Improving your mAb purification

An expanding mAb market drives the need for high-efficient protein A resins

Monoclonal antibodies (mAbs) are genetically identical antibody copies used primarily in therapy and diagnostics. The main therapy areas for mAbs include cancer, inflammatory and autoimmune diseases, and viral infections. The global mAbs market accounted for approximately USD 100 billion in 2017 with an expected continued growth over the coming years. Since mAbs are mainly used in therapy, their purity is essential. Most of the therapeutic antibodies are produced *in vivo*, i.e., they are over-expressed in a biological system, which puts a high demand on downstream purification. One challenge with the mAb drugs is that repeated high therapeutic doses are often required (up to grams of mAbs per year per patient) and the drug-related impurities correlate to dosage. Even small traces of impurities may cause big cumulative effects. The purity requirements are therefore extremely stringent.

Today the most widely used main purification technique is affinity chromatography, and protein A resins are the products of choice for antibody capture. Protein A purifications generate in a single step both high yield and high purity of antibodies. For therapeutic use however, downstream chromatographic polishing steps are necessary to further enhance the purity by reducing impurities such as host cell proteins (HCP), host cell DNA (HCD), viruses and endotoxins to the very low levels required. The polishing steps should also remove any protein A leached from the protein A resin (ligand leakage) which can be toxic in a pharmaceutical product.

WorkBeads™ affimAb is a newly launched alkaline-stable protein A resin from Bio-Works designed for mAb purification from lab scale to process scale. This high-binding resin generates a higher purity of eluted mAbs from cell extracts in comparison with the present market-leading resin. The extraordinary high binding capacity also at high flow rates (short residence times) allows high productivity in downstream bioprocesses.

Protect the protein A resin

Purification of antibodies or Fc fusion proteins from mammalian host cells, such as Chinese Hamster Ovary (CHO), which is the primary mAb expression system to reduce the risk of immunogenicity, results in extensive bioburden on the protein A column. Host cell nucleic acids, together with host cell proteins in general, cause damage to the protein A column. Regular cleaning-in-place (CIP) is mandatory in the purification process, but accumulative fouling of the column will still occur. Maximized lifetime of the protein A column is thus an important requirement during the purification process development. It would be desirable to add a purification step before the protein A purification step in order to reduce the components that constitute the worst threat to the protein A column, and in the process improve the final purity of the target antibody.

WorkBeads 40 TREN is a multimodal ion exchange chromatography (IEX) resin with a ligand (tris(2-aminoethyl)amine, TAEA) that is positively charged below pH 9.

We introduced WorkBeads 40 TREN upstream of WorkBeads affimAb in the flow scheme and used it in flow through mode to adsorb undesirable impurities while letting mAb pass through the column without binding. The heavy sample load of impurities on the WorkBeads affimAb column was thereby reduced and the purity of the eluted mAb increased. The HCD and some HCP will be bound to WorkBeads 40 TREN below pH 9 due to their negative charges. Since the majority of mAbs are basic, and mainly positively charged at neutral or low pH, they will not bind to the resin in this pH interval.

When CHO cell extract with over-expressed mAbs is loaded onto a WorkBeads affimAb column, the UV trace shows a large amount of impurities in the flow through (Fig. 1). To study potential positive effects of WorkBeads 40 TREN as a guard column, a prepacked GoBio™ Mini TREN 5 mL column was placed upstream of the WorkBeads affimAb column. The WorkBeads 40 TREN column was positioned in a separate valve to allow automatic introduction and removal from the flow upstream of the WorkBeads affimAb column. This allows placing both columns in-line during sample load, to bypass the WorkBeads 40 TREN column during mAb elution, and to bypass the WorkBeads affimAb column during regeneration of the WorkBeads 40 TREN column. (Fig. 2).

