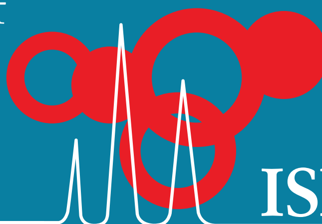
# ABSTRACT BOOK

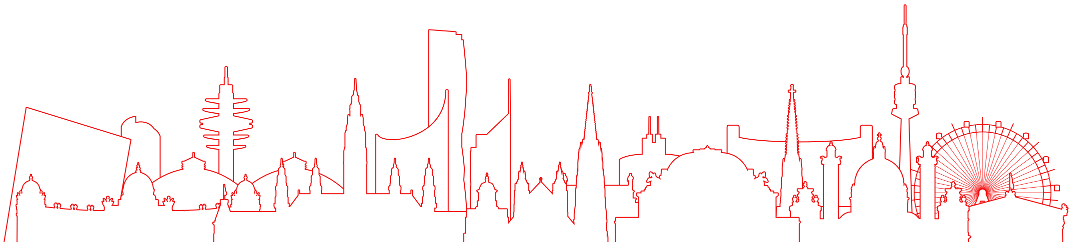
NOVEMBER 5-8, 2023

# 42nd International Symposium on the Separation of Proteins, Peptides & Polynucleotides



# 42nd International Symposium on the Purification of Proteins, Peptides & Polynucleotides

**VIENNA,
NOVEMBER 5 - 8, 2023**



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# WELCOME TO ISPPP 2023

**I am absolutely delighted to extend a warm welcome to all of you as we gather for the 42nd International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, set against the captivating backdrop of Vienna, a city renowned for its rich history and cultural vibrancy.**

**Our chosen venue, the Parkhotel Schönbrunn, provides the perfect setting for this symposium.**
**It encapsulates the essence of Vienna's imperial grandeur, offering a harmonious blend of history and modernity, mirroring the evolving landscape of the separation sciences we are here to explore.**

Our scientific program features a diverse array of presentations, including 39 oral presentations, 25 flash talks, and 38 posters. The topics cover the most recent developments in analytical and preparative separations of relevant biological macromolecules such as proteins, peptides and polynucleotides. Two full sessions deal with the characterization and separation of biological particles, signifying the increased importance of these new modalities. One can't help but wonder if a fourth "P" should be added to our conference name to reflect the evolution of our field.

Our keynote speakers will cover diverse topics, including cutting-edge proteoform analysis through MS-hyphenated separation techniques, time-dependent sorption behavior of viral vectors, and the latest developments in modeling tools for the biopharmaceutical industry. We are honored to have an impressive lineup of invited speakers who will share their expertise and perspectives with us. It's worth noting that the overwhelming number of abstract submissions we received compelled us to include flash talks, ensuring a dynamic and inclusive experience for attendees, regardless of their experience levels.

In addition to the core program, we have scheduled informative Sunday workshops and created a vendor corner, providing opportunities for hands-on learning and interactions with industry experts. I'd like to extend my heartfelt gratitude to our sponsors and exhibitors, whose support is instrumental in making this symposium possible. I also want to acknowledge the tireless efforts of our scientific committee, the symposium manager Verena Beck, and the dedicated team at Austropa, our event organizer. Special thanks go to our session chairs and session aides who will ensure the smooth flow of our sessions.

At ISPPP, we value feedback and continuous improvement. We are always open to your suggestions on how to enhance the symposium's future editions.
As you immerse yourselves in the scientific content, workshops, discussions, and networking opportunities, I hope you find solutions to your separation challenges and leave with valuable insights that will drive your research forward.

Thank you for being a part of ISPPP 2023, and I wish you an enlightening and productive symposium experience.

Warm regards,

Nico Lingg

CONFERENCE CHAIR

# CONTENT

Committees
- 4 -

Conference Programme
- 10 -

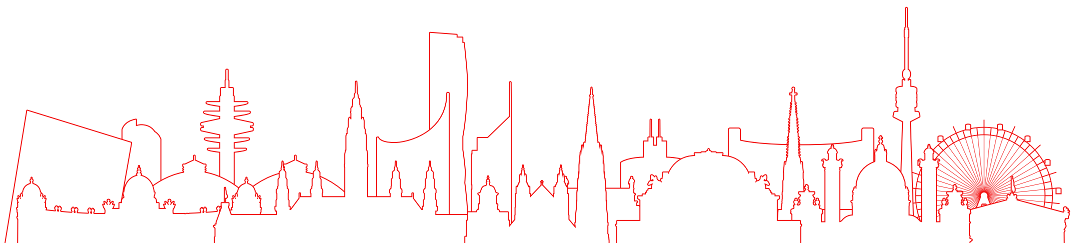
Poster Overview
- 21 -

Key Note Lecture Abstracts
- 28 -

Oral Presentation Abstracts
- 33 -

Flash Presentation Abstracts
- 70 -

Poster Abstracts
- 97 -



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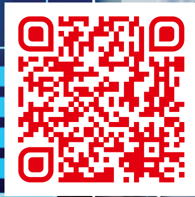

# PRE-CONFERENCE WORKSHOPS

## SUNDAY, NOVEMBER 5, 2023

| | | |
|---|---|---|
| **09:00** | **START OF REGISTRATION** | |
| **09:30** | **WORKSHOP 1**<br><br>**Sonja Berensmeier**<br>TU Munich<br><br>**Sebastian Schwaminger**<br>MedUni Graz | Magnetic separation in downstream processing |
| **11:15** | **WORKSHOP 2**<br><br>**Michel Eppink**<br>Byondis BV | Bioinformatics as a tool developing robust biotherapeutic proteins |
| **13:00** | **WORKSHOP 3**<br><br>**Cristina Cabral**<br>Univ. of Beira Interior<br><br>**Alois Jungbauer**<br>BOKU, Vienna | Mechanistic understanding of biomolecules adsorption: theory and applications |
| **14:45** | **WORKSHOP 4**<br><br>**Sonja Berensmeier**<br>TU Munich<br><br>**Michel Eppink**<br>Byondis BV<br><br>**Egbert Müller**<br>Tosoh Bioscience | Poly-/Oligonucleotide separation in biopharmaceutical processing and their quality requirements |

