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Modular high-throughput platform for the purification of scFvs and multispecific antibody-based therapeutics

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ABSTRACT

Automated ÄKTA system-based purification protocols were developed to streamline and accelerate protein production of single-chain variable fragments and multispecific antibodies. The protocols utilize ASX-560 and ALIAS autosampler systems to automate sample injections during overnight and idling times and incorporate one-, two- and three-column schemes to facilitate purification workflows. Additionally, the novel membrane affinity chromatography technology was employed to decrease residence times by increasing flowrates, shortening purification method run time per cycle. As a result, the implementation of versatile and automated multi-column purification protocols enabled the cost-effective and rapid capture of up to 51 single-chain variable fragments per day with high monomeric purities >90 %. Automated affinity chromatography protocols facilitated the daily purification of up to 22 multispecific antibodies. To increase the monomeric target purity of selected single-chain variable fragments and multispecific antibodies, size-exclusion chromatography was automated using the ALIAS Bio PREP autosampler system, enabling the polishing of up to 15 single-chain variable fragments or multispecific antibodies per day.

1. Introduction

Over the last few years multispecific antibodies (msAbs) have revolutionized the field of therapeutics, holding great promise for enhancing therapeutic efficacy and specificity in the treatment of complex diseases such as cancer, inflammatory and autoimmune diseases [6,20]. MsAbs are typically assembled from fragment antigen binding (Fab), single-chain fragment variable (scFv), fragment crystallizable (Fc) and single domain antibodies (VHH), which are interconnected by genetic fusion or heterodimerization [12]. The building blocks for msAbs at Numab Therapeutics AG (Numab) are humanized and lambda-cap-stabilized scFvs, displaying rabbit complementarity-determining regions, which can be assembled in plug-and-play formats allowing the rapid generation of hundreds of msAbs [5].

Efficient and fast manufacturing of scFvs and msAbs is crucial for screening hundreds of candidates in the early stages of drug discovery to allow pharmacodynamic and biophysical profiling with the aim to identify functional and biophysical superior molecules before entering the lead optimization phase. Various high-throughput systems for the purification of antibodies have been described in the literature [9,13]. However, the use of large, expensive and customized robotic systems make their use in drug discovery unattractive for many biotech companies and academic institutions. Additionally, conventional high-throughput methods often use plate-based or tip-column formats, which often do not provide sufficient quantities of antibodies with acceptable monomeric purity for extended biophysical characterization and stability studies to assess developability and manufacturability [1,10]. In recent years, highly efficient protein A membrane adsorbers have emerged as a promising alternative to traditional protein A resins offering faster purification cycles without compromising binding capacity [8,15]. Protein A membrane chromatography enables rapid capture purification at short residence times (RT), supporting more efficient workflows in the early stages of drug discovery.

Typically, scFvs and msAbs lacking the Fc-region are captured from the clarified cell culture fluid (CCF) by protein L affinity chromatography. Protein L shows high affinity for the variable region of the κ light chain subtypes I, III and IV [18]. For Fc containing msAbs, protein A affinity chromatography is used, as protein A binds to the Fc-region at

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the interface between the second (C_H2) and third (C_H3) constant domains of the heavy chain [2]. Over the past few years, various engineered protein A ligands were developed showing e.g., enhanced binding to the variable region of the heavy chain belonging to the V_H3 family or to the Fc, an improved alkaline stability towards sodium hydroxide (NaOH) [4,11,16,19].

Here, we describe a versatile and automated ÄKTA™ system-based high-throughput platform using mostly commercially available devices and modules that can easily be implemented in academic or industrial R&D environments to facilitate screening campaigns of transiently expressed scFvs and msAbs in a cost-effective manner without the need for big, expensive and customized robotic systems. The platform consists of (1) ASX-560 autosampler (AS) and ALIAS™ Bio PREP AS systems to automate injections and facilitate purification protocols especially during overnight and idling hours, (2) multi-column purification protocols to automate and streamline manual handling steps (i.e., sample transfer, buffer exchange) and (3) novel membrane chromatography to reduce the method run time by applying high flowrates.

Depending on the expression scale and purification workflow, our versatile platform allows for a fast and flexible purification of up to 51 scFvs with post-capture monomeric purities of >90 % or up to 22 msAbs per day and system in stabilizing buffer. A desalting column was integrated into the three-column scheme to automate buffer exchange protocols for scFvs and decrease manual handling times. To increase the monomeric target purity of some scFvs and msAbs, size-exclusion chromatography (SEC) protocols were automated using an ALIAS Bio PREP AS. This setup enables SEC polishing of up to 15 scFvs/msAbs per day on a Superdex™ Increase 10/300 GL SEC column or up to 7 scFvs/msAbs per day on a HiLoad™ 16/600 Superdex and HiLoad 26/600 Superdex SEC column, respectively.

Our approach offers a flexible solution to automate and accelerate repetitive purification protocols in academic and industrial R&D environments.

2. Material and methods

2.1. Transfection and expression of scFvs and msAbs

Transfection and expression of scFvs and msAbs were performed according to the ExpiCHO™ expression system kit user guide (Gibco) in conical TubeSpin® Bioreactors 50 (TPP) and Erlenmeyer flasks (Tri-Forest Labware, Corning) respectively. In short, ExpiCHO-S™ cells were diluted to a final cell density of 6×10^6 cells/mL for transfection. Deoxyribonucleic acid was mixed with OptiPRO™ medium and ExpiFectamine™ CHO reagent and incubated for 1-5 min at room temperature before being added to the freshly diluted cells. 18-22 h post transfection, the ExpiFectamine CHO enhancer and the ExpiCHO feed were added to the expression cultures. ScFv expression cultures were incubated at an orbital throw of 50 mm and 200 rpm at 37°C in 8 % CO_2 in a humidified atmosphere of 80 % for 8 days. Similarly, msAb expression cultures were incubated under the same conditions but with a shaking speed of 95 rpm in a non-humidified atmosphere as the expression volumes are normally higher. After 8 days, scFv and msAb expression cultures were harvested by centrifugation and sterile filtered using vacuum-driven 0.22 µm polyethersulfone (PES) membrane Sartolab® RF 50 filter units (Sartorius) and vacuum-driven 0.22 µm PES membrane Steritop™ filter units (Merck), respectively. The clarified CCF containing the scFv or msAb was then injected into the ÄKTA pure™ system.

2.2. Description of the ÄKTA system connected to the ASX-560 autosampler

The ÄKTA pure 25 (Cytiva) connected to the ASX-560 AS (Teledyne CETAC) was equipped with the following Cytiva components: injection valve (V9-Inj), inlet valve A/B (V9-IAB, 4 buffer ports), inlet valve B (V9-IB, 7 buffer ports), column valve (V9-C, 5 column ports), second column valve (V9-C2, 5 column ports), pH valve (V9-pH), outlet valve (V9-O, 12 outlet ports), versatile valve (V9-V), second versatile valve (V9-V2), UV monitor (U9-L), conductivity monitor (C9), I/O-box (E9), system pump A (P9 A), system pump B (P9 B), system pressure monitor (R9), mixer valve (V9-M), mixer (M9) and fraction collector (F9-C). This configuration allows the execution of automated purification protocols with up to three columns in series.

