Bio-Works

APPLICATION NOTE

Characteristics of WorkBeads SEC resins: porosity, selectivity and case studies

Size Exclusion Chromatography (SEC) is a powerful tool for separation of molecules, that utilizes differences in sizes and geometries. SEC is especially suited for biomolecules that are sensitive to the surrounding environment, for example, pH, ionic strength, presence of metal ions, and temperature. To select the optimal SEC resin for a specific application, it is important to understand the characteristics of resins, and that resin porosity is crucial when it comes to selectivity for different molecules. Choosing the most optimal resin for your target molecule not only depends on porosity or bead size but also on the application. In this study we have investigated how operational conditions influence the separation performance by varying parameters such as flow rates and sample volumes, with two case studies to conclude.

WorkBeads SEC resins

WorkBeads™ SEC resins are highly cross-linked, rigid agarose beads, optimized for preparative purifications. These SEC resins have good flow properties, and can tolerate extremes of pH and ionic strength, i.e. they are compatible with harsh cleaning conditions (such as 1 M NaOH). SEC is usually used as a polishing step in purification processes.

All WorkBeads 40 SEC resins described here have an average bead size of 45 µm. They are of different pore sizes, offering optimal separation for differently sized biomolecules. In this application note, the properties of WorkBeads 40 SEC resins are described along with how to choose the best resin based on resin characteristics and separation goals.

The resin of choice should have beads small enough to achieve good separation and rigid enough to tolerate a high flow rate when used in scaled-up processes. Equally important is to choose a bead with a pore size optimal for your target molecule, that exhibits a narrow pore size distribution (PSD) to achieve uniform mass transport, which ultimately leads to higher purities.

Which SEC resin to select?

Bead size

The resolution and separation characteristics of a SEC resin greatly depend on the bead size. There is an inverse correlation between separation, bead size and backpressure. Small beads cannot be scaled up to preparative scale since the backpressure will be too high. A bead size of 45 µm represents a sweet-spot to obtain good resolution at a modest backpressure. However, some applications require larger beads (> 100 µm) due to, for example, high sample viscosity, and here WorkBeads 200 SEC is a suitable option.

Pore sizes and pore size distribution (PSD)

The optimal bead pore size depends on the size of the target molecule. Large pores are required for bigger molecules, such as antibodies. Likewise, smaller pore sizes are better for smaller target molecules, such as peptides and small oligonucleotides (e.g. ASOs). The pore size distribution (PSD) is one determinant factor for efficiency, as seen by narrower peaks, i.e., narrower peaks are obtained from a narrower PSD since the molecules get a more uniform mass transport in and out of the pores. PSDs of WorkBeads 40 SEC resins were determined by inverse SEC (iSEC) using pre-defined sizes of dextrans and a refractive index (RI) detector for detection. This technique enables measurements of pore sizes in the beads in their natural environment. Figure 1 shows the PSDs for the different WorkBeads 40 SEC resins, where a correlation between the pore sizes and the spread of the PSD can be seen.

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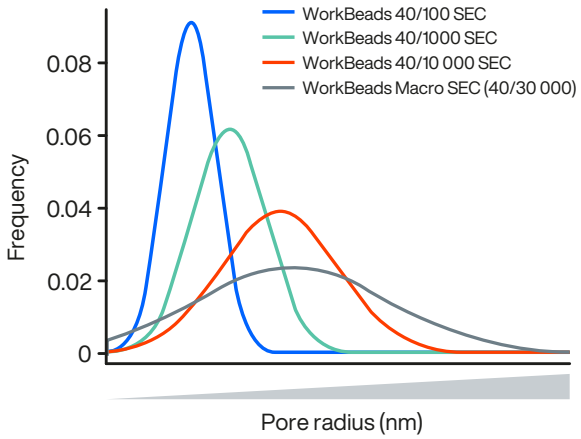

Figure 1. Pore size distribution (PSD) of different WorkBeads 40 SEC resins measured by iSEC with pre-defined sizes of dextrans. Shown SEC resin PSDs: WorkBeads 40/100 SEC (blue), WorkBeads 40/1000 SEC (green), WorkBeads 40/10 000 SEC (red) and WorkBeads Macro SEC (40/30 000) (grey).

