

Xavier LeSaout¹
Matteo Costioli¹
Lynn Jordan²
Jeremy Lambert²
Ross Beighley²
Laurel Provencher²
Brian Gerwe²
Kevin McGuire²
Nico Verlinden²
Andrew Barry²

¹Merck Serono S.A., Vevey,
Switzerland

²PerkinElmer Inc., Waltham, MA,
USA

Short Communication

Automated small-scale protein purification and analysis for accelerated development of protein therapeutics

Small-scale protein purification presents opportunities for accelerated process development of biotherapeutic molecules. Miniaturization of purification conditions reduces time and allows for parallel processing of samples, thus offering increased statistical significance and greater breadth of variables. The ability of the miniaturized platform to be predictive of larger scale purification schemes is of critical importance. The PerkinElmer JANUS BioTx Pro and Pro-Plus workstations were developed as intuitive, flexible, and automated devices capable of performing parallel small-scale analytical protein purification. Preprogrammed methods automate a variety of commercially available ion exchange and affinity chromatography solutions, including miniaturized chromatography columns, resin-packed pipette tips, and resin-filled microtiter vacuum filtration plates. Here, we present a comparison of microscale chromatography versus standard fast protein LC (FPLC) methods for process optimization. In this study, we evaluated the capabilities of the JANUS BioTx Pro-Plus robotic platform for miniaturized chromatographic purification of proteins with the GE ÄKTA Express system. We were able to demonstrate predictive analysis similar to that of larger scale purification platforms, while offering advantages in speed and number of samples processed. This approach is predictive of scale-up conditions, resulting in shorter biotherapeutic development cycles and less consumed material than traditional FPLC methods, thus reducing time-to-market from discovery to manufacturing.

Keywords: Automated protein characterization / Biotherapeutic process development / Microfluidic capillary electrophoresis / Quality by design / Small-scale protein purification

Received: February 15, 2015; *revised:* July 3, 2015; *accepted:* August 27, 2015

DOI: 10.1002/elsc.201400252

1 Introduction

Small-scale expression, purification, and downstream characterization of biomolecules are routinely performed within the development of biotherapeutics (Fig. 1). The potential process design space is immense; the relationships between process parameters and critical quality attributes of the final product must be determined experimentally. Often, process development projects utilize quality by design (QbD) or design of experiment (DoE) approaches that require the ability to screen complex

matrices of conditions critical to identity, titer, structure, and function of biotherapeutic proteins [1, 2]. In order to obtain sample numbers for statistically significant analyses, systems for rapid purification and analysis of recovered molecules are critical. Miniaturization of purification and analysis protocols reduces time and allows for parallel processing of samples offering increased statistical significance and greater breadth of variables [3]. The miniaturized protocols, however, must be predictive of scale-up conditions in order for small-scale protein purification and characterization to present opportunities for accelerated process development of biotherapeutic molecules.

It should be noted that minicolumns do have several limitations as compared to lab-scale columns. Specifically for small proteins, it has been reported that velocity-dependent mass transfer becomes the rate-limiting step, limiting the ability for miniature columns to be predictive of higher capacity purification schemes. However, it is also noted for preparative

Correspondence: Dr. Brian Gerwe (brian.gerwe@perkinelmer.com), Automation and Liquid Handling/Microfluidics, PerkinElmer Inc., 68 Elm Street, Hopkinton, MA 01748, USA

Abbreviations: CEX, cation exchange; CV, column volume; DoE, design of experiment; FPLC, fast protein LC; Micro-CE, microfluidic CE; QbD, quality by design

© 2015 The Authors. *Engineering in Life Sciences* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