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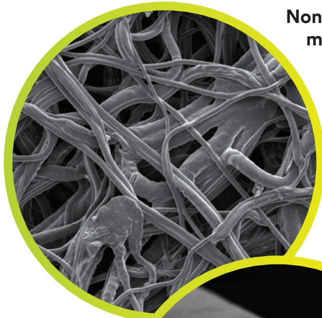


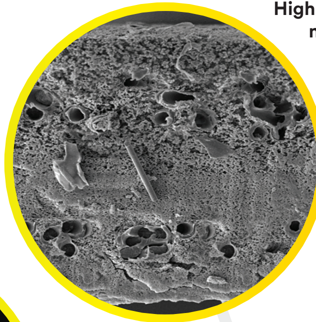


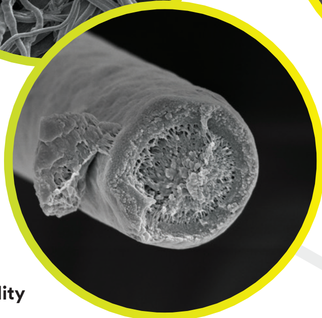


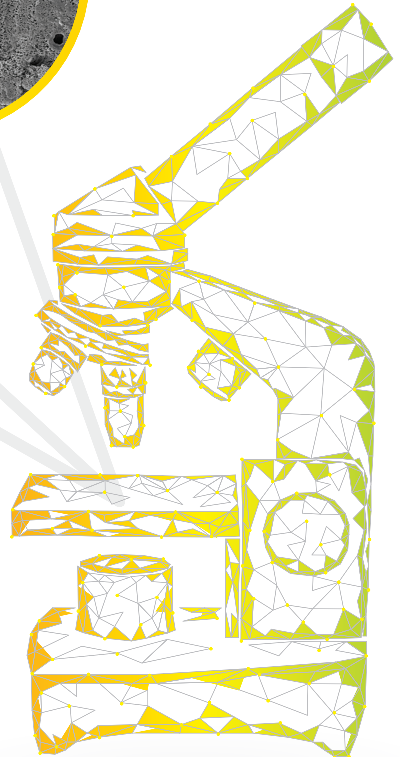

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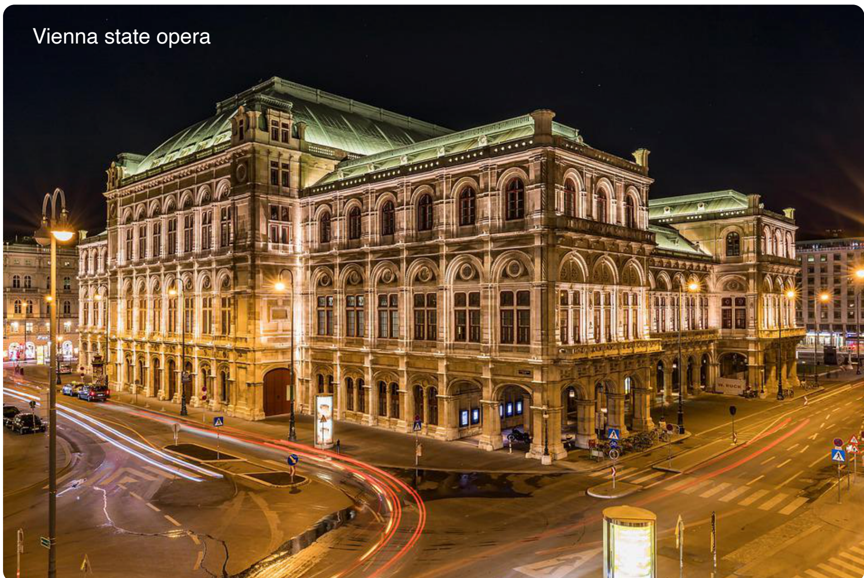
# CONFERENCE PROGRAMME

## SUNDAY, NOVEMBER 5, 2023

| | | |
|---|---|---|
| **17:00** | **WELCOMING REMARKS BY NICO LINGG** | |
| **KEY NOTE LECTURE**<br>CHAIR: NICO LINGG | | |
| **17:20** | **Elena Dominguez Vega**<br>Leiden Univ. Medical Center | **KN1:** Probing structure and function of proteoforms by MS-hyphenated separation techniques |
| **18:00** | **WELCOME RECEPTION** | |


Vienna state opera

# MONDAY, NOVEMBER 6, 2023

## KEY NOTE LECTURE
CHAIR: ALEŠ PODGORNIK

| Time | Speaker | Talk |
|---|---|---|
| **08:30** | **Nico Lingg**<br>**Alois Jungbauer** | Chairman Remarks |
| **08:45** | **Dan Bracewell**<br>University College London | **KN2:** Time-Dependent Sorption Behaviour of Viral Vectors |