Separation range

The separation range of a SEC resin defines the range of target molecular weights (M_r) that can be separated by the resin, by having different pore accessibility. Separation range is primarily determined by the pore size of the matrix. Molecules smaller than the lower limit of the separation range should elute at 1 column volume (CV) since they have access to all the pores, whilst the molecules larger than the upper limit of the separation range will elute in the void volume since they do not access the pores.

Exclusion limit of a SEC resin is used to indicate the size of molecules that cannot access the pores and thus elutes in the void volume. The exclusion limit sets the upper limit in the separation range.

WorkBeads 40 SEC resins with an exclusion limit of 10 000–30 000 kDa is suitable for purification of larger target molecules, e.g. antibodies and extracellular vesicles. SEC resin with an exclusion limit of 150 kDa is suitable for purification of small proteins, peptides, and oligonucleotides. Table 1 shows a comparison of separation ranges for WorkBeads 40 SEC resins and their exclusion limits.

For group separations, it is important to also consider the impurity profile when selecting the separation range, e.g. for SEC purifications of viruses, the aim is often to elute the virus in the void volume while retaining the impurities in the resin (see described examples in the case studies section).

Selectivity

The selectivity of a SEC resin is one of the most important properties since it defines how well the target molecule can be separated from other molecules. The selectivity can be visualized by selectivity curves, and the common way to measure selectivity is to use globular proteins with absorbance detection at 280 nm. Figure 2 shows selectivity curves of different WorkBeads 40 SEC resins using thyroglobulin, bovine serum albumin (BSA) and ribonuclease A as probes where the distribution coefficient (K_d) of the probe proteins is plotted against the M_r on a logarithmic scale. The steeper the selectivity curve, the higher the resolution between analytes would be.

The distribution coefficient K_d is usually used to compare and predict the elution volumes for molecules with different sizes using a certain SEC resin. Low molecular weight components should elute close to the column volume (K_d=1), and large molecules should elute near the void volume (K_d=0). The operational K_d range for separation is between 0.1–0.9.

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

where, V_e = Retention volume for the molecule of interest, V_0 = Retention volume for a non-retained molecule which will elute in void volume by not accessing the pores
V_t = Retention volume for a molecule that has full access to all the pores of the matrix.

Selecting the SEC resin with regard to the separation range and selectivity sets the separation foundation for the purification. Subsequently, optimizing the operational conditions is equally important as the resin selection.

Table 1. Exclusion limits of WorkBeads 40 SEC resins and illustration of comparison to different sized biomolecules

	Average bead size, µm	Separation range, kD	Exclusion limit, kD	Examples of target molecules
WorkBeads 40/100 SEC	45	10–150	150	Peptides, antisense oligonucleotides (ASOs)
WorkBeads 40/1000 SEC	45	10–1200	1200	Proteins, oligonucleotides (medium sized targets)
WorkBeads 40/10 000 SEC	45	10–10 000	10 000	Proteins, etc. (medium to large sized targets)
WorkBeads Macro SEC	45	10–30 000	30 000	Antibodies, plasmids, mRNAs, viruses

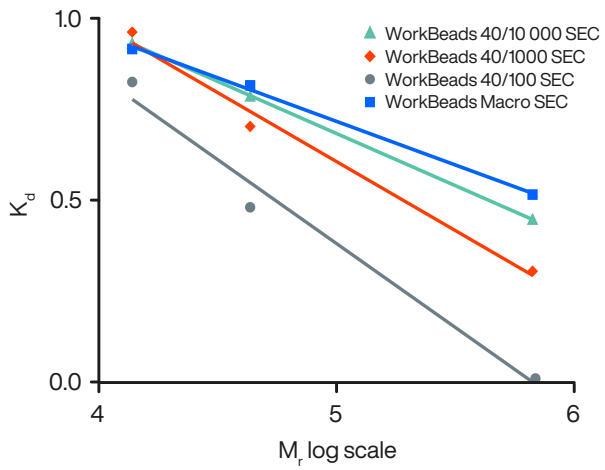

Figure 2. Selectivity curves of WorkBeads 40 SEC resins. WorkBeads 40/100 SEC (grey), WorkBeads 40/1000 SEC (red), WorkBeads 40/10 000 SEC (green) and WorkBeads Macro SEC (blue). Thyroglobulin, BSA and ribonuclease A were used in the measurements.