179

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

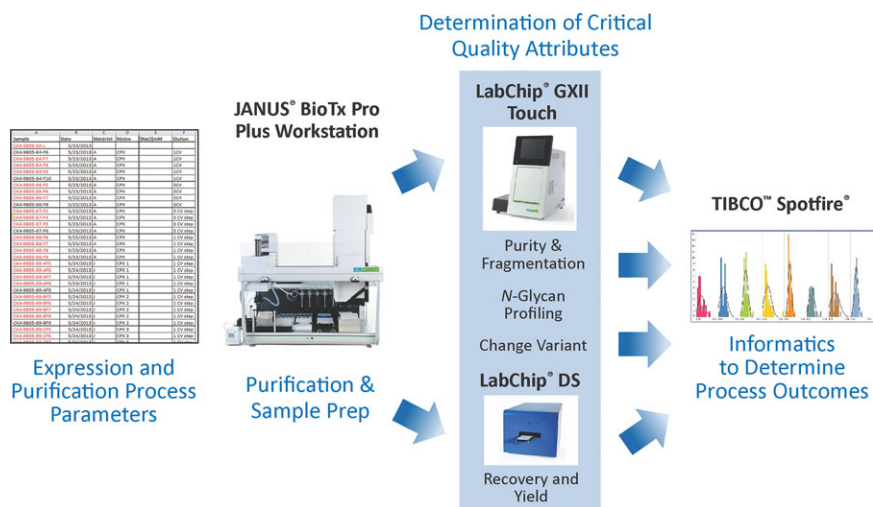


Figure 1. Small-scale high-throughput process development workflows require the testing of complex matrices of conditions to determine effects on the identity, titer, structure, and function of biotherapeutic proteins. Use of automated liquid-handlers to perform small-scale purification and automated sample analysis systems to characterize proteins significantly reduces the cost and time required to fully evaluate the process design space.

applications, velocities typically fall well within the range to be predictive of higher capacity purification schemes [4]. Additional concerns reported to impact the predictive capabilities of miniature columns include geometric factors, including bed volume heights leading to reduced separation capacities, residence times and flow rates, “wall effects” from a smaller inner-diameter, and nonlinear elution gradients. Despite these differences, transport properties are maintained for proteins > 150 kDa, across a range of chromatography resins [5].

With successfully increased throughput from small-scale purification, the bottleneck becomes protein characterization and analysis. Traditional methods such as SDS-PAGE, CE, and HPLC require manual sample handling and/or analysis times up to 20–30 min per sample. High-throughput, automated, and inexpensive analytics are needed to adequately address the sample numbers required for QbD and multifactorial DoE studies to fully achieve the benefits of miniaturization.

The JANUS[®] BioTx Pro and Pro-Plus workstations were developed as intuitive, flexible, and automated devices capable of performing parallel small-scale analytical protein purification. Preprogrammed methods automate a variety of commercially available ion exchange and affinity chromatography solutions, including miniaturized chromatography columns, resin-packed pipette tips, and resin-filled microtiter vacuum filtration plates. Microfluidic CE (micro-CE) technology enables electrophoretic separations for DNA, RNA, protein, and glycan analysis on one platform with unparalleled speed and minimal sample input. The commercialized LabChip[®] systems perform rapid analysis (<42 s per sample) of multiple protein characterizations including molecular weight, titer, fragmentation, purity assessment, and rapid analysis (<68 s per sample) of *N*-glycan profiles as well as charge heterogeneity analyses [6–8]. Microfluidic absorbance spectroscopy allows determination of protein concentration with maximum speed and minimal sample consumption and handling. The LabChip[®] DS instrument is a microliter-scale microplate reader, which provides full spectrum absorbance scans (220–750 nm) of 96 samples in less than 5 min. Additionally, the wide dynamic range of the instrument allows for a broad concentration of samples to be run with no dilution steps. The

combination of small droplet-sized volume requirements and high precision make the LabChip DS the perfect choice for total sample quantification of both nucleic acids and proteins.

The data from these instruments can be integrated with TIBCO[™] Spotfire[®] to perform further advanced statistics and use interactive visualizations to extract biologically meaningful data as well as assess operational productivity. The TIBCO[™] Spotfire[®] software’s easy-to-use interface makes it possible to quickly analyze large amounts of data that can otherwise provide challenges for interpretation, e.g. identifying outliers when assessing multiple protein characterizations, such as molecular weight, purity assessment, etc., as well as comparing profiles of different datasets. The platform’s integrative ability means that you are able to combine large datasets from both nucleic acids and proteins (among other relevant datasets) in one interactive platform and quickly drill down to interesting aspects within your data, produce enhanced visualizations to be shared with your colleagues, and build complex predictive models.

