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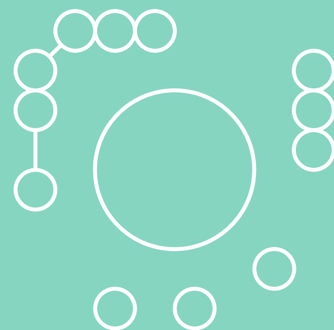

WHITE PAPER

Desalting of antisense oligonucleotides and other oligonucleotides using alkali-tolerant, small-pore SEC

Oligonucleotides as therapeutic molecules

Short oligonucleotides have become established as important molecules in therapeutics, primarily in the form of antisense oligonucleotides (ASOs), aptamers and small interfering RNAs (siRNAs). ASOs are oligonucleotide sequences designed to bind complementary RNA targets (mainly mRNAs) to affect gene expression. Antisense gene therapy is emerging as one of the most promising therapeutics for various diseases such as cancer. As of 2019, there are seven FDA-approved ASO-based therapies on the market whereof one is a siRNA, and there are many others in clinical trials.

Oligonucleotides are short sequences of deoxyribonucleic acids or ribonucleic acids that are commonly produced by solid-phase synthesis, in which the rate of failure sequences is proportional to the length of the sequence. Therapeutic oligonucleotide molecules are subject to absolute and stringent requirements for purity. This level of purity is often achieved by high resolution anion exchange chromatography (AIEX) which can separate full-length sequences from incomplete or erroneous sequences by virtue of the negatively charged phosphate backbones. Elution of the adsorbed oligonucleotides is most often done by increasing the salt concentration gradient to release the oligonucleotides in order of increasing length. An optimized AIEX step generates high purity and yield of the full-length oligonucleotide.

After ion exchange chromatography there is often a need to remove the salt used for elution before further downstream process steps, *e.g.* lyophilization, that commonly employs size exclusion chromatography (SEC), so-called desalting. The stringent purity requirements for such therapeutic molecules demands cleaning of the resins under harsh conditions.

Size exclusion resin *vs.* commonly used desalting resin

Chromatographic desalting is a fast and efficient tool for removal of salt or unwanted low-molecular weight impurities, or for buffer exchange. In this technique the large size-difference between the target molecule and salt is utilized. Since the difference between the salt and the oligonucleotide is greater than a factor of 10, this constitutes a so-called group separation of molecules. If the pore size of the beads is smaller than the molecules of interest, *i.e.* the cut-off is < target molecule size, the target molecule will elute in the void volume (does not enter the resin particles), whereas salt molecules are eluted close to the total volume of the column (they pass through most of the porous resin particles and are thus delayed). See Figure 1A. This is true for most desalting resins (cut-off: 5-10 kDa) and they are rather insensitive to flow rate. Dextran is in general included in these desalting resins to achieve this low cut-off. Figure 1B is an example of desalting of a ssDNA using a prepacked dextran-based SEC resin (GoBio™ Mini Dsalt) at a flow rate of 225 cm/h, where one third of the column volume is loaded.