For a certain component:

If $K_d > 1$, there may be non-specific interactions between the target molecule and the SEC matrix.

If $K_d < 0$, the packed resin bed might contain micro-channels and needs to be repacked.

Which application parameters to select?

When selecting the optimal resin for the separation of the target molecule, it is not only porosity and bead size that matter, but also the application itself. What does the impurity profile look like? Will the resin be used as the last polishing step in a setup where the feed is pre-concentrated? How important is the productivity, the speed of the process? These are all important and relevant questions to address to select the optimal SEC resin. Below is a study describing how two main factors, flow rate and sample volume, address these questions and affect the resolution.

Study setup

Optimized operational conditions can give sufficient selectivity and counteract any peak broadening effect. In this study, the effect of varying the flow rate and sample volume was studied. The smaller pore-sized resin WorkBeads 40/100 SEC and the medium pore-sized resin WorkBeads 40/1000 SEC were investigated in prepacked columns with an inner diameter of 16 mm and length of 600 mm (GoBio™ Prep 16x600). The advantage of using prepacked columns is the standardization of column parameters to avoid operational packing variations and it provides convenience and time saving as a ready-to-use format.

Two sets of globular proteins were used as model proteins.

- Setup 1: myoglobin (M_r 17 600) and apo-transferrin (M_r 78 300) → WorkBeads 40/100 SEC
- Setup 2: ribonuclease A (M_r 13 700) and BSA (M_r 66 300) → WorkBeads 40/1000 SEC

Flow rate

Flow velocity (cm/h) and flow rate (mL/min) are commonly used terms when describing the flow in chromatographic purifications. Lower flow rate typically improves resolution by allowing sufficient time for molecules to move in and out of the pores by diffusion. However, a very low flow rate may increase peak broadening for small molecules. At preparative scale, productivity is very important, thus an optimal flow rate needs to be balanced against resolution and process efficiency.

Resolution, R_s indicates how well the two elution peaks are separated in a chromatographic separation. It shows the selectivity of the resin and the efficiency of that resin to produce sharp, narrow peaks with minimal peak broadening. When $R_s > 1.5$, two adjacent peaks are considered as baseline resolved.

$$R_s = 2\,\frac{V_{e2}-V_{e1}}{w_1+w_2}$$

V_{e1} and V_{e2} are the elution volumes for two elution peaks measured at the center of the peaks. w_1 and w_2 are the respective peak widths.

Figures 3A-B shows the effect of flow velocity on resolution for GoBio Prep 16x600 40/100 SEC and GoBio Prep 16x600 40/1000 SEC when separating the two sets of globular of proteins described above. At a flow velocity of 15 cm/h (0.5 mL/min) and 30 cm/h (1 mL/min), both sets of globular proteins showed baseline separation with a resolution factor greater than or equal to 1.5 (calculated using UNICORN™ 5.31). When increasing the flow velocity, the resolution of the proteins continuously decreases. This decrease in resolution is due to an increase in peak widths correlating to the flow velocity, see Figures 3C-D. GoBio Prep 16x600 40/100 SEC and GoBio Prep 16x600 40/1000 SEC both exhibit good separation when the flow velocity is increased to 60 cm/h (2 mL/min).

Depending on the purity and yield requirements of the target molecule, it is important to optimize the flow rate to achieve separation of the target molecule from the impurities. For example, in group separations, a higher flow rate can be applied since the elution volume of the target molecules differs significantly from those of the impurities.