Coupling the above-mentioned approaches eliminates bottlenecks in sample preparation and characterization, resulting in shorter development cycles and increased efficiency for development of biotherapeutics compared to traditional methods. Herein, we present a comparison of microscale chromatography versus standard fast protein LC (FPLC) methods for process optimization. In this study, we evaluated the capabilities of the JANUS BioTx Pro-Plus robotic platform for miniaturized chromatographic purification of proteins with the GE ÄKTA Express system. Key attributes tested were the ability to match elution profiles, determine column dynamic binding capacity, and characterize separation resins. We were able to demonstrate predictive analysis similar to that of larger scale purification platforms, while offering advantages in speed and number of samples processed. It was shown that the JANUS BioTx Pro-Plus can obtain results of predictive of scaled-up conditions 10X faster using 6X less material as opposed to traditional FPLC methods. In addition, we illustrate how micro-CE and microfluidic absorbance spectroscopy can be used for characterization of IgG samples to provide the rapid analysis necessary for maximum productivity in process development workflows.

2 Materials and methods

A mAb was used as source protein material for the experiments performed. Experiments were performed on the JANUS BioTx Pro-Plus workstation and GE ÄKTA Explorer™ chromatography platforms. All purifications were performed with proprietary resins using 0.6 mL Atoll RoboColumns and 3.5 mL chromatographic columns (0.66 × 10 cm). Recovered fractions were analyzed using UV absorbance and the LabChip GXII microfluidic CE-SDS electrophoresis platform.

mAbs were purified on automated platforms using cation exchange (CEX) chromatography. Automated loading of sample buffers and protein was achieved through the unique four-way valve, allowing sample loops with continuous flow of >5 mL of loading material. Protein sample and buffers were preconfigured for elution profiling from microtiter plates and reservoirs, spanning a volume range of 0.2–50 mL. Precise control of flow rates allows direct control for resin screening and capacity determination studies, with minimum flow rates capable of achieving residence times of 11.83 min for 0.6-mL columns. Analytical fractions were collected in 96-well UV plates for direct analysis, or into standard 2 mL 96-DeepWell plates using the integrated plate shuttle.

The JANUS BioTx Pro and Pro-Plus workstations utilize the JANUS Application Assistant to provide a simple intuitive graphical user interface allowing the user easy control to modify parameters central to the goals of the particular study. This simple interface eliminates the need for any programming, yet it allows the user to customize protocols for the chromatography steps, such as input sample volume, and the parameters for the collected fractions. A report file is generated that couples the entered parameters with fraction collection locations to allow for seamless integration with downstream analytical data associated with each fraction; this enables more efficient analysis and accelerated protein therapeutic development.

Critical to the instrument platform comparison was that column reproducibility could be achieved. A step elution of increasing NaCl (50 mM NaCl per column volume [CV]) from 50 to 600 mM was performed in parallel on four Atoll® RoboColumns® using the same resin. UV monitoring of each collected fraction was used to evaluate system reproducibility. A similar experiment was performed on ÄKTA™ (3.5-mL scale) for comparison utilizing the same elution format. Results demonstrate a high level of reproducibility across replicates while obtaining similar results to those obtained on the ÄKTA™ platform (Fig. 2A).

To evaluate the ability of the JANUS BioTx Pro-Plus to determine and compare residence time in a similar manner to the GE ÄKTA™ 3.5-mL scale column, an mAb was purified using four strong and one weak CEX resin-packed in 0.6-mL Atoll® RoboColumns®. Flow rates for the JANUS BioTx Pro-Plus were varied between 2 and 20 $\mu\text{L/s}$, resulting in varying residence times (0.5, 1, 2, 2.5, 3.3, 5 min) in order to obtain profiles deterministic of dynamic binding capacity for the various resins analyzed (Fig. 2B). Comparisons between these profiles and those obtained on a 3.5-mL ÄKTA™ Explorer platform were drawn (R^2 value of >0.9484) to understand the predictability of these profiles to larger scale purification schemes. The required runs were accomplished in 1.5 days on the JANUS

BioTx system, compared with a 5-day run time on the ÄKTA™ platform.

To determine process yield and elution purity of recovered fractions (Fig. 2C), chromatographic experiments were conducted across five CEX resins on the JANUS BioTx workstation as well as on the GE ÄKTA Explorer™ platform. Measured capacities between the two platforms were identical. Collected fractions were analyzed for overall yield using UV absorbance at 280 nm and assessment of high and low molecular weight impurities were calculated through electrophoretic separation using the LabChip GXII platform.