## SESSION 1: DNA / VACCINES
CHAIR: ALEŠ PODGORNIK

| Time | Speaker | Talk |
|---|---|---|
| **09:25** | **Linda Gombos**<br>Biomay | **OP1:** High-Throughput Manufacturing of Personalized Plasmid DNA Cancer Vaccines |
| **09:45** | **Ana Rita Santos**<br>iBB - Institute for Bio-engineering and Biosciences | **OP2:** Towards industrial manufacturing of DNA-origami nanostructures: scalling up ssDNA scaffold purification |
| **10:05** | **Viviane Maimoni**<br>Gonçalves Instituto Butantan | **OP3:** Challenges for purification of a pneumococcal recombinant protein |
| **10:25** | **Julian Grinsted**<br>University College London | **FP1:** Design of affinity separations for the manufacture of *in vitro* transcribed mRNA |
| | **Nick Samuelson**<br>MSD | **FP2:** Increased Virus-Like Particle Recovery with Disassembly Prior to Purification |
| **10:35** | **REFRESHMENT BREAK** | |

## SESSION 2: PROCESS INTENSIFICATION
CHAIR: GIORGIO CARTA

| Time | Speaker | Title |
|---|---|---|
| 11:05 | **Michel Eppink**<br>Byondis BV | **OP4:** Cell Tolerant Radial Affinity Chromatography (cTRAC) |
| 11:25 | **Egbert Müller**<br>Tosoh Bioscience GmbH | **OP5:** Step Gradient SMB for mAb polishing using salt tolerant anion exchangers |
| 11:45 | **Mattia Sponchioni**<br>Politecnico Di Milano | **OP6:** Advantages and Opportunities of Multicolumn Countercurrent Solvent Gradient Purification Accessed by Tuning the Product Internal Recycling Phase |
| 12:05 | **Ismaele Fioretti**<br>Politecnico Di Milano | **FP3:** Process Intensification in the Purification of an Oligonucleotide Sequence by MCSGP with UV-Based Dynamic Control |
| | **Thomas Müller-Späth**<br>Chromacon AG | **FP4:** Automated two-column chromatography for the purification of Oligonucleotides and Peptides |
| | **Touraj Eslami**<br>acib Gmbh | **FP5:** Optimizing chromatography for maximum efficiency: an innovative approach to optimize productivity, resin utilization, and buffer consumption |
| 12:20 | **LUNCH BREAK** | |

## SESSION 3: NOVEL BIOSEPARATIONS & PRODUCTS
CHAIR: ANA CECILIA ROQUE

| Time | Speaker | Title |
|---|---|---|
| 13:45 | **Nils Brechmann**<br>Magic Bioprocessing | **OP7:** Scalable magnetic bead-based cell separation technology for the depletion of receptor positive cell subpopulations |
| 14:05 | **Dennis Röcker**<br>TU Munich | **FP6:** Enhancing chromatography by use of electrochemically modulated membranes |
| | **Ryan Kilgore**<br>North Carolina State University | **FP7:** Peptide ligands: a bespoke affinity platform for next-generation bio-therapeutics and gene-editing products |
| | **Staš Vrh**<br>Univ. of Ljubljana | **FP8:** Implementation of polyHIPE monoliths for preparative and analytical separation of bacteriophages and their genomic DNA |

| | | |
|---|---|---|
| **14:20** | **Noor Mujahid**<br>University College London | **OP8:** Characterising feed and membrane interactions in tangential flow filtration of lentiviral vectors: hints for recovery improvement |
| **14:40** | **Hironobu Shirataki**<br>Asahi Kasei Medical | **OP9:** Numerical calculations of membrane structure, virus removal performance, and filtration behaviours of virus filters based on a heterogeneous membrane structural model comprising multiple layers with different pore size distributions |
| **15:00** | **REFRESHMENT BREAK** | |

## SESSION 4: PROTEIN ANALYTICS
CHAIR: ELENA DOMINGUEZ VEGA

| | | |
|---|---|---|
| **15:30** | **Deepika Sarin**<br>Indian Institute of Technology, Delhi | **OP10:** Multiattribute monitoring of charge-based heterogeneity of recombinant monoclonal antibodies using 2D HIC-WCX-MS |
| **15:50** | **Tushar Savane**<br>Indian Institute of Technology Delhi | **OP11:** Quantification of concentration of mAb and excipients in a high concentration ternary mixture using ATR-FTIR spectroscopy and chemometrics |
| **16:10** | **Markus Mozgovicz**<br>Vrije Universiteit Brussels | **OP12:** Towards comprehensive SAX × RP 2D-LC-MS/MS host cell protein profiling in biopharmaceutical manufacturing |
| **16:30** | **Yehia Mechref**<br>Texas Tech University | **OP13:** Target Quantitative Analysis of Glycoproteins by Parallel Reaction Monitoring (PRM) LC-MS/MS |
| **16:50** | **Estela Giménez**<br>Univ. of Barcelona | **OP14:** In-line enzymatic digestion strategies beyond trypsin for the sensitive targeted bottom-up analysis of protein biomarkers by capillary electrophoresis-mass spectrometry |

## SESSION 5: BIOPROENG
CHAIR: ASTRID DÜRAUER

| Time | Speaker | Title |
|---|---|---|
| **17:10** | **Astrid Dürauer**<br>BOKU Vienna | Short Introduction Doctoral Programme BioProEng (BOKU) |
| **17:15** | **David Scheich**<br>BOKU Vienna | **FP9:** Purification and characterization of recombinant secretory immunoglobulin A from CHO cell culture supernatant |
| | **Anna-Carina Frank**<br>BOKU Vienna | **FP10:** Cationic flocculants assisted clarification |
| | **Alexander Zollner**<br>BOKU Vienna | **FP11:** Chromatography-based purification of enveloped virus-like particles displaying different influenza surface antigens for an immunologic study in mice |
| | **Lena Achleitner**<br>acib GmbH | **FP12:** Baculovirus working stock: the production and purification of an intermediate product for large scale VLP production in insect cells |
| | **Matthias Medl**<br>BOKU Vienna | **FP13:** Uncovering the black-box of data-driven models in biotechnological process modeling |
| **17:40** | **POSTER SESSION & NETWORKING RECEPTION** | |