Prepacked columns:	(A) GoBio Prep 16x600 40/100 SEC (B) GoBio Prep 16x600 40/1000 SEC
Column volume:	120 mL
Samples:	(A) Setup 1: Myoglobin (M_r 17 600), apo-transferrin (M_r 78 300) (B) Setup 2: Ribonuclease A (M_r 13 700), BSA (M_r 66 300)
Sample volume:	0.5 mL
Buffer:	20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Flow rates:	0.5 mL/min (15 cm/h) 2 mL/min (60 cm/h) 5 mL/min (150 cm/h)

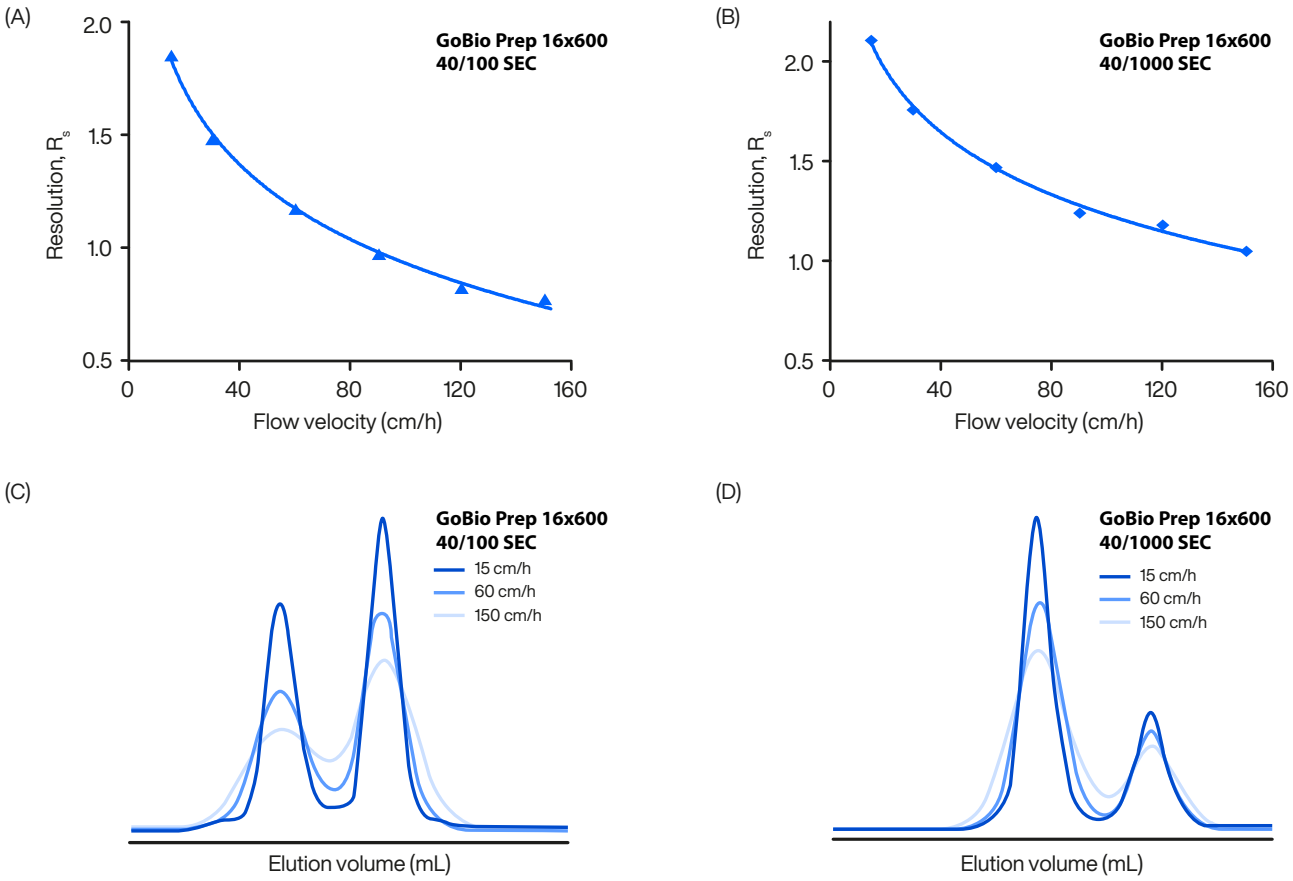

Figure 3. (A) Effect of the flow velocity on the resolution of protein set 1 on GoBio Prep 16x600 40/100 SEC. (B) Effect of the flow velocity on the resolution of protein set 2 on GoBio Prep 16x600 40/1000 SEC. (C) Effect of the flow velocity on peak width of protein set 1 on GoBio Prep 16x600 40/100 SEC. (D) Effect of the flow velocity on peak width and separation of protein set 2 on GoBio Prep 16x600 40/1000 SEC. Resolution calculated using UNICORN 5.31.

Sample volume

A small sample loading is usually preferred in SEC purification, since a large sample loading on a SEC resin causes peak broadening and thus reduces resolution. However, a higher sample volume load means less operation time for a certain process, thus improving the operational efficiency. Hence, the sample load needs to be optimized for the target molecule according to the purification goals to achieve optimal balance between resolution and sample volume.