Sample:	20 mL clarified CHO-cell extract
Resins/columns:	WorkBeads affimAb, 6.6 x 100 mm
Binding buffer:	50 mM sodium phosphate, pH 7.4
Elution buffer:	100 mM glycine-HCl, pH 2.7
Flow rate:	1.7 mL/min, 0.6 mL/min, and 0.9 mL/min (equilibration, sample load, and elution)
Gradient:	Step, 0-100% elution buffer, 10 column volumes (CV)
System:	ÄKTA™ system

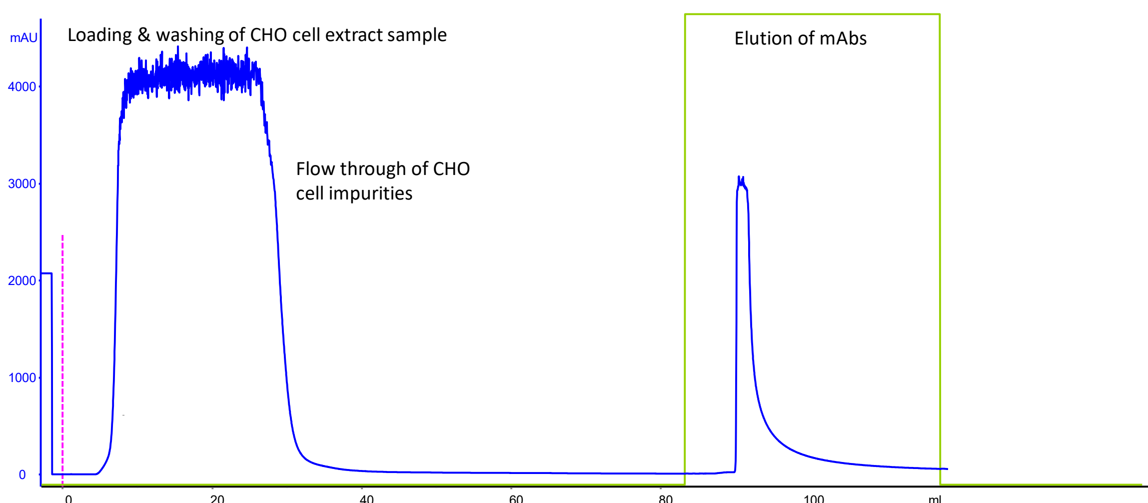

Figure 1. A purification run with only WorkBeads affimAb demonstrating the heavy mixed sample load when loading a CHO cell extract with over-expressed mAbs. Injection of sample (dotted pink line), Abs_{280} (blue line) and concentration (%) of elution buffer (green line) are shown in the chromatogram.

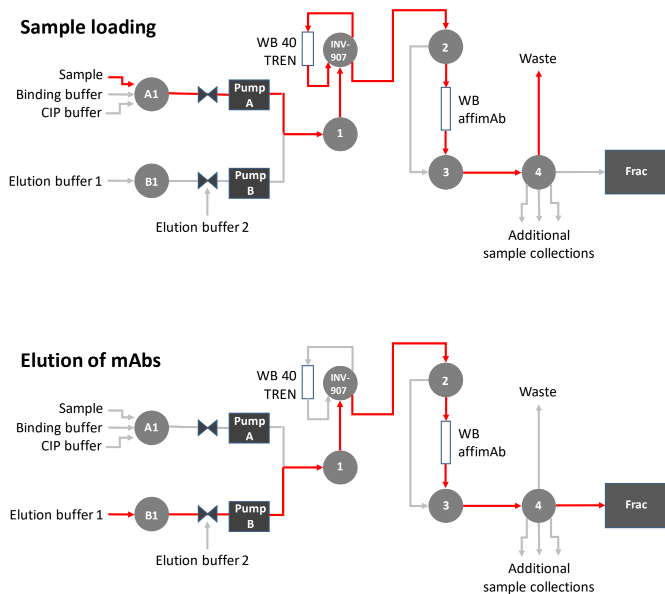

Figure 2. Flow chart of the purification method in which WorkBeads 40 TREN is acting as a guard column before WorkBeads affimAb. The WorkBeads 40 TREN column is positioned in Valve INV-907, position 2, and WorkBeads affimAb is positioned between valves 2 and 3. The top flow chart illustrates the flow during sample loading (red arrows) and the bottom flow chart illustrates the flow during the elution of mAbs (red arrows). Other steps in the process are not shown.

