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Versatile modules enable automated multi-column purifications on the ÄKTA pure chromatography system

Bastian Franke[a,*], Tuomo Frigård[b], Stephan Grzesiek[a], Shin Isogai[a,*]

[a] Focal Area Structural Biology and Biophysics, Biozentrum, University of Basel, 4056 Basel, Switzerland
[b] GE Healthcare Bio-Sciences AB, Björkgatan 30, 751 24 Uppsala, Sweden

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ABSTRACT

Protein purification processes in basic research using ÄKTA™ liquid chromatography systems are often limited to single sample injections and simple one-column purifications. Because many target proteins in structural biology require complex purification protocols the work easily becomes laborious. To streamline and accelerate downstream protein production, an ALIAS™ autosampler and a modular sample in-line dilution process coupled to ion-exchange chromatography were incorporated into the workflow to automate two of the most commonly performed purification strategies - ion-exchange to size exclusion and nickel-ion metal affinity to size exclusion. The chromatographic setup enabled purification of a large array of cytosolic and membrane proteins from small-scale expression cultures produced in insect cells necessary to develop and optimize isotope-labeling strategies for nuclear magnetic resonance spectroscopy applications, resulting in a reduction in experiment time of about 20% per run for both cytosolic and membrane protein purification schemes. However, when queuing multiple samples the throughput increased by 66% and 75%, respectively. In addition, a novel system configuration is presented, where two column valves can be operated independently. This allows for the design of purification loops to increase purity of the target protein.

1. Introduction

ÄKTA™ chromatography systems have been designed to facilitate protein purification processes, however laborious manual operations such as transfer, desalting and concentration of sample in between the purification runs are still quite common amongst research groups. The modular ÄKTAxpress instrument has simplified repetitive high-throughput processes and automated multi-column purification protocols where routine production is needed [1], but the platform lacks flexibility and versatility. Several other studies describe more tailored multi-column purification schemes for specific target use [2], or generic protein purification machinery to aim for lean downstream development processes [3–6]. In all the cases, it involves highly modified ÄKTA instruments, additional modules and convoluted flow paths that restrict rapid adaptation of automation methods for other downstream purification experiments. Therefore, it is necessary to develop various use cases and building blocks further using standard hardware to; 1. facilite a day-to-day use of automation in downstream processes, and 2. provide a development scientist means to achieve flexible implementation of automation in a limited amount of time. It is also crucial to establish closer collaborations between the academic and industrial research communities to facilitate flexible and adapted solutions for specific needs. In addition, adaptive system configurations and application dependent functionality is setting a new stretch goal for this to succeed.

The introduction of the ÄKTA 3D Kit for the old discontinued ÄKTAexplorer range offered the first commercially available platform for facilitating various multistep protocols for the purification of up to 6 different His-or GST-tagged proteins on a day-to-day basis. However, the use of desalting (DS) columns and capillary loops not only restricted sample volume during purification but also lead to dilution of sample due to long flow paths during column-to-column transfer [6]. In a more recent study, a semi-preparative multistep purification method was performed on a commonly available ÄKTA pure chromatography system using a modular in-line dilution method coupled to ion-exchange (IEX) chromatography, which allowed for intermediate sample transfer as well as volumetric operations (dilution and concentration) [7]. This way, four G protein-coupled receptors (GPCRs) were

* Corresponding authors.
E-mail addresses: bastian.franke.pub@gmail.com (B. Franke), shin.isogai.pub@gmail.com (S. Isogai).

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purified sequentially using a four-step purification scheme, which resulted in reduction in experiment time of up to ~58% and a reduction in manual labor time of ~83% [7]. The combination of in-line dilution and IEX chromatography was designed to function as a generic building block for automating other multistep purification schemes.

In the present study, the benefits of incorporating an autosampler as another modular unit and combining the aforementioned modular sample in-line dilution–IEX process were explored. An ALIAS^{TM} autosampler (ALIAS-AS) was coupled to the ÄKTA pure instrument as an additional generic modular block to further improve throughput, accuracy and reproducibility of the multistep purification protocols. The ALIAS-AS was incorporated into the workflow to automate two of the most commonly performed purification strategies in basic research – IEX to size exclusion (SEC) and nickel-ion metal affinity chromatography (IMAC) to SEC.

To evaluate the automated multistep methods and system configurations, two separate purification schemes were developed. In the first scheme, a three-step purification protocol was designed incorporating two anion-exchange (AIEX) columns followed by a high-resolution semi-analytical SEC for the purification of the model protein green fluorescent protein (GFP). GFP has been found to be a convenient protein for the development and optimization of labeling protocols [8]. 36 unlabeled GFP samples with injections ranging from 50 μg to 1500 μg as well as ^{2}H-labeled and ^{15}N-labeled GFP samples were purified. In the second scheme, both unlabeled and ^{15}N-labeled samples of the challenging GPCR, turkey β1-adrenergic receptor, were purified using a three-column procedure (IMAC to cation-exchange (CIEX) to SEC) [9]. Both proteins were expressed in different isotope-labeled media compositions and purified protein samples were subjected to mass spectrometric analysis to determine isotope incorporation and labeling efficiencies. The integration of the autosampler enabled purification of an array of small-scale expression batches produced in insect cells necessary to develop and optimize isotope-labeling strategies for nuclear magnetic resonance spectroscopy applications with high accuracy.

2. Material and methods

2.1. Bacmid and recombinant baculovirus production of GFP and tβ1-AR

The truncated, cycle3 GFP variant and the thermostabilized GPCR, turkey β1-adrenergic receptor variant AR44-SI1 (tβ1-AR), were used and expressed in baculo-infected *Sf*9 insect cells (Oxford Expression Technologies) [9,10]. Baculovirus (BV) for insect cell expression was generated in *E. coli* DH10Bac cells according to the manufacture's recommendation (Invitrogen). The recombinant BV was cryo-preserved as baculo-infected insect cells (BIICs) [8], further amplified from BIICs in an additional passage of infection, harvested at 1500 g for 15 min at 4 °C and stored at 4 °C. Optimal multiplicity of infection (MOI) was determined in small-scale cultures and expression levels were quantified either by intrinsic fluorescence for GFP or by western blot analysis for tβ1-AR.

2.2. Preparation of ^{2}H and ^{15}N labeling medium for GFP and tβ1-AR expression

For ^{2}H labeling, a commercially available 98% uniform labeled cell-free amino acid mix (Cambridge Isotope Laboratories) was supplemented to SF-4 medium (BioConcept) depleted of amino acids and yeast extract (dSF-4) at a concentration of 6.88 g/L. For ^{15}N labeling, ^{15}N-labeled yeastolate was supplemented to dSF-4 medium at a concentration of 8 g/L for GFP experiments and 16 g/L, 8 g/L and 6 g/L, respectively, for tβ1-AR experiments [8,11]. In addition, ^{14}N glutamine was supplemented at a concentration of 1 g/L to each expression medium. pH and osmolality of the expression medium were adjusted as previously described [11].

2.3. GFP expression

2.3.1. Large-scale expression of unlabeled GFP

*Sf*9 cells were grown to a density of 2.0 $\times$ 10^{6} cells/ml in SF-4 medium (BioConcept) and 500 ml insect cell cultures were infected with optimal MOI virus stock amplified from BIICs and shaken at 180 RPM on a 50 mm orbit in TubeSpin® Bioreactors 600 (TPP). Cells were harvested after 64 h post infection (hpi) at 1500 g for 15 min at 4 °C, snap frozen in liquid nitrogen and stored at −80 °C.