The sample volume applied to a SEC resin is often expressed as a percentage of the column volume (CV). For preparative SEC separations, the recommended sample volumes are between 0.5-4% of the CV, but if the application is a group separation (or desalting), the sample volume that is typically applied is ≤30% of CV.

Figure 4 illustrates how the loaded sample volume influences the resolution by affecting the peak width. With increased sample volume, the resolution of the tested globular proteins decreases because of peak broadening. Both GoBio Prep 16x600 40/100 SEC and GoBio Prep 16x600 40/1000 SEC exhibit good loadability for the tested sets of globular proteins and especially in the case of WorkBeads 40/1000 SEC where the resolution is not significantly affected by the increased sample volume, see Figure 4 C-D.

Also, note that sample concentration often has little effect on the resolution.

Prepacked columns:	(A) GoBio Prep 16x600 40/100 SEC (B) GoBio Prep 16x600 40/1000 SEC
Column volume:	120 mL
Samples:	(A) Myoglobin (M_r 17 600), apo-transferrin (M_r 78 300) (B) Ribonuclease A (M_r 13 700), BSA (M_r 66 300)
Sample volumes:	1.2 mL (1 % of CV) 3.6 mL (3 % of CV) 6 mL (5 % of CV)
Buffer:	20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Flow rate:	1 mL/min (30 cm/h)

