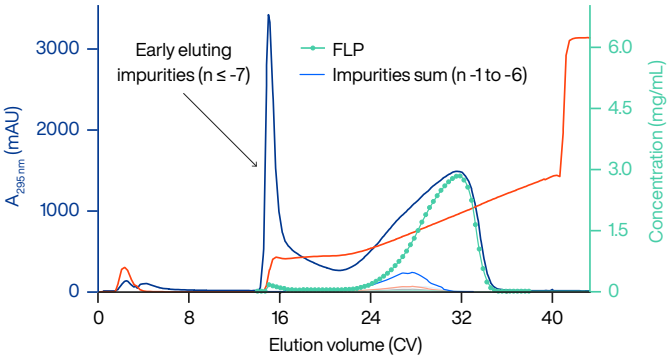

Figure 2. Chromatogram of AIEX purification. UV trace at 295 nm (dark blue) and conductivity (red) are shown as well as the FLP concentration/yield (solid green line where the dots represent each analyzed fraction) and the impurities (n-1 to n-6).

Figure 3 shows the FLP peak shapes (concentrations) for AIEX versus IPC. The plots display opposite peak profiles; the anti-Langmuirian peak shape for the AIEX separation and a Langmuirian-shaped profile, also known as tailing peak, for the IPC separation. Both profiles were obtained from the low load using a mid slope.

Since the impurities are always in the beginning of the main peaks, there is a bigger overlap between impurity yield optima with the FLP yield optima (indicated by dashed red line) for the IPC method. IPC, as a high-resolution technique will still generate a high yield at a purity of 95%, but this yield will substantially decrease when higher purity requirements are needed.

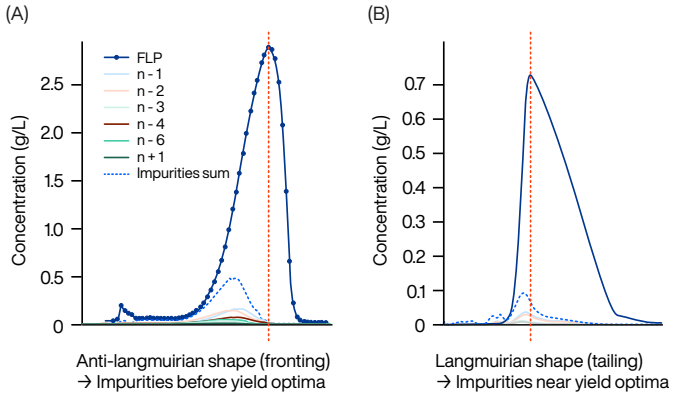

Figure 3. Purification yield profiles for FLP (solid dark blue line) and individual impurities (n-1 to n-6; colored and dashed blue lines) using either AIEX (A) or IPC (B). The profiles have been obtained using the low sample load and the mid slope.

To better understand how the peak shape affects the isolation of FLP and subsequently the yield, yield profiles from AIEX and IPC purifications are compared at different purity constraints, as shown in Figure 4. These plots represent the purifications that generated the global productivity optima, which for both methods were achieved at the highest loads. The top plots are from AIEX and the bottom ones from IPC. The collected pools (green) at purities of 95% and at 99% are shown alongside their corresponding impurities (red).

The anti-Langmuirian-shape may partially be an effect of the nature of the elution salt, the window of desorption – adsorption of ON to IEX ligands.

No such effect is expected in IPC. Langmuirian profile shapes are likely caused by non-specific binding of ON to media.

The yield is higher for the IPC compared to AIEX at the lower purity of 95% (92% yield vs. 77% yield), but it is the opposite relationship at the higher purity of 99%, where a yield of 50% is obtained for AIEX compared to 22% for IPC. This is due to the impurities eluting in the beginning of the main peak. Here, IEX has the advantage of having an anti-Langmuirian peak shape, which requires less peak shaving. The steepest elution gradient was used for all plots except IPC at 99% purity (Figure 4D), where the mid-gradient was required to isolate the FLP. For the IEX purifications at the highest sample load (Figures 4A-B), some FLP eluted together with the impurities in the early hold before the start of the gradient but was still advantageous for the productivity outcome.