# TUESDAY, NOVEMBER 7, 2023

## KEY NOTE LECTURE
CHAIR: STEFANO MENEGATTI

| Time | Speaker | Title |
| --- | --- | --- |
| 08:30 | **Arne Staby**<br>Novo Nordisk | **KN3:** Latest developments in the implementation of modelling tools in the biopharmaceutical industry |

## SESSION 6: mAbs
CHAIR: STEFANO MENEGATTI

| Time | Speaker | Title |
| --- | --- | --- |
| 09:10 | **Mariachiara Conti**<br>Univ. of Edinburgh | **OP15:** Porous platform ink for fast and high resolution 3D printing of stationary phases for affinity chromatography |
| 09:30 | **Ines Zimmermann**<br>TU Munich | **FP14:** Selective antibody capture using low-cost magnetic particles in an automated high-gradient magnetic separator |
| | **Malin Jönsson**<br>KTH Royal Institute of Technology | **FP15:** Mild purification of antibody fragments from human and mouse origin |
| | **Igor T.L. Bresolin**<br>Federal Univ. of São Paulo | **FP16:**Precipitation of monoclonal antibodies with polyethylene glycol and zinc chloride: process performance and rheological behavior |
| | **Daria Omralinov**<br>TU Darmstadt | **FP17:** 3D Printed Stationary Phases: The Future of Chromatography? |
| 09:50 | **Dan Pham**<br>TU of Denmark | **OP16:** Novel multi-modal salt-tolerant cation-exchange membrane applied for the purification of a single-chain variable fragment produced in *Pichia pastoris* |
| 10:10 | **Dorota Antos**<br>Rzeszow University of Technology | **OP17:** PEG-aided precipitation for adjusting acidic variant content in monoclonal antibody pools |

| | | |
|---|---|---|
| **10:30** | **Abraham Lenhoff**<br>University of Delaware | **OP18:** Understanding and Mitigating Persistence of CHO Host-Cell Proteins in Monoclonal Antibody Bioprocessing |
| **10:50** | **REFRESHMENT BREAK** | |

## SESSION 7: FUNDAMENTALS & MODELLING
CHAIR: CRISTINA DIAS-CABRAL

| | | |
|---|---|---|
| **11:20** | **SPONSORED TALK**<br>**Tatjana Trunzer**<br>Cytiva | **OP19:** A chromatography system modeling strategy for precise *in silico* process scaling |
| **11:40** | **Giorgio Carta**<br>Univ. of Virginia | **OP20:** Detective Stories in Chromatography: the Inseparable Pair, the Missing Peak, and the Gang of Three |
| **12:00** | **Lukas Gerstweiler**<br>Univ. of Adelaide | **OP21:** Model based process optimisation of an industrial chromatographic process for separation of lactoferrin from bovine milk |
| **12:20** | **Marcel Ottens**<br>TU Delft | **OP22:** Digital Twins for High Throughput Chromatographic Process Development |
| **12:40** | **Christian Frech**<br>Hochschule Mannheim – University of Applied Sciences | **OP23:** Mechanistic modeling of cation exchange chromatography scale-up considering packing inhomogeneities |
| **13:00** | **LUNCH BREAK** | |
| **14:15** | **POSTER SESSION** | |

## SESSION 8: PROTEIN SEPARATIONS
CHAIR: MARKUS BERG

| | | |
|---|---|---|
| **15:20** | **Sobhana Alekhya Sripada**<br>North Carolina State University | **OP24:** “Flow-through Affinity Chromatography”: a transformative approach to remove persistent and high-risk host cell proteins in Biomanufacturing |

## SESSION 8: PROTEIN SEPARATIONS
CHAIR: MARKUS BERG

| Time | Speaker | Talk |
|---|---|---|
| **15:40** | **Nico Lingg**<br>acib GmbH<br><br>**Daniel Elsner**<br>Boehringer Ingelheim RCV | **OP25:** CASPON – a platform process for non-platform proteins |
| **16:00** | **Matthias Müller**<br>BOKU Vienna | **OP26:** Purification of recombinantly produced Somatostatin-28 comparing hydrochloric acid and polyethylenimine as *E. coli* extraction aids |
| **16:20** | **Ana Cecilia Roque**<br>Nova School of Science and Technology | **OP27:** A scalable method to purify reflectins from inclusion bodies |
| **16:40** | **Preeti Saroha**<br>Indian Institute of Technology Delhi | **FP18:** Production of bioactive recombinant monoclonal antibody fragment in periplasm of *E. coli* expression system |
| | **Milan Polakovic**<br>Slovak Univ. of Technology | **FP19:** Single-pass diafiltration using a double-membrane module |
| | **Aleš Podgornik**<br>Univ. of Ljubljana | **FP20:** Determination of immobilized proteins via pH transition method |
| | **Oliver Spadiut**<br>TU Vienna | **FP21:** A Peroxidase from Inclusion Bodies as valuable Tool in Breast Cancer Treatment |
| **17:00** | **END OF SESSION** | |
| **18:30** | **CONFERENCE DINNER AT OTTAKRINGER BREWERY** | |