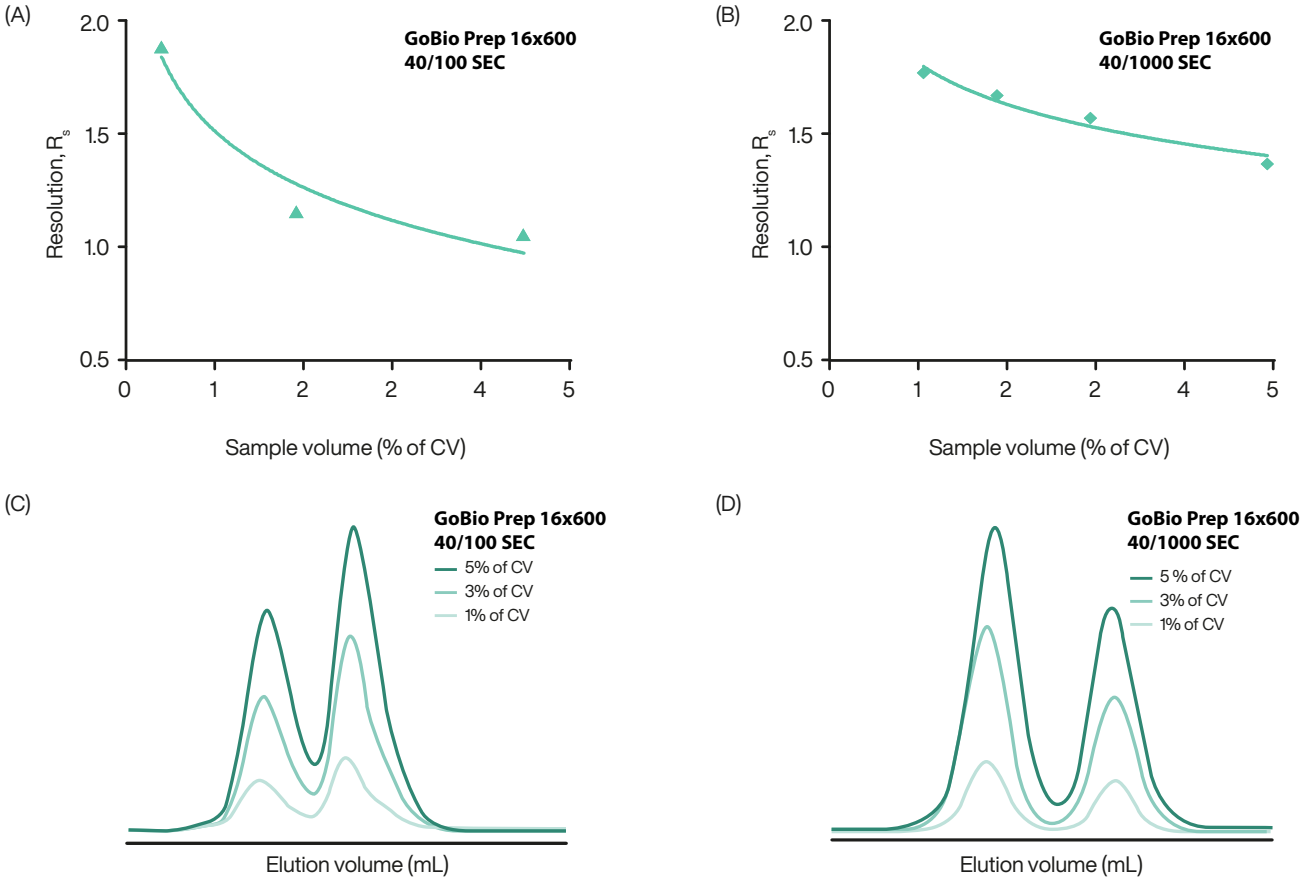

Figure 4. (A) Effect of the sample volume on the resolution of protein set 1 on GoBio Prep 16x600 40/100 SEC. (B) Effect of the sample volume on the resolution of protein set 2 on GoBio Prep 16x600 40/1000 SEC. (C) Effect of the sample volume on the peak width of protein set 1 on GoBio Prep 16x600 40/100 SEC. (D) Effect of the sample volume on the peak width of protein set 2 on GoBio Prep 16x600 40/1000 SEC. Resolution calculated using UNICORN 5.31.

Conclusion

WorkBeads 40 SEC resins exhibiting different porosities are suitable for purifications of a diverse set of biomolecules. It is important to choose a SEC resin suitable for the application purpose, for example target size, productivity requirement, purity requirement and whether scaling up is planned. By studying the characteristics of the different SEC resins, such as porosity and resolution effects under various flow rates and loading volumes, the choice of resin becomes easier. Knowing how operational conditions influence the separation performance is therefore of significant value.

WorkBeads 40 SEC properties

- Agarose beads → tolerate harsh cleaning conditions, high temperatures and autoclaving
- 45 µm bead → a sweet-spot for good resolution and modest backpressure
- Narrow pore size distribution → more uniform mass transport
- Wide range of flow rates → more flexible for optimization
- Higher flow rate for group separation → more efficient process
- High loadability → more productive SEC runs

Case studies – Virus purifications using SEC resins

Viruses (e.g. viral vectors, virus-like particles and vaccines, etc.) need to be isolated from a complex feed. Chromatographic techniques such as anion exchange chromatography (AIEX) and SEC can provide efficient and scalable approaches for virus isolations compared to the conventional methods, e.g. precipitation and sucrose density gradients. The most common approach in virus SEC purifications is to exploit the exclusion limit of the resins by eluting the macro-sized virus in the void volume while retaining the smaller impurities in the resin.