2.3. Incorporation of the ASX-560 autosampler into the purification workflow

The ASX-560 is a versatile AS that was configured for the automated injection of up to 84 samples with a volume of 50 mL per sample or up to 28 samples with a volume of 250 mL per sample. Movements of the AS injection needle to rinse or sample positions, and the peristaltic pump that is filling the rinse station, are controlled by the AScript software (Teledyne). Pumping of the sample or the rinsing solutions through the injection needle to the ÄKTA system was performed by the internal system pump B (P9 B) of the ÄKTA system via buffer inlet B7. Unidirectional communication from the ÄKTA system to the ASX-560 AS was achieved via the external equipment interface I/O-box (E9) using digital out signals triggering the move to the next AScript AS command line to operate the injection needle position and peristaltic pump of the AS.

2.4. Affinity chromatography in membrane and resin format and desalting column

The following protein A and protein L affinity chromatography in membrane and resin format and desalting column from Cytiva were used for screening and material production of scFvs: HiTrap Fibro™ PrismA (0.4 mL), HiTrap Fibro VL (0.4 mL, non-commercially available prototype containing the MabSelect™ VL ligand), HiTrap™ Protein L (5 mL) or Capto™ L resin packed into OPUS® MiniChrom® 11.3-50 column (5 mL, Repligen), MabSelect PrismA™ resin packed into OPUS MiniChrom 11.3-100 column (10 mL, Repligen), Capto L resin packed into OPUS MiniChrom 11.3-100 column (10 mL, Repligen), HiPrep™ 26/10 desalting column (53 mL).

Affinity chromatography resins in column format were used for the screening and material production of msAbs from clarified CCF with volumes ranging from 100 to 200 mL (the volumes stated correspond to volumes without ExpiCHO feed and enhancer). For molecules lacking an Fc, the following protein L resins were used: HiTrap Protein L (5 mL), HiTrap MabSelect VL (5 mL) or Capto L resin packed into OPUS MiniChrom 11.3-50/100 (5 mL/10 mL, Repligen). For molecules containing an Fc, the following protein A resins were used: HiTrap MabSelect PrismA (5 mL) or MabSelect PrismA resin packed into OPUS MiniChrom 11.3-50/100 column (5 mL/10 mL, Repligen).

2.5. Affinity chromatography buffers and steps

Affinity chromatography buffers for the purification of scFvs and msAbs consisted of buffer A1 at pH 8.5, buffer B1 at pH 2.7, and buffer B2 at pH 2.0 (all buffers were low salt low conductivity buffers). Binding and equilibration conditions were obtained by mixing buffer A1 and buffer B1 to obtain a pH of 7. After sample application, buffer A1 at pH 8.5 was used to perform a first wash step, followed by a second wash step at pH 6.0 by mixing buffer A1 and B1. Elution from protein A columns was achieved by applying a linear gradient to pH 2.7 by mixing buffer A1 and buffer B1. The elution from protein L columns was performed using a step at pH 2.0 with buffer B2. Cleaning-in-place (CIP) was performed with diluted NaOH solutions at concentrations and contact times recommended by Cytiva for each column type.

The final buffer used for equilibration and isocratic elution from the HiPrep 26/10 desalting column was selected based on the requirements of the subsequent internal assays. The CIP of the column was performed

with a 0.2 M NaOH solution.

2.6. Description of the ÄKTA system connected to the ALIAS Bio PREP autosampler

The ÄKTA pure 25 connected to the ALIAS Bio PREP AS (Spark Holland) was equipped with the following Cytiva components: injection valve (V9-Inj), inlet valve A/B (V9-IAB, 4 buffer ports), column valve (V9-C, 5 column ports), pH valve (V9-pH), outlet valve (V9-O, 12 outlet ports), UV monitor (U9-L), conductivity monitor (C9), I/O-box (E9), system pump A (P9 A), system pump B (P9 B), system pressure monitor (R9), mixer (M9) and fraction collector (F9-C).

2.7. Incorporation of the ALIAS Bio PREP autosampler into the purification workflow

The ALIAS Bio PREP AS was integrated into the flow path of the ÄKTA pure 25 via the injection valve V9-Inj connected to the ports loopE and loopF. The AS was controlled using the software SampLink and was synchronized with UNICORN™ via the I/O-box (E9). The scFv screening campaign was performed using the now outdated control software SparkLink, however the below stated AS parameters in this paragraph apply to both SparkLink and SampLink. The Bio PREP configuration has an internal 2.5 mL syringe, a 10 mL loop and can hold up to 24 V-shaped high-recovery vials (Infochroma vials: G006-23/045-H/VMμ; Infochroma caps: G007-ACC-SWFB15). V-shaped high-recovery vials can hold up to 9.5 mL and were used to minimize the dead volume and to allow for a safety margin of 100 μL per injection. The injection mode was set to partial loop-fill, sample speed was set to low, headspace pressure was turned on and the needle height was adjusted to 2 mm. After each injection step, the syringe and injection flow path in the AS were cleaned with 0.5 M NaOH (external wash bottle) and equilibrated with running buffer (internal wash bottle).

2.8. Protein concentration measurement

The concentration of the purified scFvs and msAbs was determined using an Infinite 200 Pro plate reader (Tecan) and a NanoQuant plate (Tecan). The buffer was used as blank and was subtracted from the absorbance measured at 280 nm. Each measurement was corrected for scattering caused by visible particles determined at 310 nm. The corrected value was normalized to a path length of 1 cm and the protein concentration was calculated using the theoretical extinction coefficient of the corresponding molecule.

2.9. SE-HPLC analysis

The monomeric content of the purified scFvs and msAbs was determined by analytical size-exclusion high-performance liquid chromatography (SE-HPLC). For scFvs, 5 μg were injected onto a PROTEIN KW402.5-4F column (Shodex, 4.6 × 300 mm, product code F6989201) using a HPLC system (Hitachi Chromaster). The mobile phase consisted of 50 mM sodium acetate, 250 mM sodium chloride, pH 6.0. For msAbs, 5 μg were injected onto a PROTEIN KW403-4F column (Shodex, 4.6 × 300 mm, product code F6989202) using a HPLC system (Hitachi Chromaster). The mobile phase consisted of 50 mM sodium phosphate, 300 mM sodium chloride, pH 6.5. In both cases the SE-HPLC analysis was performed at 20°C and a flowrate of 0.35 mL/min. The elution profile was monitored at a wavelength of 280 nm.

3. Results

3.1. Development of automated protocols for the purification of scFvs

3.1.1. Traditional purification of scFvs

Traditionally, scFvs are purified by protein L affinity chromatography followed by SEC polishing to achieve the desired monomeric target purity of >90 %. The sample pump of an ÄKTA pure system features 7 sample inlets, enabling the capture of up to 7 clarified CCF on a e.g., 5 mL resin-based protein L column (i.e., Capto L or MabSelect VL) in one workday or overnight. The elution fractions containing the target scFv are manually concentrated before being sequentially loaded into the sample loop for SEC polishing. Up to 6 scFvs can be SEC-polished per workday on a Superdex Increase 10/300 GL column, however the operator is fully occupied to keep the system running. To process a total of 21 scFvs (3 sets of 7 scFvs), the operator would need 2-3 days to perform protein L affinity chromatography and 1-2 days for SEC polishing depending on the monomeric purity achieved after the initial protein L capture step. Typically, an initial clarified CCF volume of approximately 50-100 mL is sufficient, depending on the titer, for provision of material for functional and biophysical characterization with accommodation of losses during manual concentration, loading and SEC polishing steps.