3 Results and discussion

Results show comparable profiles for eluates across conditions tested (e.g. yield, high and low molecular weight eluates) irrespective of platform. However, the seven chromatography runs were performed within a 1.5-h period on the JANUS BioTx workstation as compared to a 36-h period on the GE ÄKTA Explorer™ platform. Because of the decreased run times on the JANUS instrument, the results were performed in triplicate, showing statistical similarity across replicates. The improvement of time values observed can be accounted for through the equilibrium and regeneration times required for GE ÄKTA Explorer™ platform (20 cm bed height) versus RoboColumns (3 cm bed height); load and wash steps had comparable residence times since it would affect dynamic binding capacity.

A stepwise gradient elution study was performed on the JANUS BioTx Pro-Plus platform to determine optimal salt concentration for maximum yield of a desired protein. A single CEX resin was used to perform the purification. For the elution step, the instrument loaded equal volumes of prepared solutions ranging from 250 to 400 mM NaCl in 50-mM steps. Collected fractions were analyzed for overall yield using UV absorbance at 280 nm. The presence, abundance, and characterization of impurities in the eluted material were analyzed by electrophoresis on the LabChip GXII system. This allowed for the direct comparison of the relative peak areas of the target protein versus both high and low molecular weight impurities (Fig. 2D), providing a profile for each eluted sample.

The LabChip GXII Protein Express Assay utilizes high-throughput micro-CE analysis with SDS-denatured samples to determine the size and relative concentration of proteins. The microfluidic chip sequentially performs automated loading, staining, and destaining of up to 384 samples per run via a combination of pressure-driven and electrokinetic-driven sample movement. A diagram of the microfluidic chip is shown in Fig. 3A. Negative pressure applied to well 1 pulls sample into the chip, simultaneously mixing it with a marker dye provided in well 4 of the chip. The negative pressure achieves flow only in the channels colored black, as these channels have greater depth and therefore present less resistance to flow. Once the sample has been loaded onto the chip, an electric field is applied briefly to wells 3 and 8 to move proteins and marker via electrokinetic flow to the base of the separation channel. The electric field is then applied to wells 7 and 10 to inject the proteins and move them through the separation channel. While the SDS-coated proteins are moving through the gel-filled channels, they are stained with