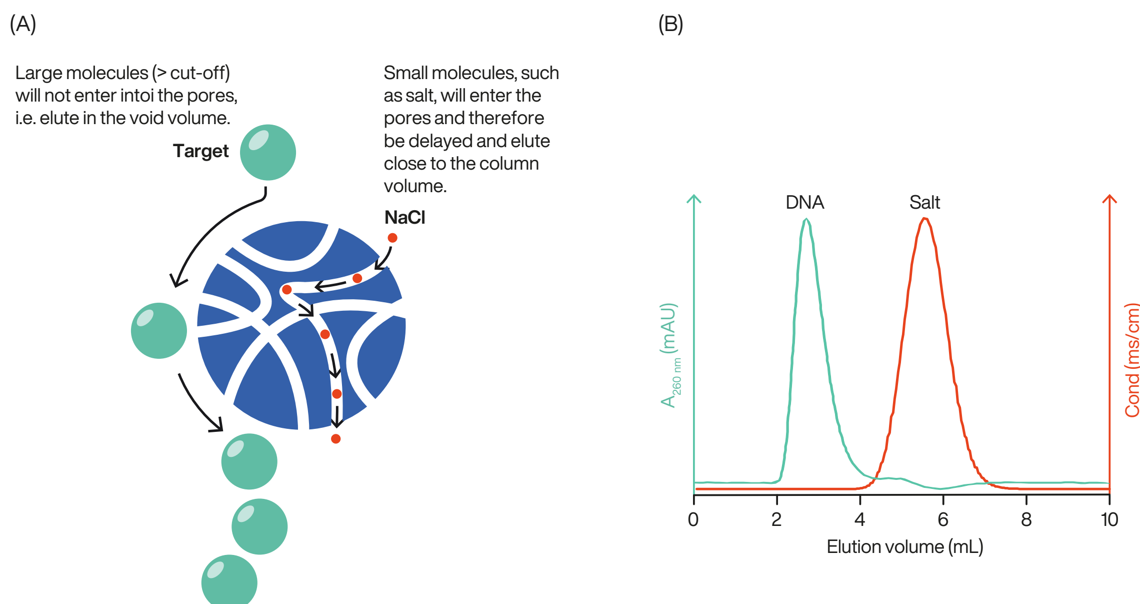

Figure 1. (A) Theory of how desalting works. (B) Example of a desalting profile of a 20 nt ssDNA with loading of one third of the column volume using a flow rate of 225 cm/h. GoBio Mini Dsalt 5 mL was used as desalting column.

The main disadvantage of these dextran-based desalting resins is the sensitivity to high concentrations of sodium hydroxide (NaOH) for cleaning-in-place (CIP). They tolerate up to 0.2 M NaOH while CIP after running oligonucleotides is preferentially performed with 1 M NaOH.

Pure agarose-based resins with no dextran attached are alkali-stable in 1 M NaOH and are therefore a preferred choice when desalting ASO/oligonucleotides. Hence, we present an application where we have used a pure agarose-based SEC resin with a cut-off of 150 kDa to desalt oligonucleotides of two different sizes; 20 nucleotide ssDNA (6.6 kDa) and 45 nucleotide RNA (14.7 kDa).

WorkBeads 40/100 SEC

Pure agarose-based SEC resins have larger pores than dextran-based SEC resins. WorkBeads™ 40/100 SEC is a non-dextran coated agarose-based resin with an estimated cut-off of approximately 150 kDa. See Table 1 for properties of the resin. WorkBeads resins are crosslinked using a proprietary method that results in a very rigid structure.

Table 1. WorkBeads 40/100 SEC

Average particle size (D_{v50})[1]	45 µm
Agarose content (%)	9
Exclusion limit	150 kDa
Max flow rate (20 cm bed height, 5 bar)	600 cm/h
pH stability	2 – 13
Alkali-tolerance	1 M NaOH

[1] The average particle size of the cumulative volume distribution

Although the general recommendation for SEC is to use low flow rate for best purification, the rigidity and tight particle size distribution allow purification of viruses and other large substance at high flow rate for fast processing and high yields. Since ASOs are in the size range of approximately 6-8 kDa, the molecules will partially enter the pore volume, indicating a limitation in usable flow rates. This also means that there is a balance at different flow rates between the resolution and the dilution factor of the target molecules.

Flow rates *vs.* resolution and volume dependence

We applied 1 mL of an AIEX-eluted RNA feed to a column (23.6 mL) packed with WorkBeads 40/100 SEC resin at several flow rates. The feed concentration was 300 OD/mL, *i.e.* 12 mg/mL in a neutral phosphate buffer. At 50 cm/h the salt was completely removed from the RNA, as shown by the non-overlapping UV absorbance trace for the oligonucleotide and the conductivity trace for salt (Fig. 2A). When the flow rate was increased to 150 cm/h a slight overlap could be seen, and when the flow rate was further increased to 300 cm/h the overlap started to become more significant, even though it is a small loss of OD in reality. This is in clear contrast to a dextran-based desalting resin where the separation is less effected by flow rates, as the large target molecules do not enter the pores in the beads.