2.3.2. Small-scale expression of ^{2}H-labeled and ^{15}N-labeled GFP

*Sf*9 cells were grown to a density of 2.0 $\times$ 10^{6} cells/ml in SF-4 medium and 30 ml insect cell cultures were infected with optimal MOI virus stock amplified from BIICs and shaken at 250 RPM on a 25 mm orbit for 16 h in TubeSpin® Bioreactors 50 (TPP). To wash the cell pellet, cells were centrifuged at 300 g for 4 min at room temperature and cell pellets were resuspended in dSF-4. Osmolality of the medium was adjusted with sodium chloride. Cells were centrifuged at 300 g for 4 min at room temperature and cell pellet resuspended in ^{2}H and ^{15}N labeling medium, respectively. Cells were harvested after 64 hpi at 1500 g for 15 min at 4 °C, snap frozen in liquid nitrogen and stored at −80 °C.

2.4. tβ1-AR expression

*Sf*9 cells were grown to a density of 2.5 $\times$ 10^{6} cells/ml in SF-4 medium and 100 ml insect cell cultures were infected with optimal MOI virus stock amplified from BIICs and shaken at 180 RPM on a 50 mm orbit in 225 ml tubes (Falcon-Corning). Cell pellets were washed and transferred into ^{15}N labeling medium and SF-4 full medium as described above. Cells were harvested after 48 hpi and resuspended in 3 ml resuspension buffer containing 20 mM TRIS, 1 mM EDTA before being snap frozen in liquid nitrogen and stored at −80 °C.

2.5. GFP purification

GFP cell pellets from insect cell cultures were resuspended in lysis buffer (50 mM TRIS, pH 8 at 4 °C, one cOmplete^{TM} mini EDTA-free protease inhibitor tablet (Roche) was added), sonicated, centrifuged at 13,000 RPM for 15 min at 4 °C and microfiltered through a 0.20 μm filter unit (Sarstedt). The protein concentration was determined by fluorescence using a NanoDrop^{TM} 3300 Fluorospectrometer (Thermo Scientific) and the GFP amount was adjusted to yield the following, which were injected in triplicates using ALIAS-AS: 50 μg, 100 μg, 150 μg, 200 μg, 300 μg, 400 μg, 500 μg, 700 μg, 900 μg, 1100 μg, 1300 μg, 1500 μg. The SEC Superdex 75 10/300 GL column (GE Healthcare) was pre-equilibrated with SEC buffer (sodium phosphate pH 7.4, 4 °C). 3.7 ml of each sample were injected using ALIAS-AS and GFP was captured onto the first with lysis buffer pre-equilibrated anion-exchange column (AIEX1, 1 ml, self-packed with GE Healthcare Q Sepharose Fast Flow resin) and eluted in reverse direction (50 mM TRIS, 2 M NaCl, pH 8 at 4 °C) from the AIEX1 onto a second pre-equilibrated anion-exchange column (AIEX2, 1 ml, self-packed with GE Healthcare Source 15Q resin). Subsequently, GFP was eluted from the AIEX2 and further refined on the SEC column.

2.6. tβ1-AR purification

tβ1-AR cell pellets from 100 ml insect cell cultures were solubilized to a final concentration of 20 mM TRIS pH 7.5, 400 mM

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NaCl, 2.5 mM imidazole, 2% *n*-decyl-β-D-maltopyranoside (DM, Anatrace). A flake of DNAse I (AppliChem) and one cOmplete mini EDTA-free protease inhibitor tablet (Roche) were added to the solubilization buffer. Cell lysis was performed using a dounce homogenizer (loose gap) and the solubilized membrane fraction was separated from the cell lysate by ultracentrifugation at 140,000 g for 45 min at 4 °C, before being microfiltered through a 0.20 μm filter unit (Sarstedt). Superdex 75 Increase 10/300 GL column (GE Healthcare) was pre-equilibrated with CIEX buffers at a buffer A (20 mM TRIS, 0.1% DM, pH 8, 4 °C) to buffer B (20 mM TRIS, 2 M NaCl, 0.1% DM, pH 8, 4 °C) ratio of 90:10. 4.3 ml of solubilized tβ1-AR were injected twice using ALIAS-AS and captured on an IMAC column (HisTrap Excel 1 ml (GE Healthcare)) pre-equilibrated with IMAC buffer A (20 mM TRIS, 350 mM NaCl, 2.5 mM imidazole, 0.15% DM, pH 8, 4 °C). tβ1-AR was eluted with IMAC buffer B (20 mM TRIS, 350 mM NaCl, 250 mM imidazole, 0.15% DM, pH 8, 4 °C), in-line diluted with CIEX buffer A [7], and bound onto a CIEX column (1 ml, self-packed with GE Healthcare SP Sepharose Fast Flow resin) pre-equilibrated with CIEX buffer A. Subsequently, tβ1-AR was eluted from the CIEX column with CIEX buffer B and further refined on the SEC column.

2.7. SDS-PAGE

10 μl of purified GFP sample was mixed with an equal volume of reduced 2x Laemmli sample buffer (Bio-Rad) and heated to 95 °C for 1 min. 10 μl of purified tβ1-AR sample was mixed with an equal volume of non-reduced 2x Laemmli sample buffer (Bio-Rad). 10 μl of each sample were loaded onto 4–20% Mini-PROTEAN® TGX precast gels (Bio-Rad) and 1-D gel electrophoresis was performed at room temperature for 30 min at constant 200 V. Gels were stained in Coomassie Brilliant Blue G-250 to visualize bands.

2.8. Analysis of isotope incorporation by mass spectrometry

Purified GFP samples were desalted according to the C4 MicroSpin column protocol (Nest Group). To determine the average mass of the GFP samples a volume of 35 μl was injected at a flow rate of 0.15 ml/min and a buffer A to buffer B ratio of 50:50 into a high performance liquid chromatography system (Agilent) coupled to a micrOTOF mass spectrometer (Bruker Daltonics). Buffer A consisted of 0.05% formic acid (FA) in water and buffer B consisted of 0.05% FA in 100% acetonitrile. Mass spectra were processed and deconvoluted using the HyStar™ data analysis software package (Bruker Daltonics).

Purified tβ1-AR samples were concentrated to ~250 μl using 15 ml centrifugal tubes (Merck Millipore, Amicon-Ultra-15, 50 kDa) at 4 °C. Concentrators were topped up with 5 mM ammonium acetate buffer containing 0.1% DM and concentrated to ~250 μl at 4 °C. Concentrated receptor samples were dried on a speed vac for 2 h, washed with 200 μl chloroform: methanol: 0.5% trifluoroacetic acid (1:3:1) and dried again for 1 h before being subjected to acidic hydrolysis (6 N HCl) for 24 h at 110 °C. Hydrolyzed tβ1-AR samples were derivatized by phenylisothiocyanate (PITC) and separated on an EASY-nLC™ nano-HPLC system (ThermoFisher) coupled to an Orbitrap Elite™ (ThermoFisher) mass spectrometer as described in Franke et al. [8].

3. Theory and design

3.1. ÄKTA pure instrument

The basic ÄKTA pure 25 M2 instrument (GE Healthcare) was customized and fitted with the following GE Healthcare hardware components: a fraction collector (F9-C), inlet valves (V9-IA/IB/IS), a sample pump (P9-S), a UV monitor for multi-wavelength detection (U9-M), a second UV monitor for single wavelength detection at 280 nm (U9-L), a mixer (M9), a conductivity monitor (C9), an outlet valve (V9-O), a loop valve (V9-L), a first versatile valve (V9-V1), a second versatile valve (V9-V2), a first column valve (V9-C1) and a second column valve (V9-C2). For simplicity, we refer to V9-C1 and V9-C2 as V9-C. The system configuration file version 1.10.0.6 was used unless otherwise specified. The instrument was kept at 8 °C in a chromatography cooling cabinet (UNICHROMAT 1500, Uni-Equip).