# WEDNESDAY, NOVEMBER 8, 2023

## SESSION 9: PARTICLE ANALYTICS
CHAIR: PATRICIA PEREIRA AGUILAR

| Time | Speaker | Presentation |
| --- | --- | --- |
| **09:00** | **Christian Hill**<br>Medical University of Graz | **OP28:** Optofluidic Force Induction (OF2i) - a BRAVE new way in time-resolved particle characterization |
| **09:20** | **Roland Drexel**<br>Postnova Analytics GmbH | **OP29:** Multi-detector Field-Flow Fractionation for quality assessment of nano-sized drug delivery systems |
| **09:40** | **Leo Jakob**<br>acib GmbH | **FP22:** Accelerating Virus-Like Particle Downstream Process Development Using Asymmetric Flow Field-Flow Fractionation (AF4) |
| | **Ricardo Silva**<br>iBB - Institute for Bioengineering and Biosciences | **FP23:** Anion exchange chromatography for extracellular vesicles purification |
| | **Rashmi Sharma**<br>Indian Institute of Technology, Delhi | **FP24:** Downstream Process Development for intact Virus-Like Particles (VLPs)<br>from yeast expression system<br>*Pichia pastoris* |
| | **Jorge João**<br>Instituto Superior Técnico - Universidade de Lisboa | **FP25:** Downstream processing of non-viral protein nanocages for biotechnological and biomedical applications: development of chromatography-based purification strategies |
| **10:00** | **Christoph Gstoettner**<br>Leiden University Medical Center | **OP30:** Novel Approaches for recombinant AAV genome and capsid characterization |
| **10:20** | **REFRESHMENT BREAK** | |

## SESSION 10: PARTICLE SEPARATIONS
CHAIR: DAN BRACEWELL

| | | |
|---|---|---|
| **10:45** | **Shuichi Yamamoto**<br>Yamaguchi University | **OP31:** Process modelling of chromatography of bio-nanoparticles based on linear gradient elution data |
| **11:05** | **Rebecca Hochstein**<br>3M | **OP32:** Advanced Approaches to Gene Therapy Viral Vector Separations |
| **11:25** | **Rita Fernandes**<br>Ibet | **OP33:** Development of a robust workflow for purification of a fusogenic oncolytic virus |
| **11:45** | **Patricia Pereira Aguilar**<br>acib GmbH | **OP34:** Functionalized non-woven fibers for purification of large labile enveloped viruses |
| **12:05** | **Stefano Menegatti**<br>North Carolina State University | **OP35:** Novel affinity ligands for Adenoassociated virus (AAV) and Lentivirus (LV) purification |
| **12:20** | **PRESENTATION OF POSTER AWARDS & CONCLUDING REMARKS** | |
| **12:35** | **END OF CONFERENCE** | |

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# POSTER LIST

## Poster Session 1

**SUNDAY, NOV. 5 – MONDAY, NOV. 6, 2023**

| | | |
|---|---|---|
| **P1** | **Monika Antosova**<br>Slovak University of Technology | Effect of conditions on the separation of proteins on a multimodal cation exchange adsorbent |
| **P2** | **Jonghwan Lee**<br>Korea Institute of Ceramic Engineering And Technology | Using $Ca^{2+}$ - dependent fusion protein with affinity precipitation for advanced antibody purification |
| **P3** | **Jinho Bang**<br>Korea Institute of Ceramic Engineering And Technology | Screening of hydroxyapatite binding peptides for protein purification-tag |
| **P4** | **Emily Berckman**<br>MSD | High-capacity purification of therapeutic mRNA with OligodT immobilized Fibro prototype chromatography media |
| **P5** | **Carly Catella**<br>North Carolina State University | Development of Peptide Glucosyltransferase Inhibitors with Comprehensive Coverage Across Clostridioides difficile Toxin B Sub-Types |
| **P6** | **Christian Fiedler**<br>Takeda Pharmaceuticals | Development of an affinity purification step for the implementation in a continuous process setup |
| **P7** | **Duc M. Do**<br>Hochschule Mannheim - University of Applied Sciences | Anion exchange membrane chromatography as capture step in plasmid DNA purification: Beneficial effect of salts on binding and elution |
| **P8** | **Linda Gombos**<br>Biomay | Recombinant Nuclease Cas9 for Therapeutic Genome-Editing – the Manufacturer´s Point of View |
| **P9** | **Fabrice Gritti**<br>Waters Corporation | Identification of Resolution Limits and Recycling Solutions for the Characterization of Monoclonal Antibodies by Size Exclusion Chromatography. |
| **P10** | **Sanket Jadhav**<br>Sartorius | Process Intensification using connected process for purification of mAbs: PD to Scale Up for Robust, cost effective, and agile manufacturing |
| **P11** | **Johann Kaiser**<br>Novo Nordisk A/S | Improving Efficiency in Monoclonal Antibody Purification: An Experimental Evaluation of Membranes for Single-Pass Tangential Flow Filtration |

| | | |
|---|---|---|
| **P12** | **Benjamin Kiss**<br>TargetEx Ltd. | Prediction of adsorption model parameters for cation exchange chromatography of proteins using molecular dynamics simulation and a self-developed coarse-grained modeling method |
| **P13** | **Tomáš Kurák**<br>Slovak University of Technology | Influence of chromatographic conditions on the adsorption of therapeutic antibodies and aggregates on multimodal adsorbents |
| **P14** | **Jacob Lebarre**<br>North Carolina State University | Mixed-mode size-exclusion silica resin for polishing human antibodies in flow-through mode |
| **P15** | **Marina Y. Linova**<br>Technical University of Denmark | Development of perfusion processes for *Pichia pastoris*: Opportunities for integrated purification of biopharmaceuticals |
| **P16** | **Tomáš Molnár**<br>Slovak University of Technology | Preparation and characterization of multimodal chromatography resins for antibody purification: A comparative study with Capto Adhere |
| **P17** | **Egbert Müller**<br>Tosoh Bioscience GmbH | Use of Immobilized Recombinant FcGamma III Receptor for Fractionation and Characterization of Antibody Preparations |
| **P18** | **Thomas Müller-Späth**<br>Chromacon AG | Accelerating chromatographic isolation and concentration of impurities with the twin-column continuous technique N-Rich |
| **P19** | **Marc Noverraz**<br>Sartorius Stedim Switzerland AG | Filtrations in mRNA Purification Processes. Studies of Tangential Flow Filtration and Sterilizing Grade Filtration. |
| **P20** | **Marius Segl**<br>Knauer Wissenschaftliche Geräte Gmbh | Maximize flexibility and throughput. Scalable and efficient purification of synthetic peptides |
| **P21** | **Ferdinando Sereno**<br>University College London | Extreme proteins require extreme purifications: a scalable and effective bioprocess for nanocompartment production. |
| **P22** | **Hironobu Shirataki**<br>Asahi Kasei Medical | Viral clearance in end-to-end integrated process for mAb purification: Total flow-through integrated polishing on two columns connected to virus filtration |
| **P23** | **Daniel Some**<br>Wyatt Technology | Advances in Downstream PAT for Biologics, Vaccines and Gene Vectors |
| **P24** | **Toru Tanaka**<br>Tosoh Corporation | Development of Novel Protein L Resin with Selective Binding to Kappa 1 Light Chain |