3.1.2. Automated tandem purification of scFvs

To automate injections of clarified CCFs containing scFvs, we integrated an ASX-560 AS connected to the ÄKTA pure system into our purification workflow (Fig. 1A). Additionally, the ÄKTA pure system was modified by a second column valve V9-C2 and a versatile valve V9-V. The tandem purification was achieved by operating a membrane protein A column (i.e., HiTrap Fibro PrismA) and a 5 mL resin-based protein L column (i.e., Capto L resin) in series (the benefits of using a protein A-L tandem purification scheme over the traditional approach are discussed in greater detail in paragraph 3.1.3). A versatile valve was placed in between the two column valves to allow for an independent column operation. For sample application, clarified CCFs were loaded via the ASX-560 AS onto both columns switched in-line, which was followed by consecutive wash, elution, CIP and equilibration steps of each column (Fig. 1B). The resin-based protein L column dictated the slow flowrate of 1.25 mL/min at a RT of 4 min during sample application, resulting in a long sample application phase (phase 2). A UV watch function with peak detection was used for automated peak collection for both columns during the elution step. The fractionated eluate was analyzed to determine its protein concentration and monomeric purity. Due to the high flowrate of 16 mL/min and a short RT of 1.5 s used during the wash, elution, CIP and equilibration steps, phases 3-6 are much shorter with the membrane protein A column compared to the resin-based protein L column (phases 7-10). The total purification time per cycle is approximately 55 min for a clarified CCF sample with a volume of approximately 15 mL. Using this automated tandem approach up to 23 scFvs can be captured with a post-capture purity >90 % in one day. To further reduce the total purification time, the resin-based protein L column was replaced by a membrane protein L column prototype containing the MabSelect VL ligand obtained from Cytiva in the HiTrap format. This way, the slow sample application flowrate as well as the slow flowrate of the resin-based protein L wash, elution, CIP and equilibration phases could be increased, which reduced the total purification time to approximately 25 min. A much smaller clarified CCF volume can be used in the automated tandem approach compared to the traditional approach, as manual concentration and loading steps are absent and approximately 90 % of scFvs have a post-capture purity >90 %. Only approximately 10 % of scFvs required SEC polishing to reach the target purity.

3.1.3. ScFv screening campaign in automated tandem mode

138 scFv expression cultures at 15 mL scale (representing a high clone-based binder diversity) were harvested and the clarified CCFs were injected using the ASX-560 AS on a membrane protein A column (i.e., HiTrap Fibro PrismA) and protein L column (i.e., Capto L resin or HiTrap Fibro VL prototype) in series as previously described. The protein concentration and monomeric purity of the fractionated protein A and L eluates were determined as described in paragraphs 2.8 and 2.9.

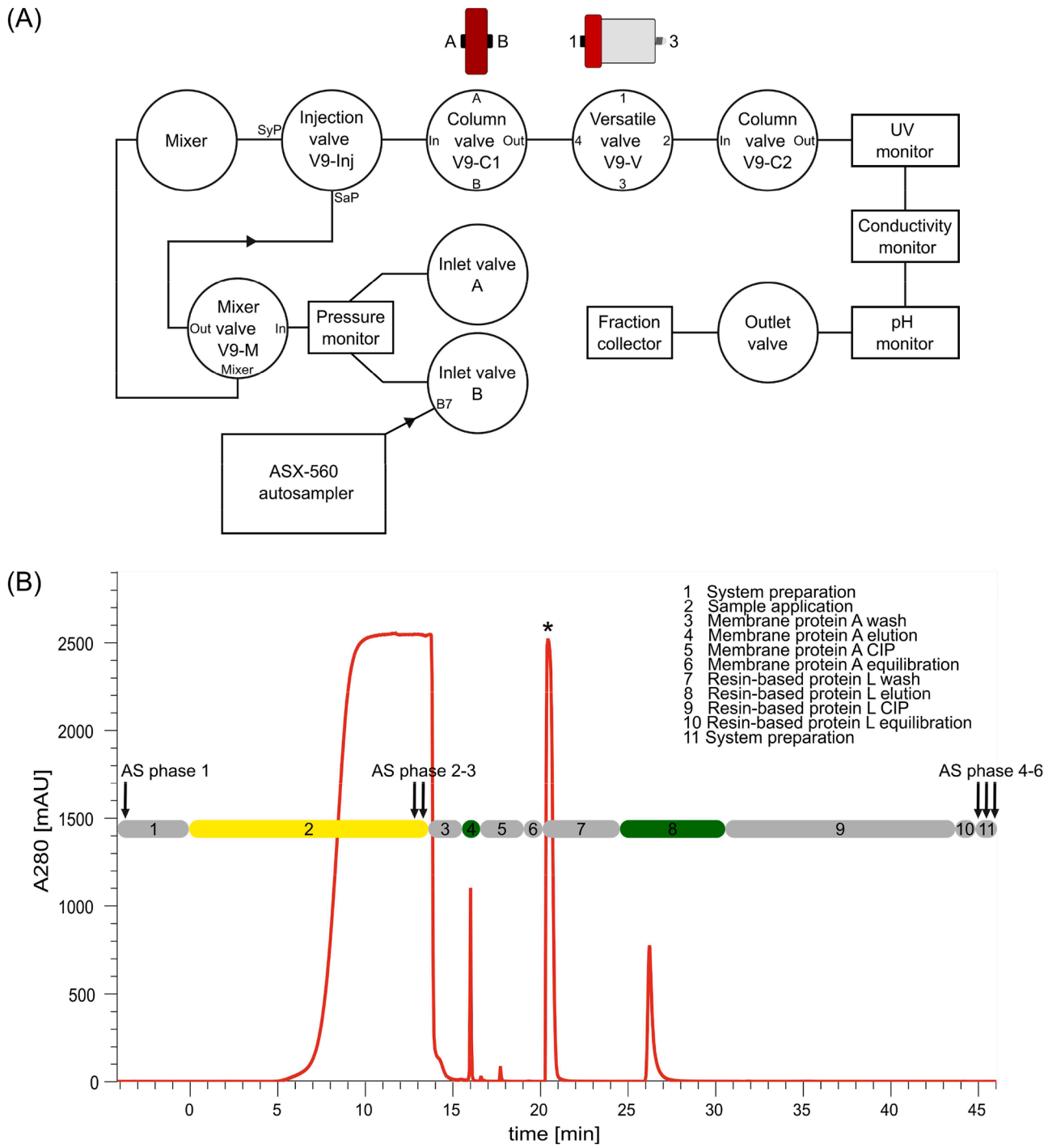

Fig. 1. (A) Fluidic scheme of the automated tandem purification on an ÄKTA pure system connected to an ASX-560 AS. Crucial modules are labeled in full name and abbreviated according to nomenclature used by Cytiva. Membrane protein A column was connected to the column valve V9-C1 at port A and B in A-B direction and resin-based protein L column was connected to the versatile valve V9-V at port 1 and port 3. For in-line applications V9-C1 and V9-V were switched from bypass to A-B and 1-4 2-3 direction, respectively. (B) Representative ÄKTA chromatogram from one automated tandem purification run, with the A_{280} trace shown in red. Numbered horizontal bars correspond to operational steps according to the UNICORN method sequence. Sample was applied in phase 2 (highlighted in yellow) and protein A and L elution occurred in phase 4 and 8, respectively (highlighted in green). The asterisk corresponds to clarified CCF present in the protein L path. Vertical arrows indicate the time points of the six AS phases. Here, a clarified CCF of approximately 15 mL was purified, which took in total approximately 55 min for an automated tandem purification.