3.2. ALIAS Bio PREP autosampler

The ALIAS Bio PREP autosampler (Spark Holland) configuration uses a 2.5 ml syringe and a 10 ml internal sample loop. It is connected to the loop valve of the ÄKTA pure instrument and can be synchronized with UNICORN™ 7.3 via the I/O-box E9 (GE Healthcare) and fully controlled with the SparkLink 5.3 PC control software package. V-shaped high-recovery vials (Infochroma) were used to minimize the dead volume to 100 μl per injection. The needle height of ALIAS-AS was adjusted to 2 mm and injection was set to partial loop mode.

The sample transfer from the vial to the internal loop was initialized by the SparkLink control software and took ~4 min for one vial with a volume of 7 ml. Duplicate UNICORN method sequences (phases) were used to achieve volume injections > 7 ml.

3.3. Design of automated three-column purification of GFP

The GFP model protein was purified using a three-column purification protocol (AIEX1 to AIEX2 to SEC). The setup in Fig. 1 shows ALIAS-AS connected to the customized ÄKTA pure instrument and the detailed schematic fluidic flow path. ALIAS-AS was connected to the loop valve of the ÄKTA pure instrument and utilizes the system pump of the ÄKTA pure to inject the sample stored in the internal sample loop. A volume of 3.7 ml of each sample was injected and captured on the AIEX1 column connected to the V9-V2 at port 2 and port 4. The AIEX1 column was washed with 5 column volumes (CV) 1% elution buffer at a flow rate of 1 ml/min. A linear gradient of 1–35% over 10 CV was applied and V9-V2 was set to a position using reverse-elution of GFP from AIEX1 onto AIEX2 connected to V9-V1 at port 2 and port 4. During AIEX1 elution the flow from V9-V1 was directed to the second UV (U9-L) placed between the two versatile valves. During AIEX2 elution, a UV watch function (peak detection) triggered a change in V9-C from bypass, allowing injection of GFP onto the SEC column (downflow) (Fig. 2). The watch function condition was met when U9-M set to 397 nm observed a peak increase greater than 10 mAU. 820 μl of the elution peak were injected onto the SEC column. To minimize the chance of column damage by excessive delta-column pressure and overall system pressure during SEC loading, the flow rate was decreased to 0.2 ml/min. Subsequently, GFP was further refined on the SEC column. CIP of AIEX1/2 columns were performed in between each purification segment to allow for optimal column performance. CIP of the SEC column was performed after a set of twelve samples.

3.4. Design of automated three-column purification of tβ1-AR

tβ1-AR was purified using a three-column purification scheme (IMAC to CIEX to SEC) with the integrated in-line dilution protocol as previously described in Delgado et al. [7]. The schematic fluidic chart is shown in Fig. 3. V9-V2 was not required in this method and was switched to the default (bypass) position. 8.6 ml of each sample was injected into the ÄKTA pure instrument and captured on an IMAC column connected to V9-C. The initial sample was split

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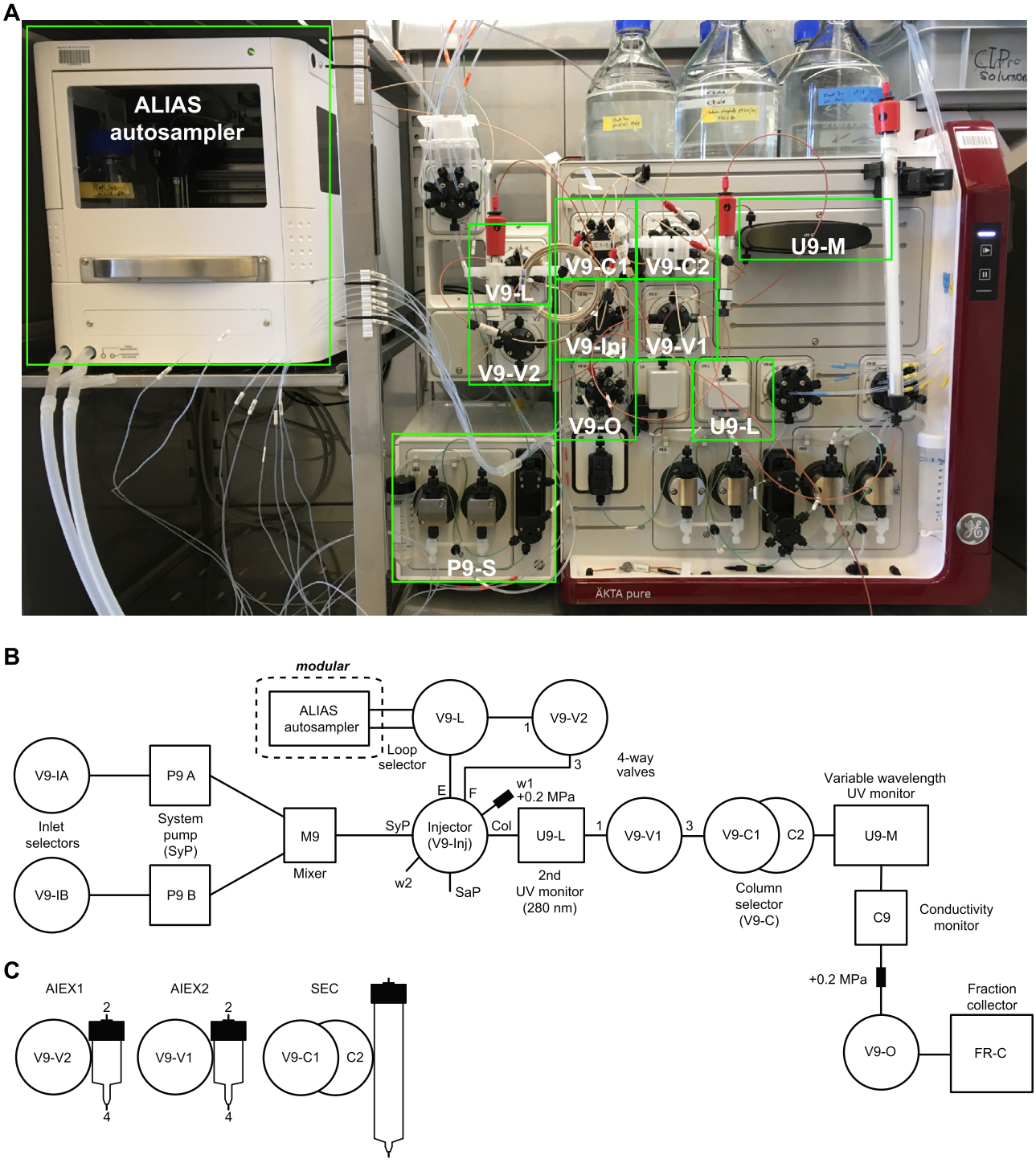