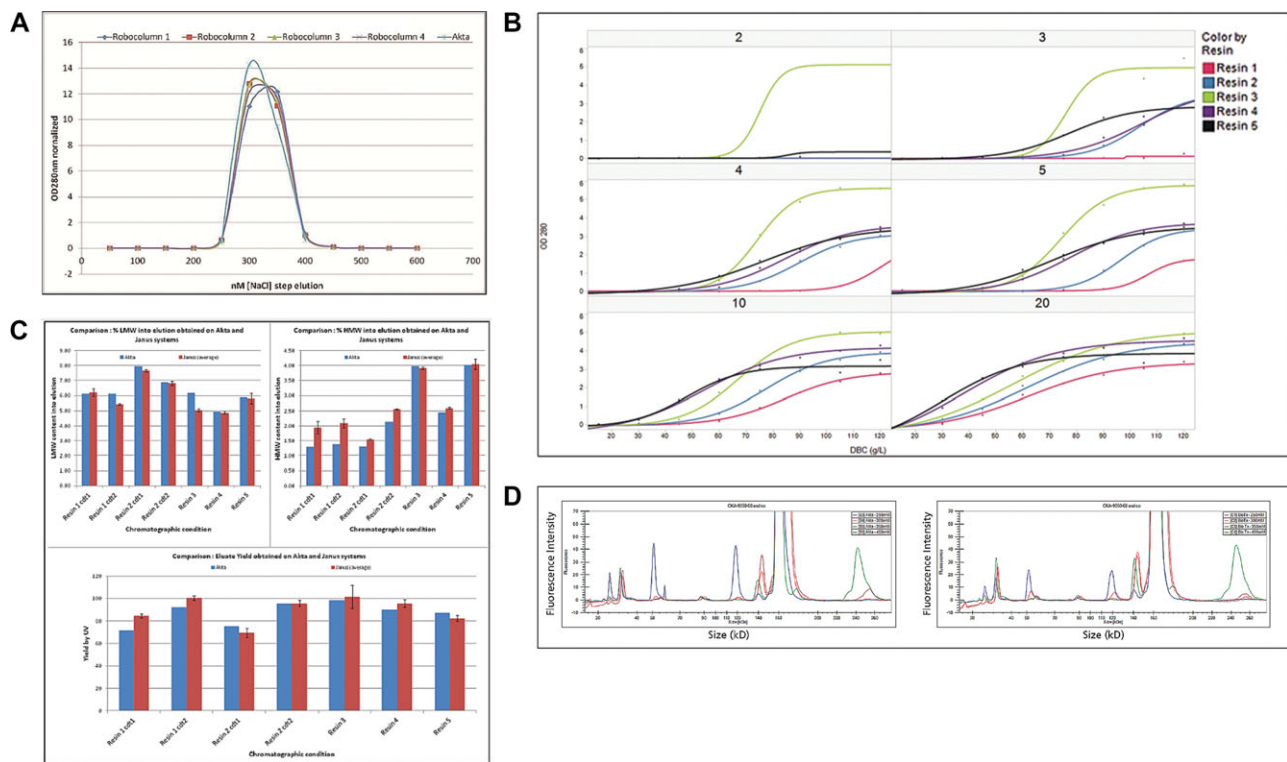


Figure 2. Performance comparison of the JANUS BioTx Pro-Plus and GE ÄKTA™ Explorer platforms. (A) Column reproducibility was evaluated during step elution, 50 mM NaCl per CV. (B) Capacity determination for several CEX columns with varying flow rates and residence times; dynamic binding capacity profiles were compared. (C and D) Process yield and elution purity from CEX column recovered fractions. Error bars represent SD ($n = 8$). Stepwise gradient elution profiling results in comparable profiles across various molecular weight impurities.

a dye that fluoresces when exposed to hydrophobic environments created by protein–SDS association or free SDS micelles. Destaining is achieved by flowing current from wells 2 and 9 to reduce the concentration of SDS below the critical micelle concentration to eliminate SDS micelles and background fluorescence. Detection is achieved by recording fluorescence versus time immediately downstream of the destain junction (Figs. 2D and 3B). While this article only describes results from analysis with the Protein Express assay, the LabChip GXII system also offers additional assays for the analysis of charge variance and *N*-glycan profiles.

Samples eluted from the JANUS BioTX and the GE ÄKTA Explorer™ systems were prepared for LabChip GXII analysis with the Protein Express Assay Kit according to the recommended protocol for IgG analysis. A sample of 5 μ L was mixed with 35 μ L of sample buffer spiked with either DTT (reducing sample buffer) or iodoacetamide (nonreducing sample buffer). Samples were denatured in sealed wells of microtiter wells in a thermocycler for 10 min at 70°C with a heated lid. After heating, each sample was mixed with 70 μ L molecular biology grade water and centrifuged at 1000 relative centrifugal force for 2 min. The microplate was placed directly on the LabChip instrument for processing. Chips were prepared according to the standard protocol for high-throughput analysis. Although sample prepara-

tion for LabChip GXII analysis was done manually for this particular project, the JANUS BioTX system could easily be adapted to allow automation of this step.

In addition to analyzing samples from the purification process, a standard IgG molecule, adalimumab, was used to show the suitability of the micro-CE system for percent purity analysis of a purified antibody product. Adalimumab (CAS number 331731-18-10) was obtained from BOC Sciences (Creative Dynamics, Inc., Shirley, NY, USA). Samples were prepared in microplates by heat denaturation in the presence of SDS as described earlier in this section. A typical data trace for adalimumab, in which impurities arising primarily from fragmentation of the intact IgG or a nonglycosylated form of the IgG, is shown in Fig. 3B. The impurity peaks range from <0.5 to 1.5% of the total protein in the sample, and are reproducibly detected and resolved in 40 s per analysis. Across 12 independent runs, the RSD for purity of the main IgG peak was <1% (data not shown).