Three possible scenarios can occur: (1) In the first scenario, scFv monomers and multimers bind strongly to the protein A column and in some cases additionally to the protein L column. As a result, no purity gain is achieved by tandem operation. If the high molecular weight species (HMWS) content of the samples is low, no SEC is needed to obtain the monomeric target purity. If the HMWS content is high, SEC is required to reach the monomeric target purity. (2) In the second scenario, scFv monomers bind weakly to the protein A column. Due to the avidity-driven effect, multimers of the scFv bind stronger to the hexameric MabSelect PrismA protein A ligand than scFv monomers. As a result, the weakly bound scFv monomers are found in flow-through of the protein A column and highly pure scFv monomers are captured and

recovered from the protein L column. (3) In the third scenario, both scFv monomers and multimers do not bind to the protein A column but to the protein L column instead. If the HMWS content of those samples is high, the needed purity gain is achieved by SEC. If the HMWS content is low, no SEC is required (Fig. 2). A summary of the 138 scFv capture runs is shown in Supplementary Table 1, stating the post-capture material amount and monomeric purity of protein A and L eluates. Each run is assigned to one of the three scenarios described above. Using this automated tandem approach, 123 scFvs (equivalent to approximately 90 % of the total) could be purified with a final post-capture monomeric purity of >90 %. 15 scFvs needed SEC polishing (equivalent to approximately 10 % of the total) to reach the desired monomeric target purity. Compared to the traditional approach, 21 lengthy, time-consuming and labor-intensive SEC polishing runs could be avoided. As seen from Supplementary Table 1, the majority of scFvs containing a high amount of HMWS benefited from the protein A and L tandem approach.

3.1.4. Operation of the ASX-560 autosampler

The ASX-560 AS is controlled by AScript, a step-based software using line by line commands. Six AS phases were implemented into the UNICORN method sequence to operate the injection needle position and peristaltic pump of the AS (Figs. 1B and 3). Digital out 1-4 were set to 0 in the system settings and reset to 0 in UNICORN at the start of each method sequence (AS phase 1). In addition, AS phases 2-6 were reset from 1 to 0 after the corresponding operation was triggered. As soon as AScript was started, the injection needle moved to the first sample. After sample application, the first signal was sent from the I/O-box to the AS to set digital out 1 from 0 to 1, to move and place the injection needle of the AS into the buffer compartment and to start the peristaltic pump to finalize the sample loading after sample application (signal 1, AS phase 2). The second signal set digital out 2 from 0 to 1 to turn off the peristaltic pump and to move and place the injection needle into the NaOH compartment (signal 2, AS phase 3). After wash, elution, CIP and equilibration steps of both columns were performed, the third signal set digital out 3 from 0 to 1. The peristaltic pump on the AS was started and the injection valve and mixer valve on the ÄKTA pure system were switched to sample pump waste and bypass, respectively, to allow CIP of the B7 inlet tubing via the SaP port (signal 3, AS phase 4). The fourth signal set digital out 4 from 0 to 1 to turn off the peristaltic pump, to move and place the injection needle into the buffer compartment and to turn on the peristaltic pump on the AS. The injection valve and mixer valve on the ÄKTA pure system remained in sample pump waste and bypass position, respectively, to allow equilibration of the B7 inlet tubing via the SaP port (signal 4, AS phase 5). The fifth signal set digital out 2 from 0 to 1 to turn off the peristaltic pump on the AS and to move and place the injection needle into the next sample (signal 5, AS phase 6). The UNICORN method can be easily adapted for single and multi-column purification without altering the AS phases by adding multiple repeats of column wash, elution, CIP and equilibration phases. This facilitates the rapid design and implementation of single and multi-column schemes.

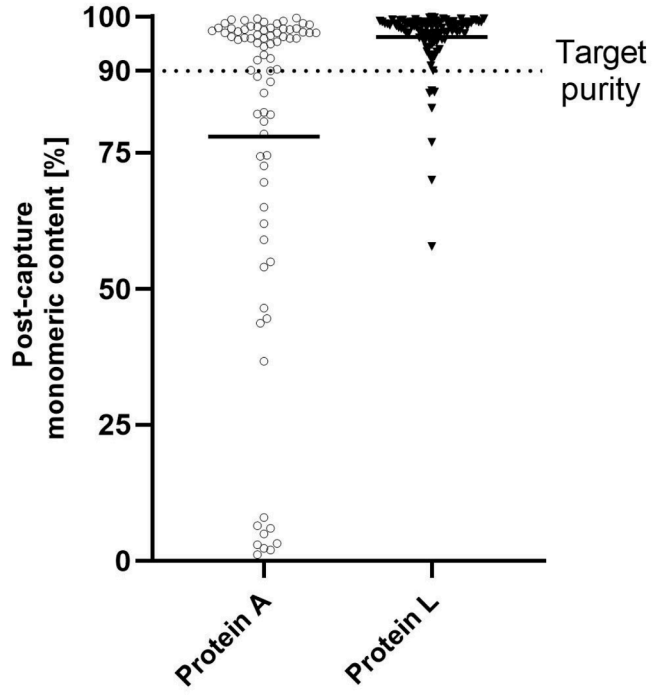

Fig. 2. ScFv screening campaign in automated tandem mode. 138 scFvs were captured in series on a membrane protein A and protein L column. Approximately 90 % of scFvs reached a monomeric purity >90 % post-capture, with approximately 10 % requiring SEC polishing to reach the desired monomeric target purity. For each dataset the mean value is shown.

3.1.5. Valve operation in automated tandem mode

The system pump B of the ÄKTA pure was used to transfer the sample from the AS to the columns via the B7 inlet tubing and the SaP port of the injection valve V9-Inj (Fig. 1A). During sample application, the mixer valve V9-M was set to bypass to avoid mixing and filtering of the sample in the mixer. The sample was captured on both membrane protein A column (i.e., HiTrap Fibro PrismA) and protein L column (i.e., 5 mL Capto L resin or 0.4 mL HiTrap Fibro VL prototype). The protein A column was connected to the column valve V9-C1 at port A and B in A-B direction, the protein L column was connected to the versatile valve V9-V at port 1 and port 3 in 1-4 2-3 position (Fig. 4A). After sample application, first V9-M was switched to the mixer path and V9-V to bypass to allow wash, elution, CIP and equilibration of the membrane protein A column. Second, V9-C1 was switched from downflow to bypass and V9-V to 1-4 2-3 position to allow wash, elution, CIP and equilibration of the protein L column. V9-C2 stayed in bypass throughout the entire purification process.