Fig. 1. ÄKTA ALIAS-AS setup to automate three-column GFP purification. (A) Crucial modules of the ÄKTA ALIAS-AS setup are framed in green and labeled with the abbreviations according to the instrument's manual. (B) Fluidics of the automated three-column purification protocol with integrated ALIAS-autosampler. P9-S was not used in this scheme but was highlighted due to its necessity in the second tβ1-AR purification protocol to achieve in-line dilution depicted in Fig. 3A. Black filled rectangles represent pressure regulators labeled with rated pressures. Pressure sensors are not drawn. The dashed rectangle represents the modular autosampler building block. (C) Columns used in this purification scheme. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

into two vials of 4.3 ml each due to the limited volume of 7 ml per vial. The IMAC column was washed with 20% IMAC buffer B for 9.9 CV and tβ1-AR was eluted with 100% IMAC buffer B. To reduce the high salt concentration and allow binding to the CIEX, the in-line dilution protocol was applied. During IMAC elution the flow (via port 10 at V9-O) was directed towards the mixing tee together with the CIEX buffer (via V9-V1) introduced using a sample pump (P9-S) allowing for dilution at a mixing ratio of 1:1. The diluted IMAC eluate was captured in upflow direction on the CIEX column connected to the V9-L. The CIEX column was then washed with 5 CV 10% CIEX buffer B in downflow direction. A linear gradient of 10–100% over 5 CV was applied to elute tβ1-AR from the CIEX column. During elution, a watch function by peak detection switched V9-C from bypass to the SEC column position to allow the injection

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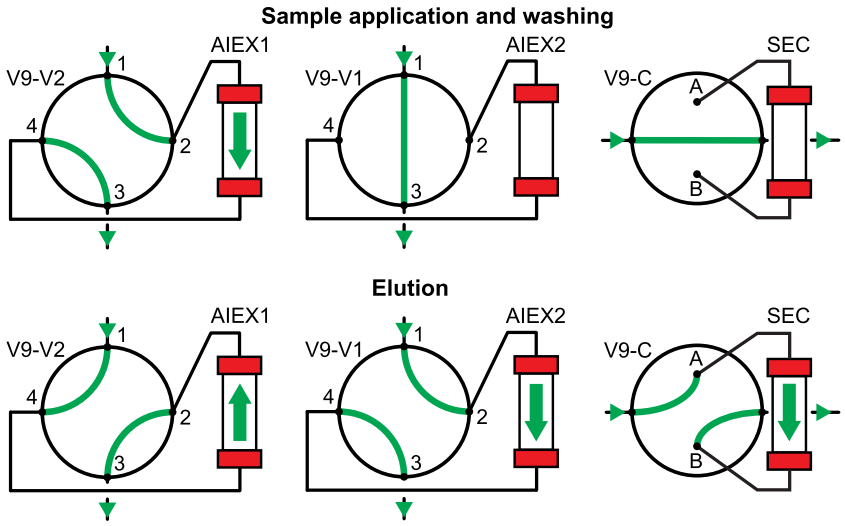

Fig. 2. Fluidic path and valve positions during automated three-column purification of GFP. Sample loading and washing on AIEX column is achieved in downflow direction. During elution, AIEX1 is switched to upflow and AIEX2 and SEC to downflow direction.

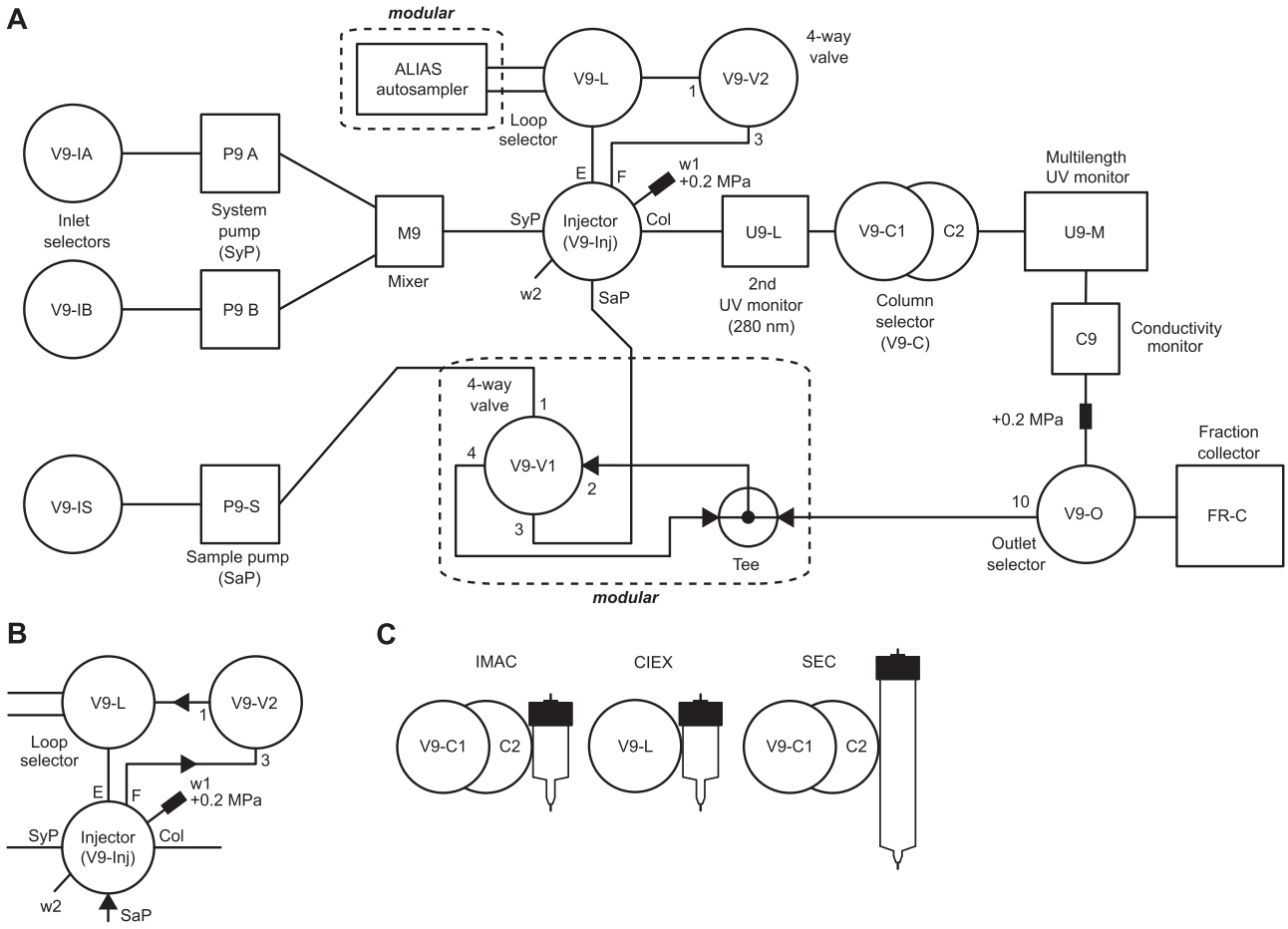

Fig. 3. Fluidics of automated three-column tβ1-AR purification with integrated autosampler and in-line dilution protocol. (A) The modules are labeled with abbreviations according to the instrument's manual. The flow directions during in-line dilution are indicated as black triangles. The dashed rectangles represent the modular autosampler and the in-line dilution building block, respectively. (B) The diluted sample gets captured in upflow direction on the CIEX column attached to V9-L. (C) Columns used in this purification scheme.