In Figure 2B the RNA feed was loaded on the SEC resin in increasing volumes at 150 cm/h. A lower flow rate is preferred for optimal resolution, but the process time is shorter at a higher flow rate. When the sample volume is increased there is a bigger overlap between the UV trace and the conductivity trace, and for this sample, 5 mL load was too high at 150 cm/h. There was no significant effect on resolution when lower sample concentrations were loaded (dilutions of 1:15), thus higher concentrated feeds can be loaded, when solubility allows, without compromising the separation.

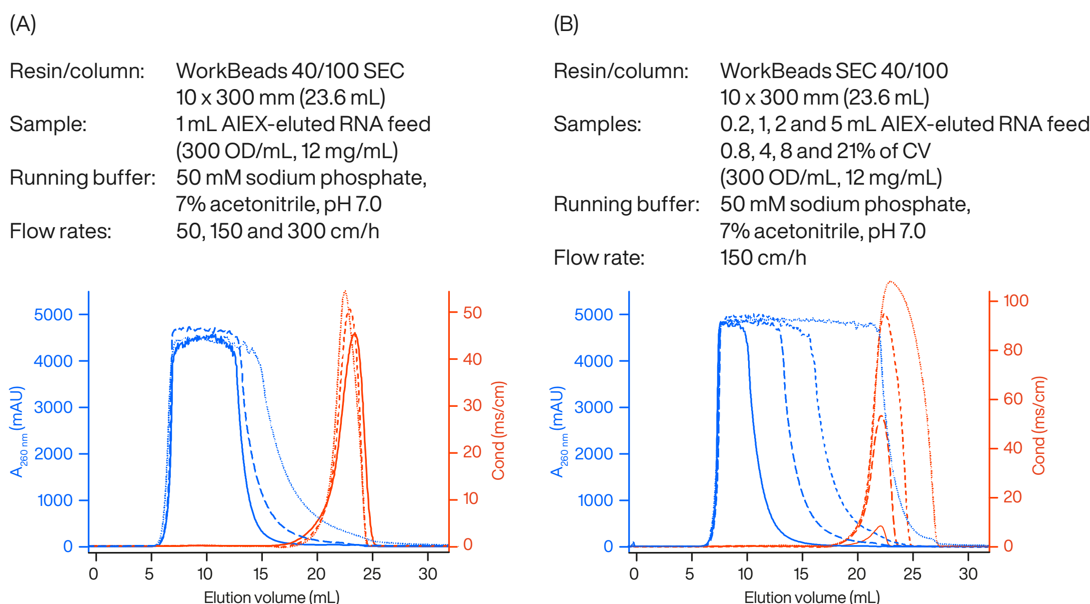

Figure 2. (A) 1 mL load of a 45 nt RNA feed on WorkBeads 40/100 SEC at different flow rates. UV traces are blue (solid: 50 cm/h, dashed: 150 cm/h and dotted: 300 cm/h) and corresponding conductivity traces are red (solid: 50 cm/h, big dotted: 150 cm/h and small dotted: 300 cm/h). (B) Different volumes of RNA feed loaded on WorkBeads 40/100 SEC at 150 cm/h. UV traces are blue (solid: 0.2 mL, large dashed: 1 mL, short dashed: 2 mL and dotted: 5 mL) and corresponding conductivity traces are brown.

DNA 20 nt-oligomer (ASO)

To test the feasibility of desalting an oligonucleotide of 20 nt with a molecular weight (M_w) of 6600 Da, *i.e.* a 100-fold difference in M_w compared to NaCl, we loaded different sample volumes of the feed (20 OD/mL) onto the SEC resin at 50 cm/h to obtain maximal resolution. Figure 3A shows that almost up to 20% of the column volume can be loaded and successfully desalted under these conditions (5 mL equals 22% and is slightly over loaded). K_D analysis was also performed to assess the pore usage of the oligonucleotide, which revealed that it behaves as a much bigger molecule (30 kDa), than predicted from its actual molecular weight (6.6 kDa), see Figure 3B. The 20-nt DNA, which in size represents a typical therapeutic ASO, had a K_D of approximately 0.5, meaning that it can access 50% of the pore volume (but a value of 0.93 is predicted from its Mw).