To monitor yield in fractions eluted from the CEX columns under different purification schemes, high-throughput absorbance analysis was used for determination of total protein concentration. Absorbance spectrum analysis was done using the LabChip DS instrument on DropPlate D+ plates (Fig. 2C, bottom graph). The microfluidic plates used by the LabChip DS are shown in Fig. 3C inset. Only 2 μ L of sample

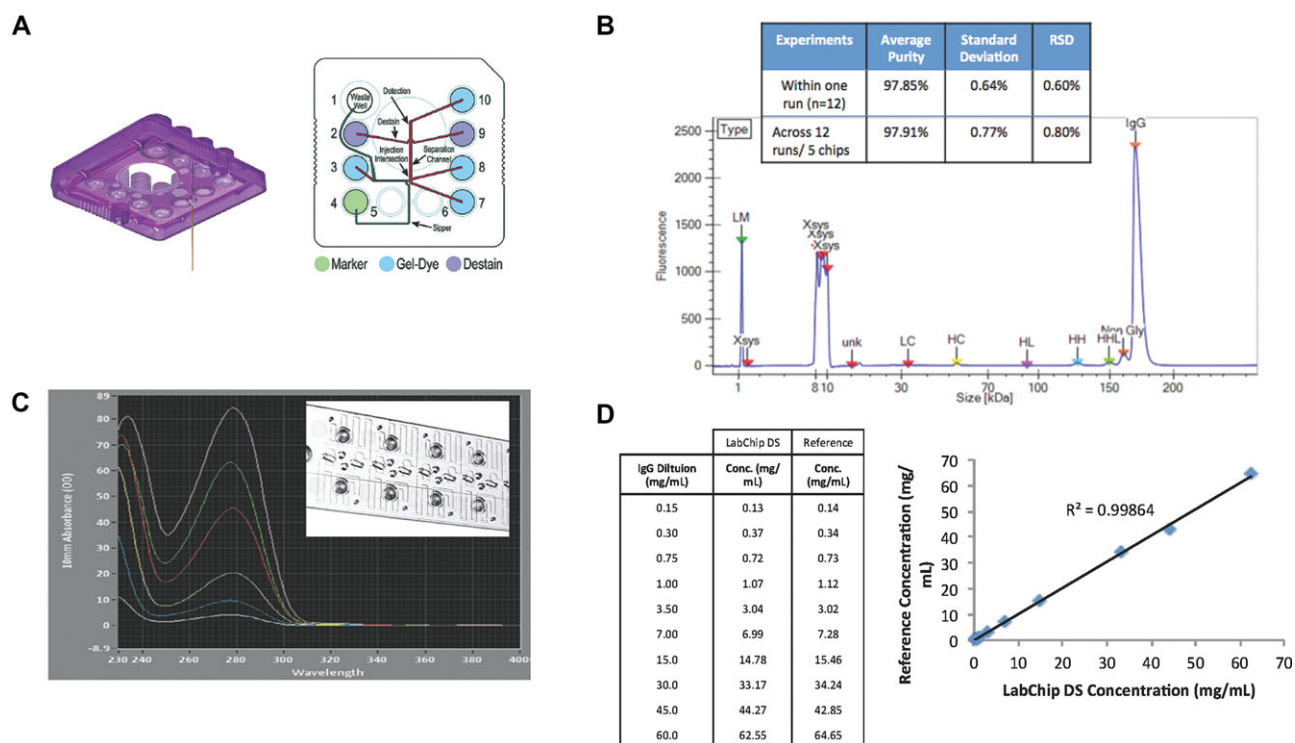


Figure 3. Microfluidic tools for high-throughput analysis of protein samples. (A) Design and layout of LabChip GXII Protein Express chips for rapid, automated CE-SDS analysis of protein samples. (B) Representative data trace from percent purity analysis of nonreduced adalimumab showing low-level contamination of the sample with antibody fragments. Inset shows reproducibility of percent purity values across runs. (C) Absorbance spectrum traces from a titration of IgG sample analyzed on the LabChip DS system. Inset shows design and layout of microfluidic chips for rapid, low volume absorbance spectrum measurements. (D) Concentration values derived from the data shown in panel (C) illustrating accuracy of the absorbance measurements across the concentration range. RSD, relative standard deviation.

is used to fill each channel of the plate. The sample is pipetted into a shallow well, from which capillary flow draws the sample into the microfluidic channel. The sample is thus protected from any effects of evaporation, allowing the user to obtain reliable results from very small volumes. The extinction coefficient value of 1.39 was used to calculate the concentration of IgG.

The suitability of the LabChip DS microfluidic absorbance spectrum analysis platform for accurate protein quantification was also tested by analyzing a dilution series of IgG. Figure 3C shows the spectra obtained from IgG samples ranging in concentration from 0.15 to 60 mg/mL. Plotting the known concentration of each sample versus the experimental results produced the linear relationship shown in Fig. 3D, with an R^2 value >0.998 .