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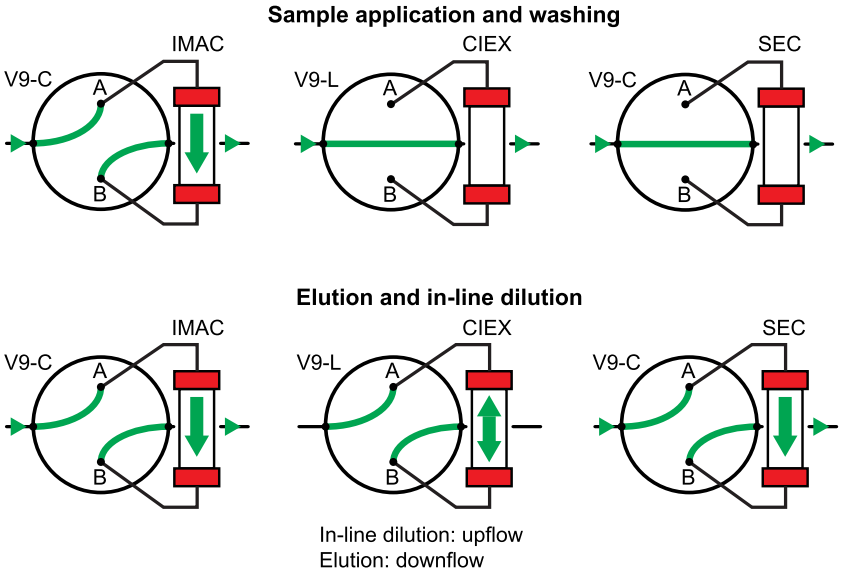

Fig. 4. Fluidic path and valve positions during automated three-column purification of tβ1-AR. Sample loading and washing on IMAC column is achieved in downflow direction. During in-line dilution sample from IMAC is stored in upflow direction on CIEX and further eluted in downflow direction on SEC.

of tβ1-AR onto the SEC column (Fig. 4). The watch function condition was met when the UV monitor U9-M set to 280 nm observed a peak height greater than 15 mAU. 2.5 ml were injected onto the SEC column. During SEC loading the flow rate was decreased to 0.5 ml/min. After loading, V9-C was switched back to bypass position. tβ1-AR was further polished on the SEC column. CIP of IMAC and CIEX columns as well as mixing tee and the ALIAS-AS loop was performed between samples.

4. Results

4.1. Automated multi-column purification of GFP

To evaluate the ÄKTA ALIAS-AS setup, 36 unlabeled GFP samples (twelve samples ranging from 50 μg to 1500 μg in triplicates) expressed in insect cells were purified using this fully automated three-column purification scheme (AIEX1 to AIEX2 to SEC). GFP samples were purified in sets of twelve due to limited capacity of the fraction collector to hold enough 96-well plates. One purification cycle including CIP ran for ~126 min. The 36 runs were separated into three sets of twelve automated runs, each of which ran in total ~25 h. Between each set, all buffers were refilled and the next set of twelve samples were prepared and placed in the autosampler. The SEC peak of each A_{397} trace was integrated and the recovered GFP yield in μg was calculated. The highest recovery >75% was obtained when injecting between 300 μg and 700 μg of sample (Fig. 5A and Supplementary Table 1). Beyond 700 μg, the protein recovery decreased steadily to 60%. Higher recovery could be achieved by increasing the injection volume onto the SEC column, however at the expense of purity due to other co-eluting proteins.

Triplicates of ^{2}H- and ^{15}N-labeled GFP samples were purified and the protein recovery was calculated in the same manner as described for the unlabeled samples (Supplementary Table 2). The recovered yield was ~70% for both ^{2}H- and ^{15}N-labeled GFP samples. 1-D gel electrophoresis was performed to show the purity of purified unlabeled GFP samples (50 μg, 700 μg and 1500 μg injections) (Fig. 5B), as well as the purity of purified ^{2}H- and ^{15}N-labeled GFP samples (Fig. 5D). The bands at 50 kDa in both gels were identified as GFP dimers by in-gel digestion and mass spectrometry (Supplementary Table 3). SEC chromatogram traces (A_{397}) of selected unlabeled, ^{2}H- and ^{15}N-labeled GFP samples were overlaid to show the reproducibility and accuracy of the developed purification method (Fig. 5C).

Purified unlabeled, ^{2}H- and ^{15}N-labeled GFP samples (each in triplicates) were subjected to mass spectrometry to determine the isotope incorporation into the protein. The ^{15}N-labeled sample showed an average uniform ^{15}N labeling of 75%, whereas the ^{2}H sample showed an average uniform ^{2}H labeling of 82% (Fig. 5E and Supplementary Table 4).

4.2. Automated multi-column purification of tβ1-AR

Unlabeled and ^{15}N-labeled tβ1-ARs were purified using a fully automated three-column purification scheme (IMAC to CIEX to SEC). Because tβ1-AR is prone to degradation, only three samples were used in each automated purification run. One purification cycle ran for ~4 h 40 min including CIP. Recoveries of unlabeled and ^{15}N-labeled tβ1-AR samples are shown in Supplementary Table 5. SEC chromatogram traces (A_{280}) of unlabeled and ^{15}N-labeled tβ1-ARs samples were overlaid in Fig. 6A and B, respectively. 1-D gel electrophoresis of the first unlabeled sample and the 16 g/L yeastolate ^{15}N-labeled sample was performed to show the purity of the tβ1-AR fractions and the low molecular weight impurities below 25 kDa in the neighboring fractions. Although tβ1-AR has a molecular weight of ~35.9 kDa, it appears on the gel at ~25 kDa (Fig. 6C and D). Mass spectrometric analysis of the PITC-derivatized amino acids obtained by acidic hydrolysis revealed an amino acid sequence-weighted average uniform ^{15}N labeling of 60% for the receptor sample supplemented with 8 g/L ^{15}N-yeastolate during expression. During acid hydrolysis, asparagine and glutamine are converted to aspartate and glutamate, respectively, and tryptophan is destroyed. (Fig. 6E). 55% ^{15}N labeling was achieved when 16 g/L ^{15}N-yeastolate was supplemented and 52% ^{15}N labeling when 6 g/L ^{15}N-yeastolate was supplemented, respectively. A lower ^{15}N labeling was achieved in GFP and tβ1-AR compared to previous studies [8,11], due to the supplementation of ^{14}N glutamine instead of ^{15}N glutamine during expression and the use of lower ^{15}N-labeled yeast extract.