| | | |
|---|---|---|
| P25 | **Jakob Liderfelt**<br>Cytiva | Purifying challenging entities: capture of bispecific antibodies and removal of product-related impurities |
| P26 | **Marco Kress**<br>Valneva Austria GmbH | The influence of unspecific viral adsorption on pharmaceutical container surfaces in vaccine process development |
| P27 | **Dominik Voltmer**<br>Roche Diagnostics GmbH | Mechanistic modeling case study: The early model catches the leanest process |
| P28 | **Carsten Voss**<br>Repligen GmbH | Rapid development of caustic stable AAV affinity chromatography resins for AAV5 and AAV6 |
| P29 | **Maria Weinberger**<br>Boehringer Ingelheim RCV | Depth filtration for early recovery of soluble expressed microbials |
| P30 | **Tatsuya Yumoto**<br>Tosoh Co., Ltd. | FcRn Immobilised HPLC Affinity Column for Antibody Evaluation |

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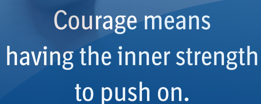
# POSTER LIST

## Poster Session 2

**TUESDAY, NOV. 7 – WEDNESDAY, NOV. 8, 2023**

| | | |
|---|---|---|
| **P31** | **Jürgen Beck**<br>BOKU Vienna | Challenges in parameter estimation for two-component protein adsorption using batch and small-scale column adsorption |
| **P32** | **Janos Bindics**<br>acib GmbH | Morphine Production in Genetically Engineered Poppy Cell Culture |
| **P33** | **Alexander Jurjevec**<br>BOKU Vienna | Polyethyleneimine efficiently extracts recombinant cytoplasmatic green fluorescent protein produced in *Escherichia coli* with high purity. |
| **P34** | **Rupali Kumthekar**<br>CSIR - National Chemical Laboratory | Mapping time-dependent disulfide bond formation during *in-vitro* refolding of recombinant peptibody: A Fc-fusion protein |
| **P35** | **Narges Lali**<br>acib GmbH | Fluorescently Labeled Antibody as an Inert Tracer for Characterization of Residence Time Distribution in Counter Current Protein A Affinity Chromatography |
| **P36** | **Sabrina Leigheb**<br>BOKU Vienna | Asymmetric field flow fractionation and Taylor Dispersion Analysis for separation and characterization of Adeno-Associated Viruses for gene therapy |
| **P37** | **Rashmi Sharma**<br>Indian Institiute of Technology | Optimization of the *in-vitro* refolding of biotherapeutic Fab Ranibizumab |
| **P38** | **Martina Winter**<br>BOKU Vienna | Efficient identification of optimal process conditions with Gaussian processes |
| **P39** | **Hannah Davison**<br>UCL | Evaluating novel monoclonal antibody (mAb) purification techniques |
| **P40** | **Mikael Andersson Schönn**<br>Bio-Works | Inline-tandem purification of viruses from cell lysate by agarose-based chromatography |
| **FP1** | **Julian Grinsted**<br>University College London | Design of affinity separations for the manufacture of in vitro transcribed mRNA |
| **FP2** | **Nick Samuelson**<br>MSD | Increased Virus-Like Particle Recovery with Disassembly Prior to Purification |
| **FP3** | **Ismaele Fioretti**<br>Politecnico di Milano | Process Intensification in the Purification of an Oligonucleotide Sequence by MCSGP with UV-Based Dynamic Control |
| **FP4** | **Thomas Müller-Späth**<br>Chromacon AG | Automated two-column chromatography for the purification of Oligonucleotides and Peptides |