1. Purification of PRD1 virus by AIEX and WorkBeads Macro SEC

PRD1 is an icosahedral virus of 65 nm in diameter and 66 MDa in molar mass, and it shares many similarities with the human pathogen adenovirus (AV). To improve the purification of PRD1, an efficient and scalable method was developed, an inline AIEX-SEC, in which both steps were individually optimized prior to the final tandem setup.

WorkBeads Macro SEC was chosen after a screening of porosities of WorkBeads 40 SEC resins due to its high yield of target virus (98%) and large pore size. Figure 5 shows the chromatograms with absorbances at 260 nm and 280 nm from 1 mL loading (A) and 5 mL loading (B) of AIEX-pre-purified virus feed. A sample load of 5 mL, ~20% of resin´s CV (standard load for group separations) was needed to match elution volume from AIEX step.

There was a baseline separation between the virus-containing peak (void) and the impurity peak when loading both 1 mL feed and 5 mL feed on WorkBeads Macro SEC. High virus infectivity was detected in the void peak and only minor virus activity detected in the remaining impurity peaks, as determined by plaque assays. The larger sample load of 5 mL (20% of CV) resulted in very good separation and high yield.

For the two-step inline chromatographic setup, 40 mL of virus feed was loaded onto an AIEX column, where it was washed and, after removal of loosely bound impurities, eluted onto the inline connected SEC column to be collected in the void volume. Table 2 shows the comparison of virus yield and specific infectivity between the AIEX-SEC purified virus to virus purified by the conventional method, in which viruses were precipitated from the cell lysate using a PEG precipitation step followed by rate zonal centrifugation in a sucrose gradient.

The setup using AIEX-SEC resulted in purification being completed in only 320 minutes with 3.5 times higher yield and without losing any specific activity compared to the PEG-precipitation-ultracentrifugation method. For a more detailed description see the publication (M. Andersson Schönn et al., J Chromatogr B.; 2022: 1192: 1 March, 123140).