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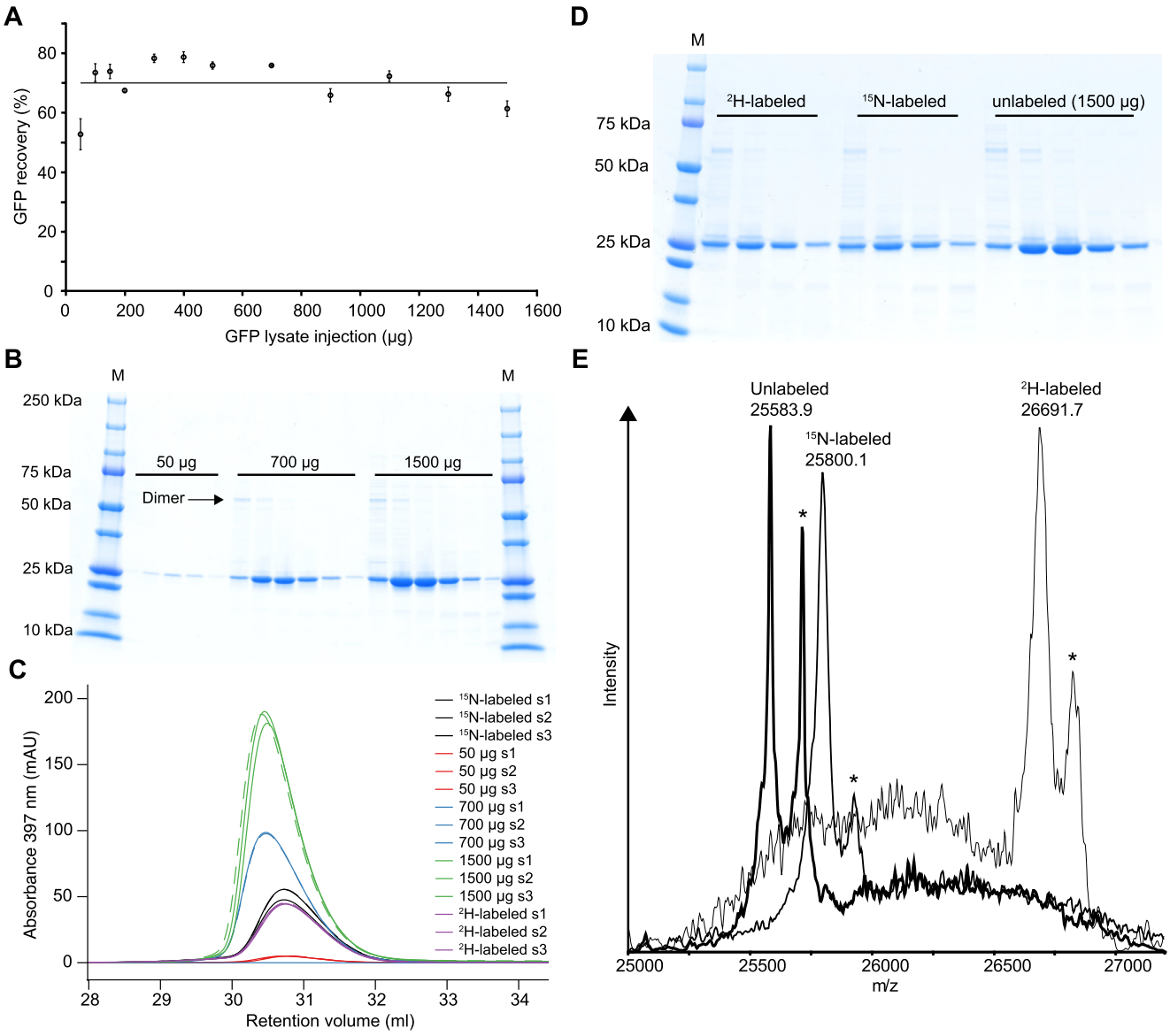

Fig. 5. Automated three-column purification of unlabeled, ^{2}H- and ^{15}N-labeled GFP samples as well as mass spectrometric analysis to determine isotope incorporation. (A) 36 unlabeled GFP samples were injected into the ÄKTA using the ALIAS-AS. The horizontal line represents the average protein yield of ~70% across all injections. (B) 1-D gel electrophoresis of purified unlabeled as well as purified ^{2}H- and ^{15}N-labeled GFP samples. (C) Overlay of SEC A_{397} traces of purified unlabeled, ^{2}H- and ^{15}N-labeled GFP samples in triplicates showing the accuracy of this purification setup. (D) 1-D gel electrophoresis of purified ^{2}H- and ^{15}N-labeled GFP samples. (E) Processed and deconvoluted mass spectra of purified unlabeled, ^{2}H- and ^{15}N-labeled GFP samples showing the *m/z* values of unlabeled and labeled GFP. The peaks marked with an asterisk contain an additional N-terminal methionine (+131.2 Da).

5. Discussion

5.1. Efficiency gains due to modular building blocks

In the current study we presented two modular building blocks, which can be incorporated into purification workflows to facilitate downstream processing applications on ÄKTA pure chromatography systems. The in-line dilution method coupled to IEX chromatography allowed for intermediate sample transfer as well as volumetric operations, whereas the ALIAS-AS improved accuracy and reproducibility of the developed automated purification protocols.

In the first AIEX1 to AIEX2 to SEC scheme an autosampler was incorporated into the work stream to purify unlabeled, ^{15}N and ^{2}H-labeled GFP samples. Manual interactions were limited to refill of autosampler with new samples and buffers, respectively. The total experiment time of ~126 min per purification cycle would allow us to screen 36 GFP samples obtained from different expression culture conditions every three days, which is more than sufficient to purify material for mass spectrometric studies, an efficiency gain of about 20% per run when comparing automated and traditional methods (Table 1). However, this efficiency gain and throughput can be increased by 75% when queuing multiple samples over a three-day period. In the second IMAC to CIEX to SEC scheme, unlabeled and ^{15}N-labeled tβ1-ARs were purified using both modular blocks combined. tβ1-AR purification should be limited to three samples per set, due to its susceptibility to degradation, resulting in 3 purified receptor samples every 14 h. Traditional schemes would allow us to perform one purification cycle per day. The average recovery of unlabeled tβ1-AR was 1.7 mg/L culture, an increase of about 65% in comparison to the four-step tβ1-AR purification [7]. The efficiency and recovery gains are associated with the removal of the affinity chromatography step [12].

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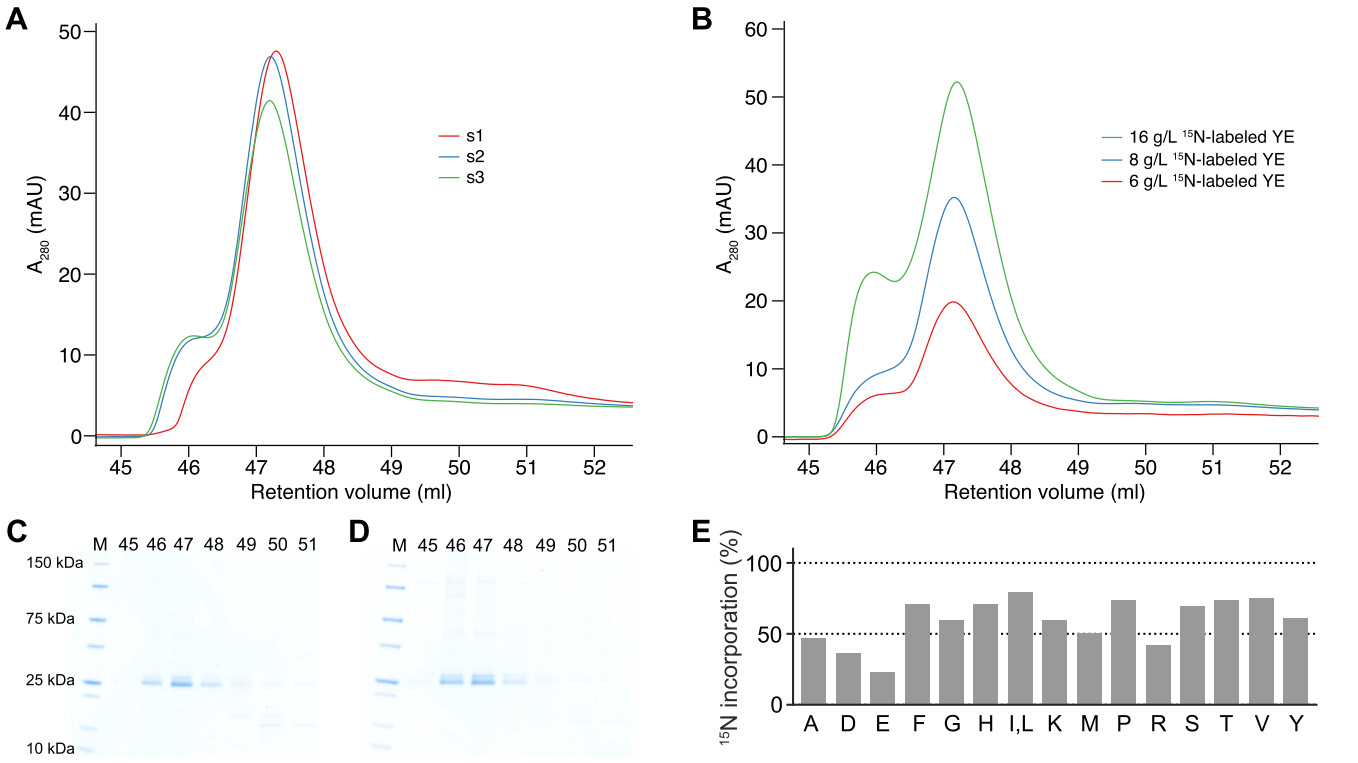