Valve · Pump · Fraction collector · Pump inlet · Column

The SDS-PAGE analyses demonstrated that a significant amount of host cell proteins was adsorbed to WorkBeads 40 TREN and thus removed from the sample before loading onto the WorkBeads affimAb column (see flow through lanes +/- WorkBeads 40 TREN in Fig. 3B). These analyses also demonstrated the non-binding of mAbs on WorkBeads 40 TREN at pH 7.4 resulting in no loss of recovery (see lane 6 in Fig. 3B).

Sample:	20 mL clarified CHO-cell extract
Resins/columns:	GoBio Mini TREN, 5 mL + WorkBeads affimAb, 6.6 x 100 mm
Binding buffer:	50 mM sodium phosphate, pH 7.4
Elution buffer:	100 mM glycine-HCl, pH 2.7
Flow rate:	3.5 mL/min (GoBio Mini TREN only/WorkBeads affimAb column bypassed), 1.7 mL/min, 0.6 mL/min, and 0.9 mL/min (equilibration, sample load, and elution)
Gradient:	Step, 0-100% elution buffer, 10 CV
System:	ÄKTA system

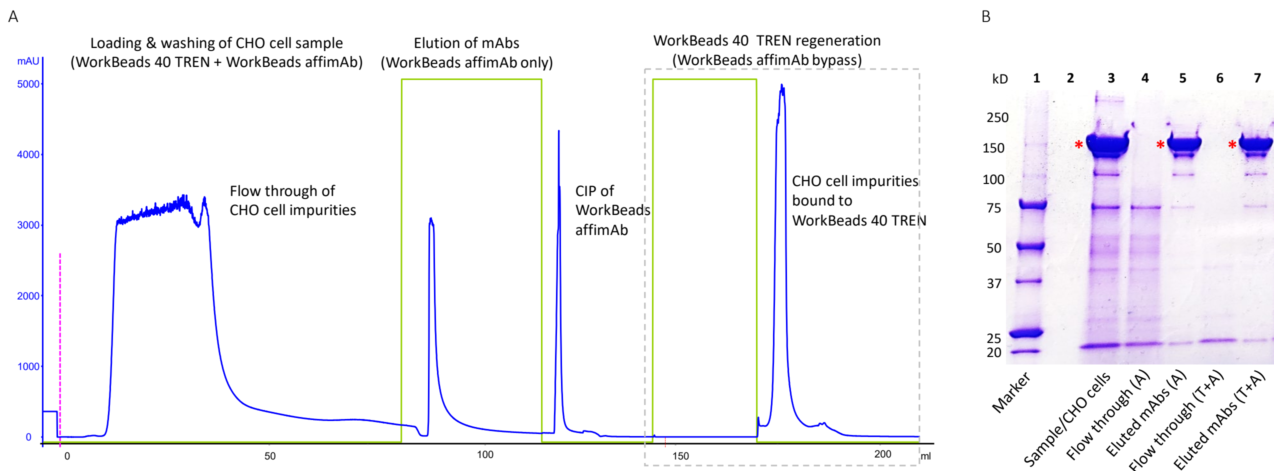

Figure 3. A) A monoclonal antibody purification where 20 mL clarified CHO cell extract is loaded onto WorkBeads 40 TREN + WorkBeads affimAb connected in series. Impurities in the sample will bind to WorkBeads 40 TREN. Sample loading and washing steps are performed with WorkBeads 40 TREN in line, but when running elution and CIP steps WorkBeads 40 TREN is bypassed. Regeneration steps are run with the WorkBeads affimAb column in bypass position. Injection start of sample (dotted pink line), Abs_{280} (blue line), concentration (%) of elution buffer (green line) and regeneration of WorkBeads 40 TREN (dotted grey box) are shown in chromatogram. B) SDS-PAGE analysis of the different flow throughs and eluates from the mAb purifications on WorkBeads affimAb +/- upstream WorkBeads 40 TREN. Lane 1 is the marker; lane 2 is empty; lane 3 is the sample; lane 4 is the flow through from WorkBeads affimAb; lane 5 is the mAbs eluted from WorkBeads affimAb (1:5 dilution); lane 6 is the flow through from WorkBeads 40 TREN + WorkBeads affimAb in series; lane 7 is the mAbs eluted from WorkBeads 40 TREN + WorkBeads affimAb in series (1:5 dilution). The red asterix indicate the mAbs.