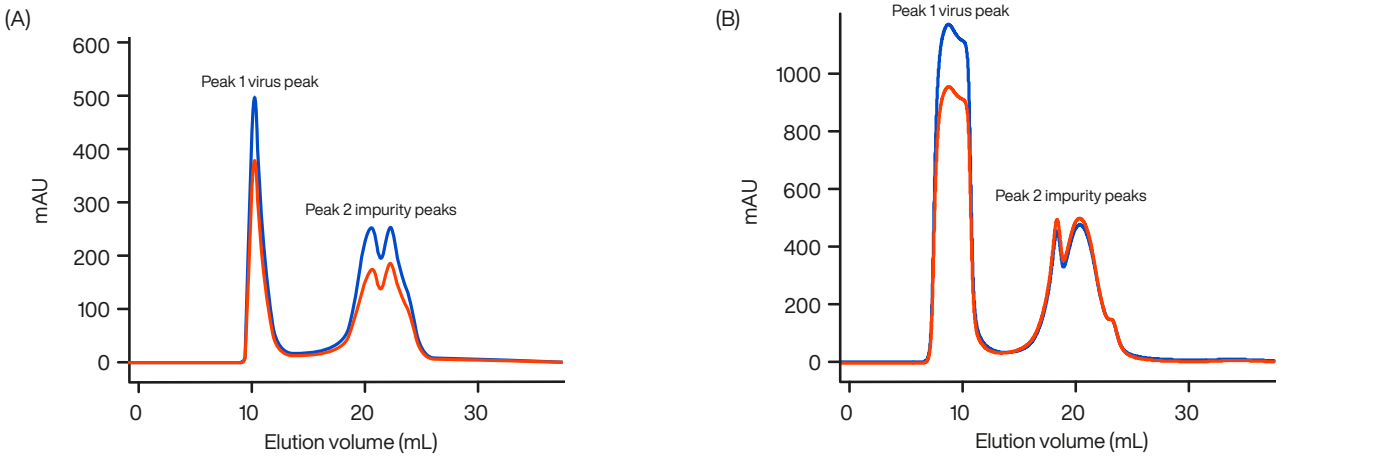

Figure 5. Size exclusion chromatograms using AIEX-pre-purified PRD1 preparation and WorkBeads Macro SEC using 1 mL feed (A) and 5 mL feed (B). The size of the column was 24 mL (10x300 mm). The flow rate was 1 mL/min (76.5 cm/h). Virus containing peaks (peak 1) and impurity containing peaks (peak 2) are indicated in the chromatograms. UV traces are shown as solid lines: Abs_{260nm} (blue) and Abs_{280nm} (red).

Table 2. Recovery and infectivity data for tandem purifications (AIEX-SEC) and conventional method based on four biological results

Method	Sample	Yield (pfu[1])	Specific infectivity (pfu/mg[2] of protein)	Time (hours)
AIEX-SEC	Lysate (feed)	100	$4.4x10^{11}$	-
	Pure virus[3] (final)	53.5	$5.8x10^{12}$	6
PEG Precipitation-ultracentrifugation	Lysate (feed)	100	$3.4x10^{11}$	-
	Pure virus[4] (final)	13	$6.6x10^{12}$	>24

[1] Determined by plaques assay analysis.
[2] Determined by Bradford assay analysis.
[3] Purified on WorkBeads 40Q and WorkBeads Macro SEC (no additional concentration step).
[4] PEG-NaCl precipitated, purified by rate zonal centrifugation in sucrose.

2. Vaccines and other viruses

The publication “Adenovirus dodecahedron, a VLP, can be purified by size exclusion chromatography instead of time-consuming sucrose density gradient centrifugation” by I. Szurgot et al. describes purification of a virus-like particle (VLP) adenoviral dodecahedron (Dd). Dd is used as a vector for delivery of small molecule anticancer agents and as a vaccination platform for foreign antigens. WorkBeads 40/10 000 SEC was used as the first step in a two-step chromatographic setup. Table 3 shows the comparison of Dd yield and recovery between the SEC-AIEX purified Dd to the more conventional method of purifying Dd which uses a sucrose density gradient centrifugation followed by AIEX.

Exchange of the centrifugation step to a SEC step significantly reduced the time consumption of the purification method from almost three days to 4–5 hours, see Table 3. When applying a three-fold larger amount of Dd-expressing cells with the same chromatographic setup, a similar yield of Dd per cell was obtained, which showed the scalability of this setup.

Table 3. Recovery from two-step chromatographic purifications and conventional method

Method combination	Recovery after 1st purification	Recovery after 2nd purification	Time (hours)
SEC - AIEX	93%	28%	4–5
Sucrose density gradient - AIEX	55%	15–18%	72

Conclusions – case studies

Both studies described above have resulted in two-step chromatographic methods which can be conducted in 4–6 hours compared to the more traditional, time-consuming virus purification techniques, such as precipitation and sucrose density gradient ultracentrifugation which takes days to complete. Additionally, the resulting yields have been reported to be high, making this chromatographic setup and the SEC technique an alternative approach for isolation of viruses as it is both efficient and cost saving.

Advantages of using WorkBeads SEC resins for purifying viruses, VLPs and vaccines

- The agarose matrix is compatible with biomolecules such as viruses and VLPs, no interference with their specific activities and infectivities was reported in the cases above
- Four different porosities and separation ranges offer a wide choice for separation of viruses and VLPs with their respective impurity profile
- Scalable approach for virus isolation and purification at larger scale
- High rigidity of the resin allows for a high flow rate for more efficient SEC purifications in group separation
- WorkBeads 40 SEC resins are tolerant to harsh chemical conditions allowing easy cleaning and re-use

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bio-works.com

Bio-Works, Virdings allé 18, 754 50 Uppsala, Sweden. For local office contact information, visit bio-works.com/contact.

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via neera 8/a 20141 Milano
Tel. +39 02.8954201 - Fax +39 02.89542022
www.cps.it-cps@cps.it