Fig. 6. Automated three-column purification of unlabeled and ^{15}N-labeled tβ1-ARs samples as well as mass spectrometric analysis to determine isotope incorporation. (A) Overlay of SEC A_{280} traces of three purified unlabeled tβ1-ARs samples (s1-s3). (B) Overlay of SEC A_{280} traces of three purified ^{15}N-labeled tβ1-AR samples, where ^{15}N-labeled yeast extract (YE) was supplemented at different concentrations during expression. (C) 1-D gel electrophoresis of purified unlabeled tβ1-AR (s1) and (D) purified ^{15}N-labeled tβ1-AR (16 g/L ^{15}N-labeled YE). Numbers above the gels indicate SEC retention time volumes. (E) LC-MS analysis of PITC derivatized ^{15}N-labeled amino acids obtained by acidic hydrolysis of ^{15}N-labeled tβ1-AR, supplemented with 8 g/L ^{15}N-labeled YE during expression.

5.2. Autosampler in fast protein liquid chromatography applications

Although autosamplers are widely incorporated into high-performance and ultra-performance liquid chromatography applications, they have not piqued the interest of the fast protein liquid chromatography (FPLC) community with a limited number of references described in the literature [13]. This is most likely due to various volume requirements depending on the scale of the purification processes. Currently there are only three main autosamplers available on the market, which can be used with ÄKTA instruments: (1) the ÄKTA-branded A-900 and A-905 models (GE Healthcare), allowing sample volume injections between 0.1 μl and 1 ml and are only compatible with the discontinued previous generation ÄKTA range (i.e. ÄKTAexplorer and ÄKTApurifier); (2) ASX-520 autosampler (CETAC), which has been tested in combination with the ÄKTApurifier to automate a two-step purification protocol and can manage up to 240 samples with variable volumes ranging from 0.5 ml to 14 ml or up to 84 samples with volumes ranging from 0.5 ml to 50 ml [13]. Based on this study by Yoo et al. [13], Teledyne CETAC developed the ASX-560 model, which offers similar specifications but with increased injection volumes (up to 1 liter) and can also be used with the ÄKTA pure chromatography system (application note by Teledyne CETAC technologies). (3) ALIAS Bio and ALIAS Bio PREP (Spark Holland). The ALIAS Bio can house two 96 well microtiter plates or 96 samples with 1.5 ml sample volume and injection volume ranging from 1 μl to 5000 μl with 1 μl increments. The ALIAS Bio PREP offers a capacity to hold 24 vials of 10 ml with sample injections ranging from 1 μl to 19,999 μl. To avoid large dead volumes, V-shaped high-recovery vials should be used. ALIAS Bio PREP can easily be modified to allow lower injection volumes by changing the injection valve, syringe, sample loop and sample holder. However, seamless change between analytical and preparative-mode operations would be desirable.

5.3. Automation of enzymatic batch reaction processes

The combination of ALIAS-AS and in-line dilution coupled to IEX chromatography as an intermediate step can be extended to automate complex purification protocols to reduce laborious sample transfers as well as concentration and buffer exchange steps. In addition to conventional high-throughput applications, our ÄKTA ALIAS-AS configuration enables us to perform purifications that involve multiple proteins or reactions. Batch reaction processes can be automated and implemented into the workflow, where enzyme and reaction components are added to an external reaction vessel from ALIAS-AS (Fig. 7). Such examples include tag cleavage by common Tobacco Etch Virus (TEV) protease and protein-protein ligation or cyclization reactions mediated by ligases such as Sortase A. This way, sophisticated purification protocols can be designed, where multiple purification steps as well as enzymatic reactions are needed.

5.4. Novel system configuration allows independent operation of two column valves

A custom system configuration file allows for a feature where researchers can operate two column valves independently from each other. This two-column valve operation can be used to e.g. create purification loops to increase purity of the target protein. Flow direction between column valves is controlled by a preceding versatile valve (Fig. 8A). Sample transfer between V9-C1 to V9-C2 and vice versa is achieved by changing the versatile valve po-

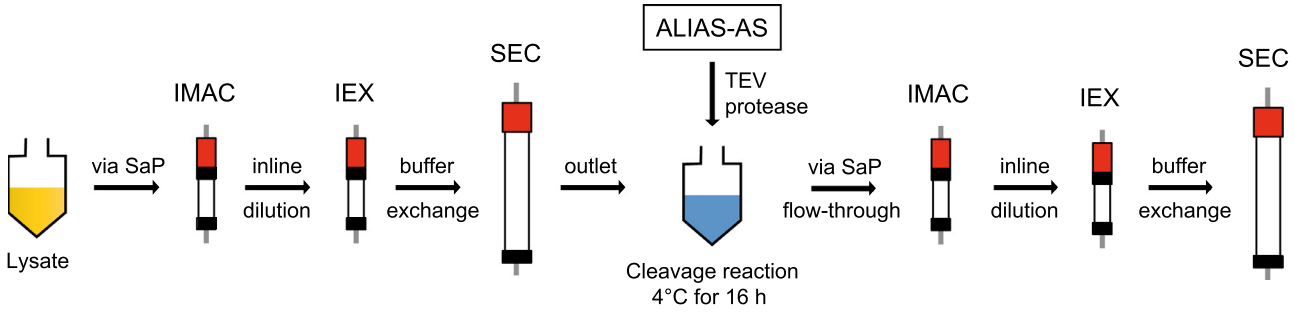

Fig. 7. Example of purification scheme involving enzymatic reactions. The His-tagged protein of interest is initially purified using an IMAC to IEX to SEC scheme. ALIAS-AS is used to inject TEV protease into an external vessel, where the cleavage reaction takes place. After cleavage, the reaction solution is re-injected into the ÄKTA system and further polished using a second set of IMAC to IEX to SEC. An in-line dilution protocol is implemented between IMAC and IEX column transfers.