| | | |
|---|---|---|
| FP5 | **Touraj Eslami**<br>acib Gmbh | Optimizing chromatography for maximum efficiency: an innovative approach to optimize productivity, resin utilization, and buffer consumption |
| FP6 | **Dennis Röcker**<br>TU Munich | Enhancing chromatography by use of electrochemically modulated membranes |
| FP7 | **Ryan Kilgore**<br>North Carolina State University | Peptide ligands: a bespoke affinity platform for next-generation biotherapeutics and gene-editing products |
| FP8 | **Staš Vrh**<br>University of Ljubljana | Implementation of polyHIPE monoliths for preparative and analytical separation of bacteriophages and their genomic DNA |
| FP9 | **David Scheich**<br>BOKU Vienna | Purification and characterization of recombinant secretory immunoglobulin A from CHO cell culture supernatant |
| FP10 | **Anna-Carina Frank**<br>BOKU Vienna | Cationic flocculants assisted clarification |
| FP11 | **Alexander Zollner**<br>BOKU Vienna | Chromatography-based purification of enveloped virus-like particles displaying different influenza surface antigens for an immunologic study in mice |
| FP12 | **Lena Achleitner**<br>acib GmbH | Baculovirus working stock: the production and purification of an intermediate product for large scale VLP production in insect cells |
| FP18 | **Preeti Saroha**<br>Indian Institute of Technology, Delhi | Production of bioactive recombinant monoclonal antibody fragment in periplasm of *E. coli* expression system |
| FP19 | **Milan Polakovic**<br>Slovak University of Technology | Single-pass diafiltration using a double-membrane module |
| FP22 | **Leo Jakob**<br>acib GmbH | Accelerating Virus-Like Particle Downstream Process Development Using Asymmetric Flow Field-Flow Fractionation (AF4) |
| FP23 | **Ricardo Silva**<br>iBB - Institute for Bioengineering and Biosciences | Anion exchange chromatography for extracellular vesicles purification |
| FP24 | **Rashmi Sharma, Pragya Prakash**<br>Indian Institute of Technology Delhi | Downstream Process Development for intact Virus-Like Particles (VLPs) from yeast expression system *Pichia pastoris* |
| FP25 | **Jorge João**<br>Instituto Superior Técnico - Universidade de Lisboa | Downstream processing of non-viral protein nanocages for biotechnological and biomedical applications: development of chromatography-based purification strategies |

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# KEY NOTE LECTURE ABSTRACTS

# Probing structure and function of proteoforms by MS-hyphenated separation techniques

**Elena Dominguez Vega[1]**

[1]*Leiden University Medical Center, Leiden, Netherlands*

Proteins are heterogeneous species comprised of genetic variants and many post-translational modifications defined as proteoforms. Proteoforms are very diverse in structure - ranging from small chemical variations to the attachment of large carbohydrates - but also in functionality. Despite their relevance, structural and functional characterization of proteoforms remains a major analytical challenge in modern biotechnology and bioanalytical laboratories. Mass spectrometry (MS) has become the primary tool for the structural characterization of proteoforms. Yet, due to the vast heterogeneity and high mass similarity of many proteoforms selective separation of proteoforms prior to MS is often required. Furthermore, while the structural characterization of proteoforms has significantly advanced recently, the proteoform-selective functional characterization is still largely neglected due to current limitations in conventional approaches, providing an overall affinity for all proteoforms.
In this lecture, designs and applications of MS coupled to liquid chromatography and capillary electrophoresis (CE) for the structural and functional characterization of proteoforms will be presented. Examples of recombinantly produced as well as endogenous proteins of clinical relevance will be showcased. Analysis of proteoforms at the intact and subunit level (i.e. without digestion) provided a more accurate proteoform representation. MS characterization of endogenous proteins benefited from the use of low separation flows and novel hyphenation strategies such as dopant-enriched nitrogen enhanced nano-electrospray ionization. For functional assessment, we have exploited for the first time the capabilities of affinity CE-MS to assess functional characteristics of proteins in a proteoform-resolved fashion. Examples on the binding between monoclonal antibodies and Fc receptors will be illustrated.

# Time-Dependent Sorption Behaviour of Viral Vectors

**Daniel Bracewell**[1]

*[1]University College London, London, United Kingdom*

Viral vector processes are still maturing. Here we study two viral vectors: adenovirus (AV) and lentivirus (LV). The labile nature of these products is a concern when using adsorption-based separation. Downstream processing sequences involving UF/DF and anion exchange-based (AIEX) chromatography were investigated.
Despite AIEX's extensive use for LV recovery, variable performance and generally low recovery is reported. This poor understanding of product loss mechanisms highlights a significant gap in our knowledge of lentiviral vector adsorption and other types of vector delivery systems. Here we show HIV-1-LV recovery over quaternary-amine membrane adsorbents is a function of time in the adsorbed state. Kinetic data for product loss in the column bound state was generated. Upon gradient elution, a two-peak elution profile implicating the presence of two distinct binding subpopulations is observed. Characterizing the loss kinetics of these two sub-populations showed a higher rate of vector loss in the weaker binding peak.
In the analogous recovery of AV, nanofiber-based ion exchange chromatography was investigated at differing ligand densities. Yield and resolution was highly sensitive to the ion exchange ligand density and time adsorbed to the solid phase. Increasing Q amine ligand density improved the separation of intact AV capsids from host cell protein impurities and product-related impurities including free hexon (a major capsid coat protein) and replication-defective AV capsids that contained DNA but was also associated with poor recovery.

In summary for both vectors minimizing the time spent in the adsorbed state was shown to be a critical parameter in retaining high yield.

# Latest developments in the implementation of modelling tools in the biopharmaceutical industry

**Arne Staby**[1]

*[1]Novo Nordisk, Bagsværd, Denmark*

Modeling for down-stream process development and control have lately changed from an academic exercise to widespread implementation and application in the biopharmaceutical industry. The use of modelling is driven by a combination of regulatory requests for improved understanding of biopharmaceutical processes through Quality by Design (QbD) approaches, as well as financial benefits through shorter development times and improved process economy. Statistical models have found widespread appliance, however, mechanistic models or hybrid modelling are increasingly being implemented now in areas where suitable descriptions based on chemical engineering principles exist. This presentation will provide a status on the current development level of advanced models in the biopharmaceutical industry among others based on findings from the most recent modelling workshop in June 2023. Examples and case studies of application of models and machine learning for various biopharmaceutical processes and other unit operations will be provided. A linking of process understanding with the general QbD approach and recent feed-back from regulatory authorities on the implementation of mechanistic models will also be discussed.