When using a second column valve V9-C2, the post-column pressure sensor of V9-C1 and the pre-column pressure sensor of V9-C2 are deactivated. Therefore, the pressure sensors at the inlet of V9-C1 and the outlet of the V9-C2 are used to measure the pressure drop across the columns, modules and tubing placed between V9-C1 and V9-C2 (Fig. 4B). The global pressure drop increases the more modules, tubing and columns are placed between V9-C1 and V9-C2.

3.1.6. Extension of tandem method by a desalting column to achieve automated three-column purification

A third column, namely a HiPrep 26/10 desalting column, was added to the automated tandem purification to prevent labor-intensive buffer exchange protocols of protein A and protein L eluates, which are normally performed manually. In order to allow a three-column operation, a second versatile valve V9-V2 was placed between V9-V and V9-C2. The desalting column was connected to V9-V2 at port 1 and port 3 and was only switched in-line in 1-4 2-3 position during equilibration, CIP and when the protein A and L eluates were transferred to the desalting column for buffer exchange. The fractionated eluate was analyzed to determine its protein concentration and monomeric purity. The description below uses the membrane protein L prototype (i.e., HiTrap Fibro VL) but the purification can also be performed with a resin-based protein L column. If a resin-based protein L column is used, changes in flowrates and column volumes (CV) need to be considered.

The AS phases 1-6 were not altered (Fig. 3). Equilibration of the HiPrep 26/10 desalting column with final buffer was carried out at a flowrate of 10 mL/min. Both membrane protein A and L columns were equilibrated in-line with running buffer at pH 7, and the sample was applied in series at a flowrate of 10 mL/min. First, the membrane protein A column was switched in-line, while the membrane protein L and

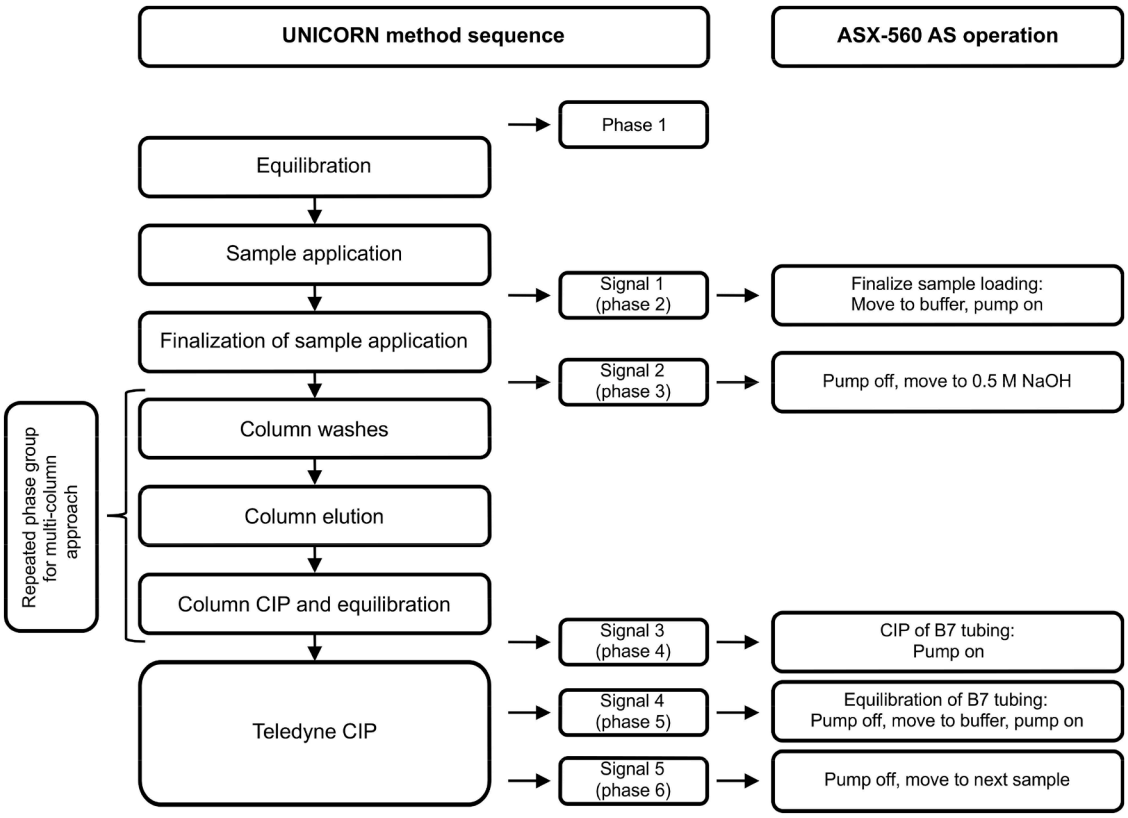

Fig. 3. Overview of UNICORN and AS method phases required for ASX-560 AS operation. In the UNICORN method sequence six AS phases were implemented to reset digital out signals and to operate the injection needle position and peristaltic pump of the AS. The AS operations were initiated by digital out signals 1-5 that were sent via the I/O-box of the ÄKTA pure to the AS.

desalting columns were switched to bypass. The membrane protein A column was washed at 16 mL/min. V9-V2 was switched to 1-4 2-3 position to place the membrane protein A and the desalting column in-line, and the flowrate was reduced to 3 mL/min to stay within the pre- and delta-column pressure limits of 0.5 MPa and 0.15 MPa, respectively. A total fixed volume of 12 mL was transferred to the desalting column (first gradient segment 41 % B to 100 % B in 6 mL, second gradient segment of 100 % in 6 mL), before the membrane protein A column was switched to bypass. Elution from the desalting column was performed at 10 mL/min using a UV watch function with peak detection for automated peak collection and fractionation volume was set to 5 mL. The total length of the elution from the desalting column was 63 mL (equivalent to 1.2 CV) to ensure full equilibration of the desalting column. Following elution, the desalting column was switched to bypass and the membrane protein A column was switched in-line for CIP and equilibration at a flowrate of 16 mL/min. Similarly, the membrane protein L column was first washed, before a total fixed volume of 12 mL was transferred to the desalting column (step gradient at 100 % B). Elution from the desalting column was performed at 10 mL/min and automated peak collection and fractionation volume was set to 5 mL. The total length of the elution from the desalting column remained at 1.2 CV to ensure the conductivity trace reached the desired value. Following elution, the desalting column was switched to bypass, and the membrane protein L column was switched in-line for CIP and equilibration at a flowrate of 16 mL/min. The HiPrep 26/10 desalting column was cleaned for 0.5 CV and equilibrated for 2 CV at 10 mL/min after each purification cycle. The total purification time for this three-column process is approximately 70 min for a clarified CCF volume of 15 mL.