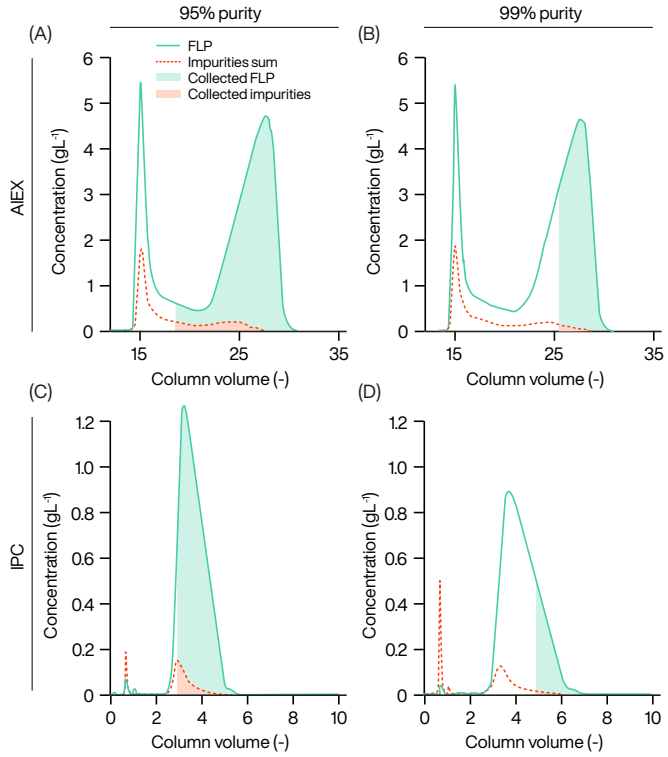

Figure 4. Yield/concentration profiles at the largest loadings (37.9 mg ON load/mL AIEX resin and 1.8 mg ON load/mL IPC media). Green area represents the collected pool at 95% purity (A, C) or at 99% purity (B, D). The red area represents the collected impurities in respective pools. AIEX: Top panel (A-B); IPC: Bottom panel (C-D).

Global productivity optima were be obtained for 95 – 99% purities, as detailed in Table 2.

The productivity, measured as produced mg FLP per minute per mL resin, is much higher for AIEX compared to IPC, even though the AIEX has a much longer total cycling time. This long cycling time is compensated by the very high binding capacities of the AIEX resin which generates a higher productivity. The highest productivity is observed at the lower purity constraints for both techniques. However, when targeting 99% purity instead of 95%, productivity decreases by only 1.5 times for AIEX, whereas this reduction is five times lower for IPC. The reduced productivity seen for IPC at a purity constraint of 99% is due to the difficulty to remove the n-1 species from the FLP, as seen in Figures 3-4.

Figure 5A illustrates the difference in productivity between IEX and IPC under the conditions used. IEX has a two-fold higher productivity at 95% purities and a seven-fold higher productivity at 99% purities.

Table 2. Yields, productivities and solvent consumptions at 95% - 99% purities at global productivity optima

	Purity (%)	Productivity (mg FLP/min/ mL resin)	Solvent consumption (mL/mg FLP)	Yield (%)	Gradient slope (mM NaCl/min for IEX; %MeCN/min for IPC)	Load (mg feed/ mL resin)	Cycle time (min)
IEX	95	1.2	1.7	76.6	120	37.9	18.2
	96	1.2	1.7	73.3		37.9	
	97	1.1	1.8	68.6		37.9	
	98	1.0	2.1	61.0		37.9	
	99	0.8	2.5	50.4		37.9	
IPC	95	0.5	5.4	92.0	7.5	1.8	2.8
	96	0.5	6.2	80.3		1.8	
	97	0.4	7.9	71.4	5	1.8	3.2
	98	0.2	12.5	45.9		1.8	
	99	0.1	26.3	21.8		1.8	

Another important aspect when it comes to sustainability is the actual solvent consumption. The solvents are not only costly to purchase but also expensive to dispose of. To calculate the solvent consumption, the obtained FLP/mL consumed solvents was considered. Even though more solvents are used in each individual AIEX purification, the obtained yield of the FLP is very high and many IPC cycles had to be run to achieve the same yield using more solvents in the end. The solvent consumption for IPC was three times higher than for AIEX at 95% purities and ten times higher at the highest purity (Figure 5B). Since the IPC solvents contain high concentration of organic solvents, this consumption has a big negative environmental effect.

Lastly, we wanted to visualize the parameter dependence for these purifications. To do empirical modelling of the productivity, the coefficients/variables (load and gradient slope) were first scaled and centered before the actual model fitting, see Figures 6A-B. For IEX, a higher load has a significant and positive effect on productivity for all purities (Figure 6A), whereas the slope is insignificant. The effect seems to decrease with increasing purity requirements. For IPC (Figure 6B), both a higher load and a steeper slope have a positive effect on productivity, but their effects diminish with increasing purity, becoming insignificant at purities above 98%.