MabSelect SuRe™ resin (GE Healthcare) was also included in our study to compare the performance of WorkBeads affimAb with a market leading protein A resin. Thus, four mAb purifications were performed, where 20 mL clarified CHO cell extract were loaded onto the two protein A columns with and without WorkBeads 40 TREN as a guard resin. The presence of impurities was determined by SDS-PAGE, CHO HCP ELISA and PicoGreen™ DNA analysis. The results are shown below in Table 1.

Table 1. Analysis of different collected flow through and eluate from four combination run on WorkBeads affimAb, WorkBeads 40 TREN + WorkBeads affimAb, MabSelect SuRe, and WorkBeads 40 TREN + MabSelect SuRe.

Resins included in flow chart for experiments 1-4	HCP (ng/mL)[1] *	HCD (ng/mL) [2] *
1a. WorkBeads affimAb (flow through)	NA[3]	10000
1b. WorkBeads affimAb (mAbs eluate)	775	40
2a. WorkBeads 40 TREN (guard); WorkBeads affimAb (flow through)	NA[3]	130
2b. WorkBeads 40 TREN (guard); WorkBeads affimAb (mAbs eluate)	610	40
3a. MabSelect SuRe (flow through)	NA[3]	10000
3b. MabSelect SuRe (mAbs eluate)	> 10000	500
4a. WorkBeads 40 TREN (guard); MabSelect SuRe (flow through)	NA[3]	130
4b. WorkBeads 40 TREN (guard); MabSelect SuRe (mAbs eluate)	950	40

[1] CHO HCP ELISA analysis.

[2] PicoGreen DNA analysis.

[3] Above detection limit.

* The values obtained in this application is based on the experimental conditions and setup as described above. Different sample batches under different conditions will generate variation in absolute numbers obtained.

Low level of HCP is one of the key quality attributes during downstream process purification development for biopharmaceuticals. HCP analysis using an enzyme-linked immunosorbent assay (ELISA) for measurement of CHO host cell proteins showed a relatively low level of HCP in the mAb eluates from WorkBeads affimAb with and without WorkBeads 40 TREN included upstream (see Table 1, second column). The eluted mAbs from MabSelect SuRe contained more than 10-fold higher levels of HCP (> 10000 ng/mL) compared to WorkBeads affimAb. When WorkBeads 40 TREN was added upstream MabSelect SuRe, the HCP level was reduced to a 10-fold lower level, indicating its ability to remove protein impurities from high bioburden samples. The HCP impurities are usually further removed by applying downstream ion exchange polishing steps to reduce the HCP level below the recommended pharmaceutical requirements.

The HCD (host cell DNA) analysis using the Pico Green assay showed superior results for WorkBeads affimAb eluates compared to MabSelect SuRe eluates with a 10-fold difference, 40 ng/mL *vs* 500 ng/mL, (see Table 1, third column, and Figure 4). However, adding an upstream WorkBeads 40 TREN column decreased the DNA content to approximately 40 ng/mL, indicating the ability of WorkBeads 40 TREN to remove DNA. Analyses of the different flow throughs showed that about 99% of HCD was removed from the sample feed when a WorkBeads 40 TREN column was connected upstream of the protein A column. Since the TREN-ligands will be positively charged at these running conditions (pH 7.4), the major part of the negatively charged host cell nucleic acids will be adsorbed to WorkBeads 40 TREN and thereby prevent loading of samples including high amount of impurities on the protein A column.