4 Concluding remarks

The automated process development platform described offers 10-fold advantages in throughput with six-fold reduction in protein mass requirement over larger scale FPLC platforms. While several technical hurdles (e.g. offline monitoring, linear gradients, and discontinuous flow) for automated process development exist, if understood, they can be addressed and minimized.

The first challenge is offline monitoring for UV, pH, and conductivity. UV measurements can be addressed with high-throughput analytical instruments for rapid results (e.g. LabChip DS and GXII). Unfortunately, pH and conductivity would still need to rely on more manual methods. Reproducing a linear gradient is not feasible on the JANUS BioTx workstation or on any other type of automated liquid-handler; however, it can be minimized by mimicking the linear gradient with multiple step-gradients of increasing salt concentration, every CV for example. Lastly, the JANUS BioTx workstation offers an innovative approach to minimize the challenge of discontinuous flow. The system is able to inject 5 mL of solution per dispense; thus, minimizing process interruptions as compared to competitors. Even with the hurdles mentioned, the capability to scale down a variety of process development experiments presents a tractable platform for acceleration of biotherapeutic protein development. Micro-CE and microfluidic absorbance spectroscopy instruments for characterization of IgG samples provide rapid analysis required for maximum efficiency within high-throughput process development workflows. The combination of these approaches aid to eliminate bottlenecks in sample preparation and characterization, resulting in shorter development cycles and increased efficiency for development of biotherapeutics compared to traditional methods.

Practical application

Critical to the utility of small-scale workflows for expression, purification, and downstream characterization are processes that are predictive of large scale-up and the subsequent throughput needed to achieve optimal process parameters. Given an immense design space, experimental determination of relationships between process parameters and critical quality attributes is necessary for product commercialization. Miniaturization of purification and analysis protocols allows for parallel processing of samples, which increases statistical significance and the breadth of variables tested. With successfully increased throughput from small-scale purification, the bottleneck becomes protein characterization and analysis. High-throughput, automated, and inexpensive analytics are needed to adequately address the sample numbers required for QbD and multifactorial DoE studies to fully achieve the benefits of miniaturization. The combination of these approaches eliminates bottlenecks in sample preparation and characterization, resulting in shorter development cycles and increased efficiency for development of biotherapeutics compared to traditional methods.

Authors L.J., J.L., R.B., L.P., B.G., K.M., N.V., and A.B. are either current or former employees of PerkinElmer Inc. or its subsidiary companies and may have nominal stock ownership in the company. Further, PerkinElmer equipment was used to conduct the research described in this publication.

The authors have declared no conflict of interest.

5 References

- [1] Swalley, S. E., Fulghum, J. R., Chambers, S. P., Screening factors effecting a response in soluble protein expression: Formalized approach using design of experiments. *Anal. Biochem.* 2006, 351, 122–127.
- [2] Chambers, S. P., Swalley, S. E., Designing experiments for high-throughput protein expression. *Methods Mol. Biol.* 2009, 498, 19–29.
- [3] Vincentelli, R., Cimino, A., Geerlof, A., Kubo, A. et al., High-throughput protein expression screening and purification in *Escherichia coli*. *Methods* 2011, 55, 65–72.
- [4] Łacki, K. M., High-throughput process development of chromatography steps: Advantages and limitations of different formats used. *Biotechnol. J.* 2012, 7, 1192–1202.
- [5] Welsh, J. P., Petroff, M. G., Rowicki, P., Bao, H. et al., A practical strategy for using miniature chromatography columns in a standardized high-throughput workflow for purification development of monoclonal antibodies. *Biotechnol. Prog.* 2014, 30, 626–635.
- [6] Chen, X., Tang, K., Lee, M., Flynn, G. C., Microchip assays for screening monoclonal antibody product quality. *Electrophoresis* 2008, 29, 4993–5002.
- [7] Primack, J., Flynn, G. C., Pan, H., A high-throughput microchip-based glycan screening assay for antibody cell culture samples. *Electrophoresis* 2011, 32, 1129–1132.
- [8] Han, H., Livingston, E., Chen, X., High throughput profiling of charge heterogeneity in antibodies by microchip electrophoresis. *Anal. Chem.* 2011, 83, 8184–8191.