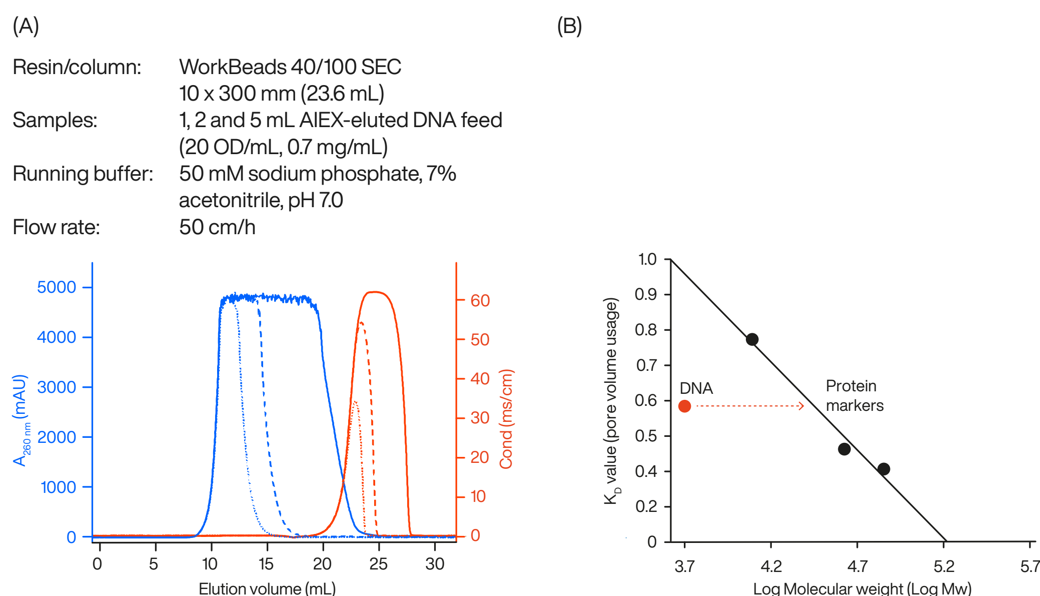

Figure 3. (A) Different volumes of DNA feed (20 nt) loaded on WorkBeads 40/100 SEC at 50 cm/h. UV traces are blue (solid: 5 mL, dashed: 2 mL, dotted: 1 mL) and corresponding conductivity traces are red. (B) K_D analysis on WorkBeads 40/100 SEC showing the difference between predicted and actual pore usage for 20 nt DNA compared to protein markers. The black dots are protein standards used to calculate trendline. Red dot represents measured 20 nt DNA K_D value and arrow indicates its predicted molecular weight (in log) accordingly to measured K_D value (30 Da instead of 6.6 kDa).

From the experiments above we can see that flow rate affects resolution on a SEC resin with a cut-off of 150 kDa, where a lower flow rate is required to obtain maximal separation between oligonucleotides and salt. The optimal and maximum loading volume is smaller than for traditional desalting using a dextran-coated resin, but the sample concentration plays a minor role. Analysis of the pore usage of the 20 nt DNA demonstrates that oligonucleotides behave as if they are much bigger than their actual molecular weights. Thus, we can conclude that the oligonucleotides partially enter the resin but can still be successfully desalted using an optimized flow rate and smaller sample volume and with the added advantage to being able to use a resin with high tolerance to harsh cleaning conditions.

Conclusions

- WorkBeads 40/100 SEC can be used to desalt oligonucleotides
- Resolution is not sensitive to sample concentration
- Loading maximum 20% of the column volume at a relatively low flow rate is required to obtain optimal resolution, and a pure desalted oligonucleotide
- WorkBeads 40/100 SEC can be cleaned with 1 M NaOH
- Oligonucleotides behave as larger molecules than is predicted from their molecular weight

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