3.1.7. Modular platform enables various purification protocols

This ÄKTA system-based modular platform allows the rapid design of multi-column purification protocols in a plug-and-play principle for screening and material production of up to 84 samples with a volume of 50 mL per sample per AS charge. Using the automated tandem approach in dual-membrane format, up to 51 scFvs can be purified per day with a post-capture monomeric purity >90 % (Fig. 5A and Table 1). The tandem mode was extended by a desalting column to automate buffer exchange protocols, which resulted in the purification of up to 18 scFvs per day with a post-capture monomeric purity >90 % (Fig. 5B and Table 1). In both screening approaches clarified CCFs with a volume of approximately 15 mL were used. For scFvs with a higher post-capture titer and for purifications where a larger CCF volume (i.e., approximately 50 mL) is processed, the membrane protein A or L column can be replaced by resin-based formats, as the dynamic binding capacity (Q_{B10}) of the 0.4 mL membrane columns is currently approximately 30 mg IgG/mL matrix. At material production scale, approximately 10 scFvs using the resin-based tandem or three-column purification scheme can be purified per day with a post-capture monomeric purity >90 % (Fig. 5C-D, Table 1).

3.2. Development of automated protocols for the purification of multispecific molecules

Fc containing msAbs are typically captured on protein A, whereas msAbs lacking an Fc are typically captured on protein L. To automate injections of affinity chromatography protocols at screening and material production scale, the ASX-560 AS was employed. The identical ÄKTA pure AS setup and AS phases as previously described for scFvs (see paragraphs 2.3 and 3.1.4) were used for the msAb purifications. All affinity columns were connected to the first column valve V9-C1 and the second column valve V9-C2 as well as all versatile valves remained in bypass during the entire purification. Since the AS can accommodate up to 28 samples, each with a volume of up to 250 mL, the selection of CV depends on the volume of the clarified CCF and the choice of resin is

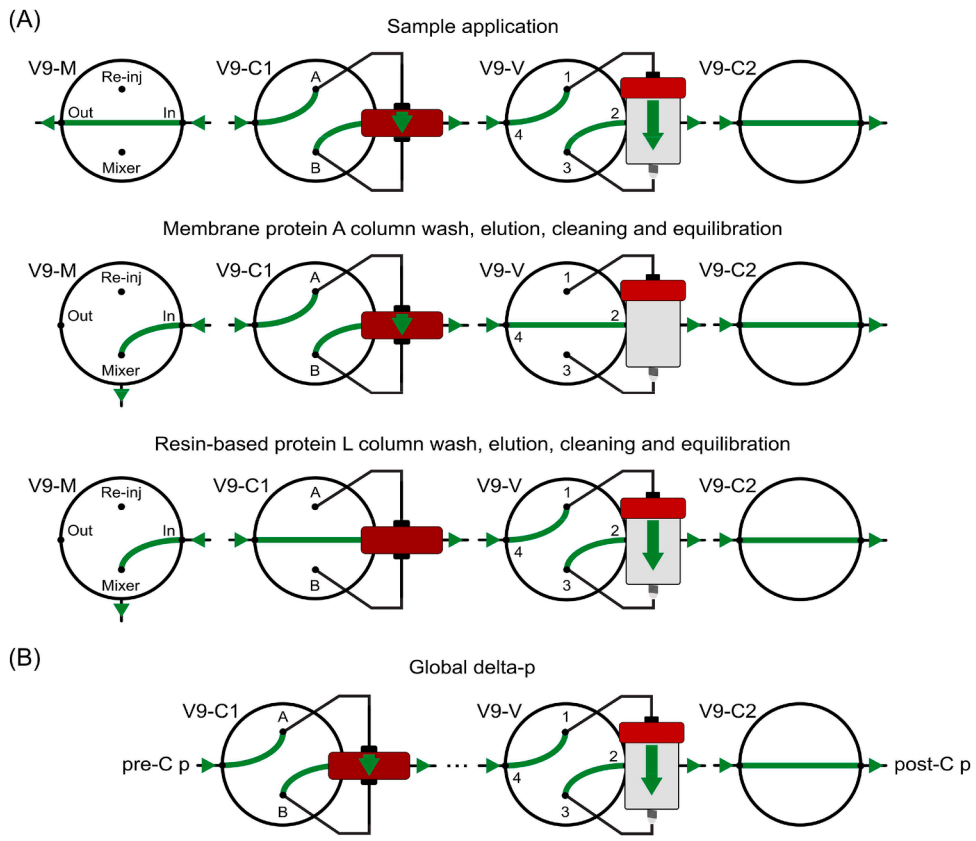

Fig. 4. (A) Valve operation during automated tandem operation. Sample is applied in-line on both membrane protein A and resin-based protein L columns. Wash, elution, CIP and equilibration steps are performed consecutively on each column. (B) Pressure calculation during multi-column operation. Pre-column pressure (pre-C p) on V9-C1 and post-column pressure (post-C p) on V9-C2 are used to calculate the global pressure drop (delta-p) between the two column valves.

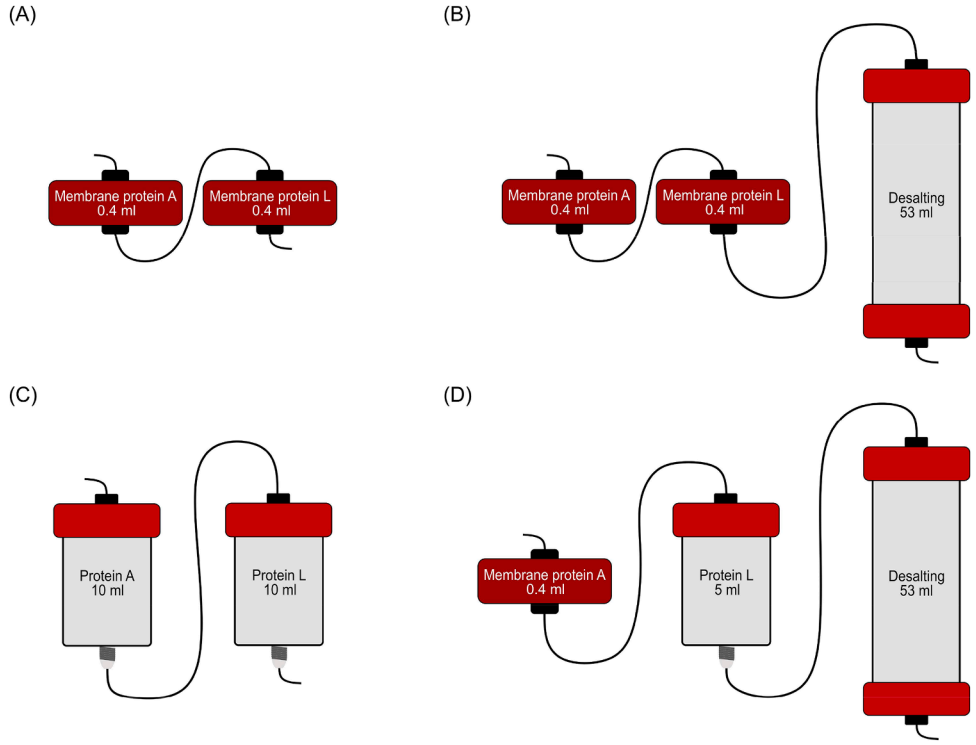

Fig. 5. Example overview of multi-column schemes currently used at Numab for the screening and material production of scFvs. (A) Automated tandem mode in dual-membrane format at screening scale. (B) Automated tandem mode in dual-membrane format extended by a desalting column to facilitate buffer exchange protocols at screening scale. (C-D) Automated tandem and three-column purification schemes at screening or material production scale.