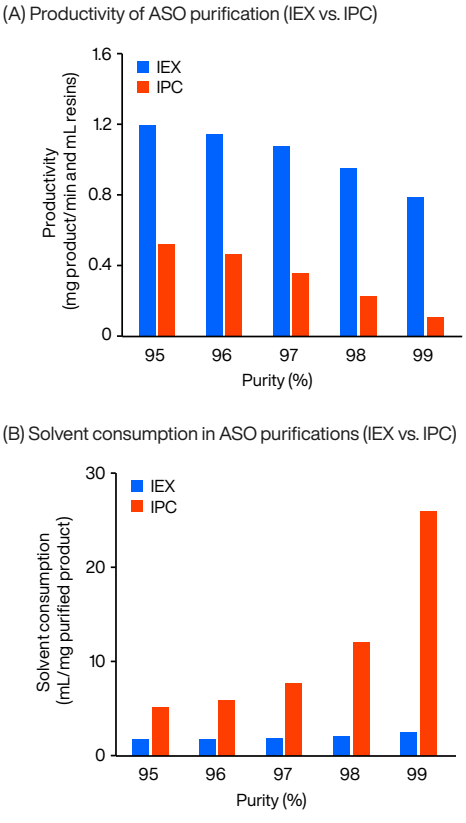

Figure 5. Productivity (A) and solvent consumption (B) for IEX and IPC at global productivity optima.

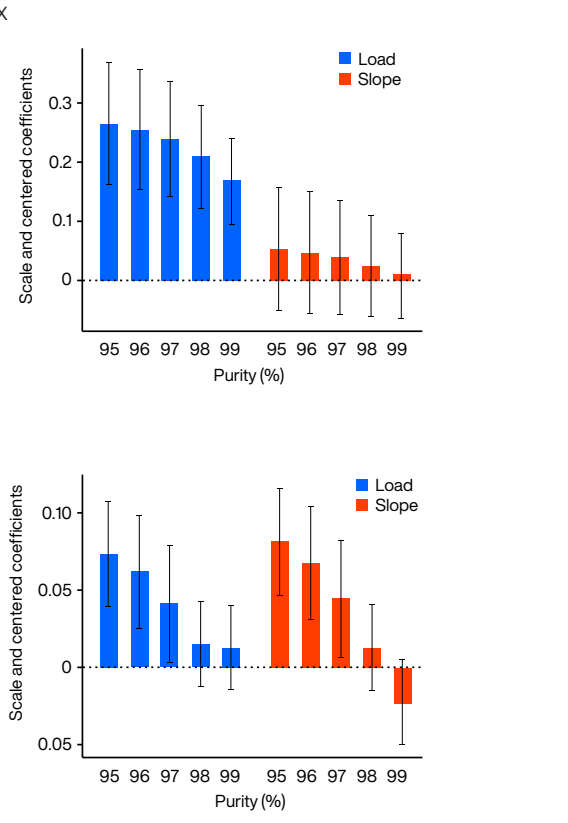

Figure 6. Model coefficients and response surfaces. Panels (A) and (B) present the scaled and centered model coefficients of IEX and IPC, respectively. Error bars represent 95% confidence intervals.

A shallow slope (length of gradient) improves the yield for IEX, especially at higher purities, but at the cost of increased time, which negatively impacts productivity. This trade-off diminishes the positive effect on separation due to the longer process time. Since this study is aimed at production scale, productivity is essential.

Final words

The productivity data for IEX shown here are reached using environmental-friendly buffers without applying any heat, organic solvents or chaotropic salts, which is applicable to the native PO ON used in this study. However, if the feed consists of modified ONs, such as PS ONs, additives and optimized running conditions will be needed.

Another important aspect is the counter ions. IEX-eluted ON feed commonly contains the correct counter ions. However, post IPC, the ion-pairing reagents in the eluted ON feed need to be removed, requiring an additional counter ion exchange step, which can be performed by IEX.

Conclusions

From this comparative study a conclusion can be drawn – due to the high loading capacities, AIEX offers much higher productivity and reduced solvent consumption, making it an attractive, sustainable alternative or complement to IPC. AIEX has the ability to handle a feed of a native 20-mer, qualifying it as a high-resolution technique. Under preparative loadings, it can achieve higher yields compared to IPC at a set purity of 99%.

AIEX compared to IPC for phosphodiester oligonucleotide purifications:

- Higher productivity (2-7x)
- Lower solvent consumption (30-90% lower)
- Environmental-friendly running conditions
- Lower energy consumption (runs at ambient temperatures)
- Higher product yield in a single cycle (high loadability)
- More sustainable

For more details about this study, see: Enmark, Martin, *et al. A Comparative Study of Ion Exchange vs. Ion Pair Chromatography for Preparative Separation of Oligonucleotides. Journal of Chromatography A*, vol. 1746, 12 Apr. 2025, p. 465790.

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