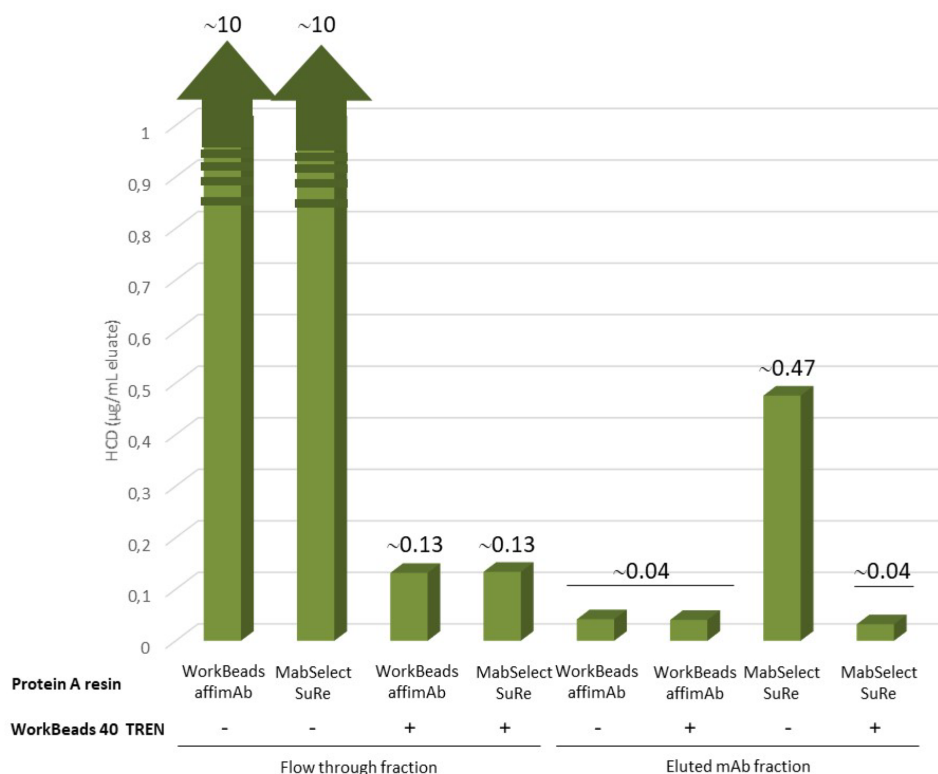

Figure 4. A diagram showing the HCD contents (µg/mL eluate) in the collected flow through fractions and the eluted mAb fractions. The flow through fractions from both protein A columns in the absence of an upstream protecting WorkBeads 40 TREN column contained more than 10 µg/mL HCD (off scale in this diagram).

Endotoxin removal

The CHO cell extracts before and after passing the WorkBeads 40 TREN column were analyzed for endotoxin content. The result showed no endotoxins present (as expected). In a separate experiment a sample containing known endotoxins was passed through WorkBeads 40 TREN and the result showed a clearance of > 93% of the endotoxins in phosphate buffer, pH 6 condition, indicating the advantage of employing WorkBeads 40 TREN as a guard column if samples containing endotoxins are purified, e.g., mAbs over-expressed in bacterial cells.

TREN is the new TRENd

WorkBeads 40 TREN is a new important tool for process purification of monoclonal antibodies. Clarified cell extract can be passed through the resin to remove a majority of the impurities such as host cell DNA and host cell proteins. Early removal of these impurities eliminates bioburden on the protein A column and extends its lifetime. Reduction of impurities early in the purification process further enhances the final purity of the product. This is essential for pharmaceuticals and diagnostic mAb based products. If mAbs are expressed in bacterial systems where endotoxins are present, WorkBeads 40 TREN has an even more important role due to its demonstrated ability to remove these impurities.

In this study, WorkBeads 40 TREN has been used as a guard column to protect the downstream protein A resin. WorkBeads 40 TREN can also be placed downstream of the protein A resin as a polishing column in flow

through mode to collect impurities coeluting with the mAb from the protein A column at low pH. This scheme will however not protect the protein A resin. Due to its orthogonal functionality, regardless of where in the mAb purification process it is used, WorkBeads 40 will significantly improve the purity of the final mAb.

Conclusions

- Protein A resins are the product of choice for monoclonal antibody purifications
- WorkBeads affimAb, as a single purification step, produced a mAb with higher purity compared to another supplier's resin which is extremely important to consider for pharmaceutical production
- WorkBeads 40 TREN, as a guard column, is an excellent tool for increasing the lifetime usage of protein A resins
    - Reduction of 99% of host cell DNA impurities
    - Reduction of host cell protein impurities
    - The eluted mAb showed increased purity and no loss of yield

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