Table 1
Summary of post-capture qualities and total process times of automated single and multi-column schemes, respectively, and SEC polishing protocols used at Numab for the screening and material production of scFvs and msAbs.

Format	Chromatography technique	Purification scheme	Approximate expression scale (without ExpiCHO feed and enhancer) [mL]	Post-capture titer [mg/L CCF]	Post-capture monomer content [%]	Approximate purification time per cycle [min]	Approximate number of purified molecules [day]
scFv	Affinity	Membrane protein A (0.4 mL) and resin-based protein L (5 mL)	15	383	100	55	23 with a post-capture purity >90 %
		Membrane protein A (0.4 mL) and membrane protein L (0.4 mL)	15	318	99	25	51 with a post-capture purity >90 %
		Membrane protein A (0.4 mL) and membrane protein L (0.4 mL) and desalting (53 mL)	15	317	98	70	18 with a post-capture purity >90 %
		Membrane protein A (0.4 mL) and resin-based protein L (5 mL) and desalting (53 mL)	50	370	100	120	10 with a post-capture purity >90 %
		Resin-based protein A (10 mL) and resin-based protein L (10 mL)	100	272	90	110	11 with a post-capture purity >90 %
msAb		Resin-based protein A or L (10 mL)	100	254	87	65	22
		Resin-based protein A or L (10 mL)	200	254	87	80	18
scFv/ msAb	Size exclusion	Superdex Increase 10/300 GL (24 mL)	N/A			95	15
		HiLoad 16/600 Superdex (120 mL)	N/A			200	7
		HiLoad 26/600 Superdex (320 mL)	N/A			200	7

N/A = not available

determined by the molecule format. In the following example, an Fc containing multispecific molecule was captured on a 10 mL column packed with MabSelect PrismA resin. After equilibration, the sample was applied at a RT of 1.5 min. The wash step was performed at 10 ml/min for 12 CV. Elution from the protein A column was performed at a flowrate corresponding to the RT of 1.5 min and automated fractionation was achieved by fixed volume fractionation. The fractionated eluate was analyzed to determine its protein concentration and monomeric purity. After elution, CIP and equilibration of the column were performed.

One purification cycle took approximately 65 min for a clarified CCF volume of approximately 100 mL on a 10 mL protein A or L column and approximately 1 h 20 min for a clarified CCF volume of approximately 200 mL on a 10 mL protein A or L column (Table 1). Using the automated injection approach, up to 22 msAbs can be captured per day. MsAbs that did not attain the desired monomeric purity were polished by SEC using an ÄKTA system coupled to an ALIAS Bio PREP AS as described below.

3.3. *ALIAS autosampler facilitates preparative SEC polishing*

The ALIAS Bio PREP AS was utilized to automate SEC polishing protocols for both scFvs and msAbs (Fig. 6A). SEC polishing was required for approximately 10 % of scFvs purified using the tandem or three-column purification protocols to achieve the desired monomeric target purity of >90 %. In addition, msAbs that did not attain the monomeric target purity post-capture of 95 % for *in-vitro* and 98 % for *in-vivo* application were polished by SEC. At Numab, we implemented SEC polishing protocols using the AS for three SEC columns namely Superdex Increase 10/300 GL, HiLoad 16/600 Superdex, HiLoad 26/600 Superdex. One SEC cycle on the Superdex Increase 10/300 GL took approximately 1 h 35 min, whereas one SEC cycle on the HiLoad column took approximately 3 h 20 min (Table 1). This allows us to polish up to 15 scFvs or msAbs per day in a fully automated way. Two AS phases are required in the UNICORN method sequence to operate the ALIAS Bio PREP AS, which in the example below is controlled by the SampLink software.

The ALIAS Bio PREP loading phase (first AS phase) is placed before the equilibration phase in the UNICORN method sequence, which is followed by the ALIAS Bio PREP injection phase (second AS phase) and the sample injection phase (Fig. 6B). In brief, digital out 1 and 2 values were changed in the UNICORN system configuration to 0. In the first AS phase, a watch function in UNICORN monitors the continuous digital signal sent from SampLink until the condition digital in 1 = 1 in UNICORN is met. As a result, UNICORN sends a 0.5 s digital out 1 pulse and SampLink changes the value of the continuous digital signal (detected as digital in 1 = 0 in UNICORN), initiating the sample loading from the vial into the internal 10 mL loop of the AS. During sample loading, the ÄKTA system remains in pause. Once the sample is loaded, SampLink changes the value of the continuous digital signal (detected as digital in 1 = 1 in UNICORN) and waits for the second UNICORN pulse. UNICORN sends a 0.5 s digital out 2 pulse and SampLink changes the value of the continuous digital signal (detected as digital in 1 = 0 in UNICORN). As a result, the AS turns the internal injection valve to inject and starts the CIP and equilibration of the AS syringe and flow path. The sample injection phase in UNICORN starts the application of the sample onto the column via V9-Inj using the system pump of the ÄKTA pure system. V-shaped high-recovery vials were used to minimize the dead volume. These vials have a maximum capacity of 9.5 mL, allowing the safe injection of up to 9.4 mL of sample into the AS loop.

4. Discussion

In the current study, we presented a versatile ÄKTA system-based platform to automate and streamline repetitive purification protocols for scFvs and msAbs. Standard ÄKTA pure and ÄKTA avant systems can easily be modified in a cost-effective way with the addition of a second column valve and one or two versatile valves to enable the implementation of two-column and three-column purification schemes (Table 2). For automated sample injections, the ÄKTA system can be further extended with an I/O-box to control the ASX-560 and ALIAS Bio