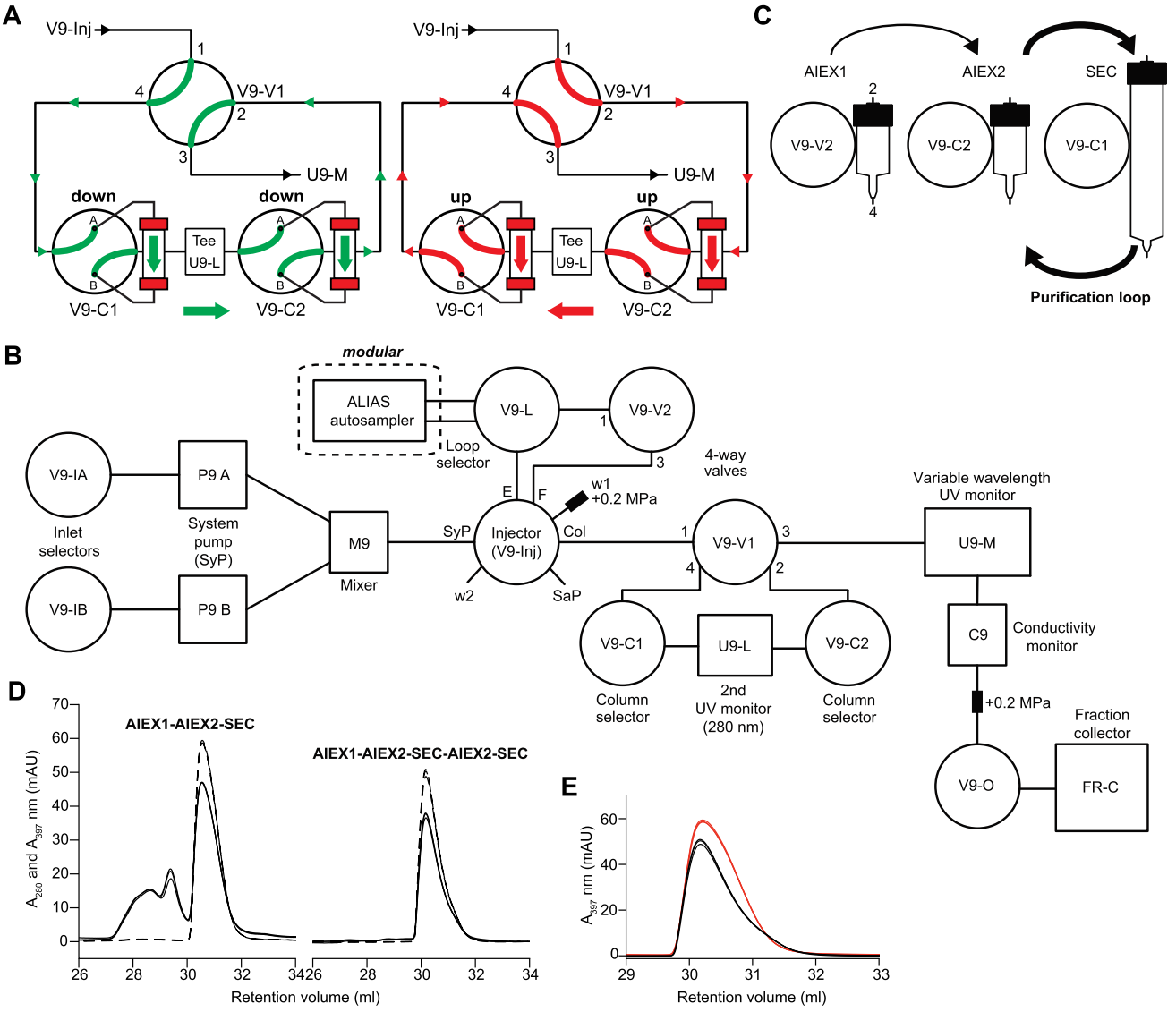

Fig. 8. Independent two-column valve operation allows design of purification loops. (A) V9-V1 was placed upstream of column valves to control flow directions. Sample transfer between V9-C1 to V9-C2 and vice versa is achieved by changing the versatile valve position and operating both column valves in downflow or upflow direction, respectively. (B) Fluidics of independent two-column valve configuration setup. (C) Columns used in this purification scheme. (D) Overlay of A_{280} (continuous line) and A_{397} (dashed line) SEC traces of one- and two-cycle purifications performed in triplicates. (E) A_{397} SEC traces of one-cycle (red) and two-cycles (black) were overlaid to show minimal sample loss during transfer. One-cycle chromatograms were slightly shifted in the x-direction for overlay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 1
Comparison of purification methods (process time, purity, recovery and process reproducibility).

Target	Mode	Purification scheme	Time per cycle[a]	Purification runs performed per 3 days	Recovery	Purity	Process Reproducibility[f]	Comparison to other literature values	Reference
tβ1-AR	Automated	IMAC–IEX–Affinity–IEX	19 h	4	1.1 mg/l culture[e]	High[b]	High	–	Delgado et al.
tβ1-AR	Manual	IMAC–IEX–Affinity–IEX	3 days	1	0.8 mg/l culture[e]	High[b]	High	–	Delgado et al.
tβ1-AR	Automated	IMAC–CIEX–SEC	4 h 40 min	9	1.7 mg/l culture[e]	Mid[c]	High	–	This study
tβ1-AR	Manual	IMAC–Manual operation[g]–SEC	6 h[d]	3	ND	ND	High	–	This study
GFP	Automated	AIEX–AIEX–SEC	2 h	36	53%	Mid[c]	High	His_6-GFP IMAC–DS–IEX 2 h 10 min	Sigrell et al. (2003)
GFP	Manual	AIEX–Manual operation[g]–SEC	2 h 30 min	9	18%	Mid[c]	High	His_6-GFP IMAC–DS–IEX 2 h 30 min	Sigrell et al. (2003)

[a] Cycle includes CIP.
[b] SDS-PAGE, no visible impurities.
[c] SDS-PAGE, >90% purity.
[d] Estimate.
[e] Determined using the same batch of culture.
[f] Overall reproducibility (yield, recovery, retention time and purity).
[g] Manual operations include buffer exchange and concentration of sample; DS: desalting; Manual operations include buffer exchange and concentration of sample.

sition and operating both column valves in downflow or upflow direction, respectively (Fig. 8A). This way, purification loops can be generated where the sample is transferred back and forth between two columns. In addition, a mixing tee or U9-L monitor can be placed between the column valves for in-line dilution or for monitoring the sample during transfer. To evaluate this novel system configuration, the purification scheme in Fig. 1B was altered and GFP was re-cycled, meaning the GFP SEC eluate was recaptured on the AIEX2 column before being polished again on the SEC column (Fig. 8C). Sample loss between each cycle was kept to a minimum of ~9% when the watch function by peak detection was replaced by simple peak fractionation at predefined retention volume in the 2nd cycle (Fig. 8D and E). Overall, the operator can combine appropriate pairs of purification columns, not only different SEC and IEX columns steps but also specialized affinity and hybrid columns according to the specification of the later analyses. Multiple combinations of two-step purifications are possible due to the availability of five ports on each column valve. If in-line dilution is required, a mixing tee needs to be placed between the two column valves.

6. Conclusions

In conclusion, the commonly available modular ÄKTA pure platform provides a solid scaffold for the flexible development of complex protein purification strategies for researcher on a daily basis. The modular in-line dilution method coupled to IEX chromatography can be rapidly implemented into different purification workflows to perform dilution and concentration operations. Manual single injections were replaced by an ALIAS-AS to automate the purification of large arrays of protein lysate samples. In combination with the ÄKTA pure system, the ALIAS-AS offers a reliable and robust module to increase throughput, accuracy and reproducibility of protein purification methods.

Declaration of Competing Interest

Tuomo Frigård works for GE Healthcare Bio-Sciences AB, which produces MabSelect SuRe, MabSelect PrismA, Capto adhere, and ÄKTA chromatographic systems.

CRediT authorship contribution statement

Bastian Franke: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Tuomo Frigård:** Conceptualization, Software, Writing - review & editing. **Stephan Grzesiek:** Resources, Writing - review & editing, Funding acquisition. **Shin Isogai:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2019.460846.

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