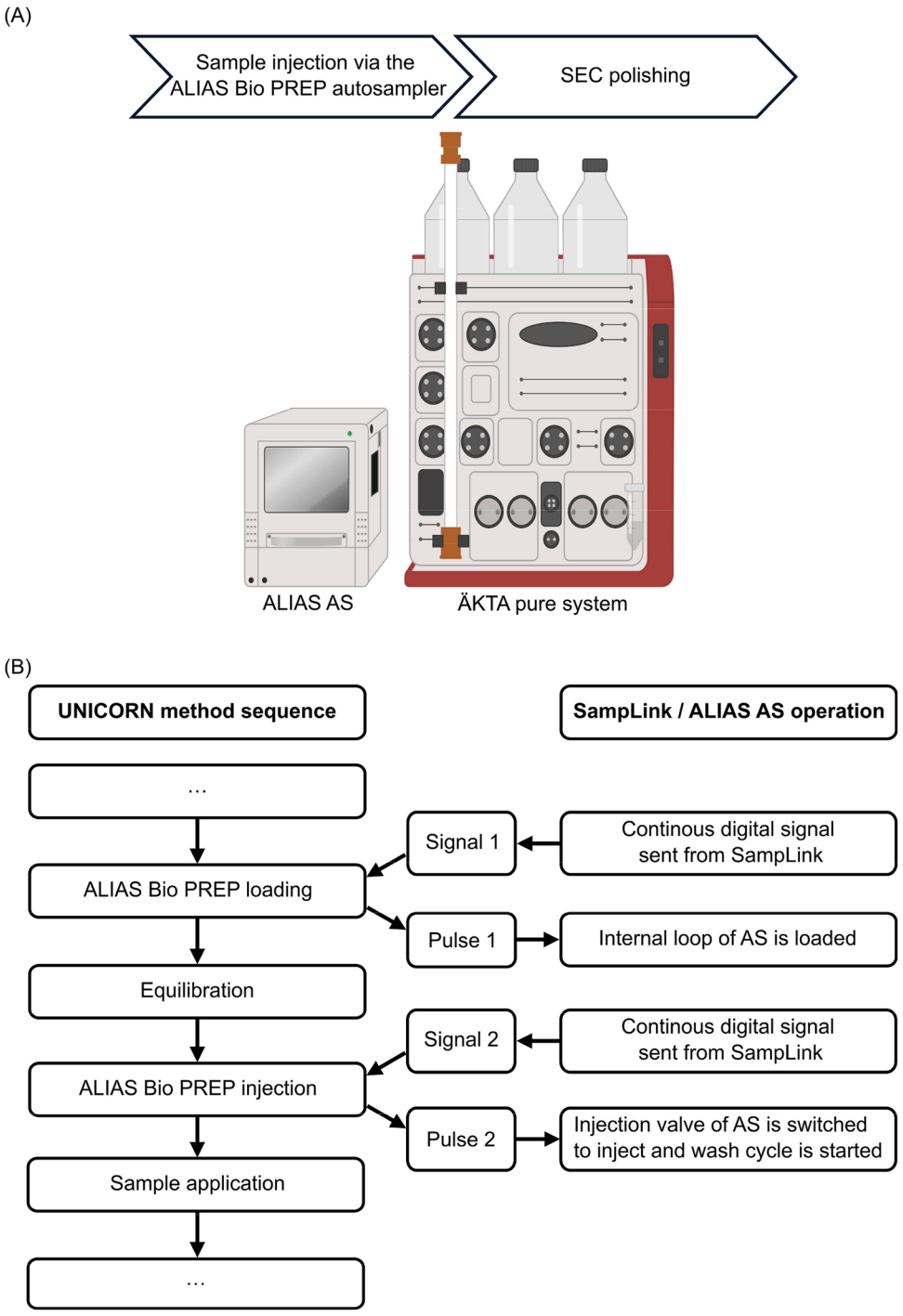

Fig. 6. (A) Graphical representation of the ALIAS AS ÄKTA pure setup for the automated SEC polishing of scFvs and msAbs. (B) Overview of UNICORN method phases required for ALIAS Bio PREP AS operation. In the UNICORN method sequence two AS phases were implemented to operate sample loading and injection of the AS.

PREP AS systems. Once the AS phases are integrated into the UNICORN method sequence, the method template can be transferred in a plug-and-play principle to automate purification protocols of up to three columns in series.

Automated injection protocols using AS systems are not limited to multi-column purification workflows described in this study. The integration of sophisticated workflows can be expanded to other resin modalities in two-column formats (e.g., affinity- and ion-exchange chromatography, affinity- and multi-modal chromatography) and three-column formats (e.g., affinity-, ion-exchange chromatography and desalting; affinity-, multi-modal chromatography and desalting).

The use of the ÄKTA system-based platform to develop versatile and automated multi-column purification schemes has been shown previously [3,7,14,17]. In a study by Delgado *et al.* [3], a four-column purification protocol was established using a modular in-line dilution method coupled to an ion-exchange chromatography step to automate the purification of thermostabilized turkey β1-adrenergic receptor variants, which resulted in the reduction in experiment time of approximately 58 % and the reduction in manual labor of approximately 83 %. In another study, Franke *et al.* [7] incorporated an ALIAS Bio PREP AS

Table 2
Overview of additional ÄKTA modules and AS required to allow for automated injections via AS system and the design and operation of multi-column purification protocols for the screening and material production of scFvs and msAbs.

ÄKTA modules/AS	List price* [US$]
Column valve V9-C	8,734.00
Versatile valve V9-V	3,224.00
Inlet valve B (V9-IB)**	6,516.00
I/O-box E9	2,842.00
ASX-560 AS***	13,000.00-15,000.00
ALIAS Bio PREP AS	21,400.00

* List price as per 28.08.2024: https://www.cytivalifesciences.com/en/us/shop, https://store.teledyneacs.com/collections/asx-560
** required when ÄKTA system-based platform is extended by an ASX-560 AS and only the inlet valve A/B (V9-IAB, 4 buffer ports) is present
*** Price depends on region

into the workflow to improve throughput, accuracy, and reproducibility of multi-step purification protocols. The use of the ASX-560 AS to fully automate two-step purification protocols of different antibodies at a high-throughput medium scale has been demonstrated by Madura *et al.* [14] published in an application note (Teledyne CETAC Technologies).

To increase productivity, it is highly desirable to employ the novel membrane affinity chromatography products, as the capture step purification can be performed with high flowrates and RT in the seconds range. We observed a decrease of two-fold in total purification time per cycle when the scFv tandem purification was operated in dual-membrane column mode (i.e., HiTrap Fibro PrismA and HiTrap Fibro VL prototype) compared to the membrane-resin column mode (i.e., HiTrap Fibro PrismA and 5 mL resin-based protein L column). Due to the faster blockage of fiber matrix in membrane columns, it is recommended to use ligands with enhanced high alkaline stability for CIP. The HiTrap Fibro PrismA membrane column is based on a cellulose fiber matrix and uses the same protein A PrismA ligand as the chromatography resin MabSelect PrismA. MabSelect PrismA is highly resistant to alkaline conditions, tolerating CIP procedures using 0.5 M NaOH and higher, making it an excellent candidate for high-throughput platforms. Osuofa and Husson [15] conducted a comparative analysis of the performance and physical properties of commercially available protein A membrane columns for antibody purification. Each membrane protein A column offers distinct characteristics and selecting the appropriate supplier depends on the specific requirements of the purification protocol.

In our opinion, there is a growing need for membrane columns at different scales with stable novel ligands to automate single or multi-column purification schemes in the early stages of drug discovery. The development and commercialization of stable protein L and A ligands with e.g., affinity for VH3 or Fc could further tailor the design of high-throughput purification methods for variable fragments and complex multispecific molecule formats.

5. Conclusions

In conclusion, we show that Numab successfully developed and implemented advanced purification processes for both scFvs and multispecific antibody-based therapeutics. The use of the ÄKTA system-based platform provides a fast and flexible solution, paired with limited capital expenditures and no need for dedicated automation personnel. The integration of ASX-560 and ALIAS AS systems and multi-column schemes streamline the workflow, particularly during overnight and idling operation and enhance throughput, accuracy, and reproducibility. Membrane affinity chromatography columns further increase the throughput and shorten purification run times. The ÄKTA system-based platform offers a modular, reliable, and cost-effective scaffold for the design of complex purification methods.

CRediT authorship contribution statement

Bastian Franke: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Conceptualization. **Julien Québatte:** Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Investigation. **Sebastian Wolniak:** Writing – review & editing, Visualization, Software, Methodology, Investigation. **Amélie Terreaux:** Writing – review & editing, Software, Investigation. **Cheryl Erne:** Writing – review & editing, Software, Investigation. **Christian Hess:** Conceptualization. **Ronnie Palmgren:** Writing – review & editing, Resources. **Stefan Warmuth:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466096.

Data availability

The data that has been used is confidential.

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