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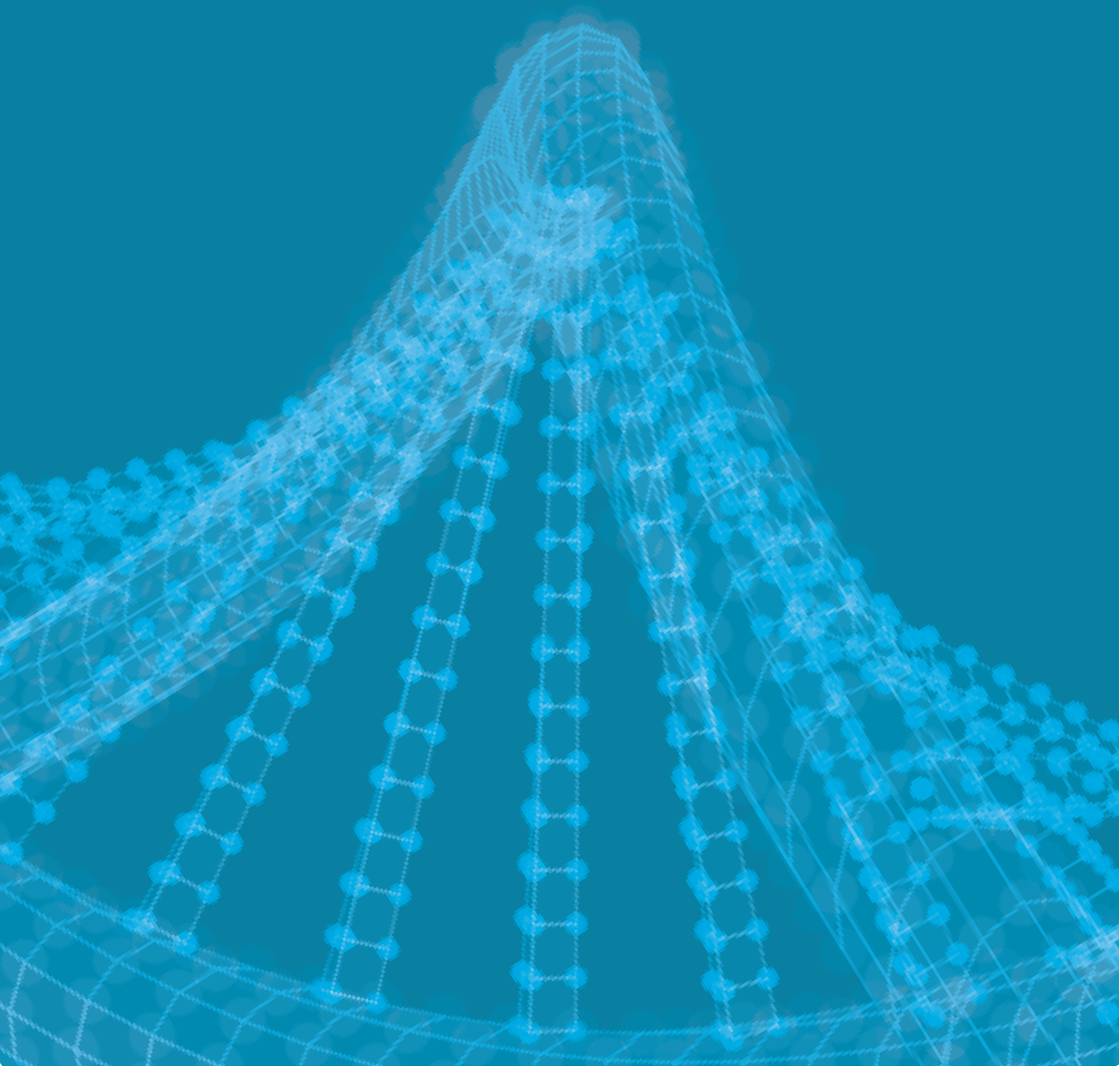
# ORAL PRESENTATION ABSTRACTS



# High-Throughput Manufacturing of Personalized Plasmid DNA Cancer Vaccines

**Linda Gombos[1],** Elijahu Babaev[1], Ernst Böhm[1], Hauke Hüttmann[1], Angela Neubauer[1], Gottfried Stegfellner[1], Hans Huber[1]

[1]*Biomay, Wien, Austria*

Personalized medicine is an emerging therapeutic approach based on the unique genetic makeup of each patient. One of the most rapidly advancing areas of application has been individualized cancer therapy, where the mutational alterations found in each patient's tumor cells are evaluated and a customized therapeutic cancer vaccine specific for that individual's tumor antigens is developed.
Here we present our manufacturing platform including a specialized manufacturing facility for individualized plasmid DNA vaccines according to Good Manufacturing Practice (GMP). Over 100 patient-specific batches have been manufactured to support a clinical trial to treat different types of cancer.
Manufacturing individualized vaccines requires rapid turnaround times and robust processes across the complex value chain including tumor biopsy, identification of tumor-specific biomarkers, vaccine design, generation of the patient-specific plasmid DNA starting material and manufacturing of the individualized drug product.
During the parallelized small-scale manufacturing process, a personalized E. coli cell bank is generated, which is then used in a fed-batch fermentation to produce the plasmid DNA. After harvesting and lysing the biomass, the product is purified in a two-step chromatography process, formulated by UF/DF and sterile filled. Product quality is characterized by a comprehensive set of analytical methods.
We will present our approaches to facility design, process optimization using a combination of different methods (e.g. design of experiment) as well as the evaluation of trending data to monitor manufacturing performance to ensure a small footprint, rapid turnaround times and cost effectiveness of our manufacturing platform for personalizes plasmid DNA.

# Towards industrial manufacturing of DNA-origami nanostructures: scalling up ssDNA scaffold purification

**Ana Rita Santos[1],** Joana Mendes[1], Miguel Prazeres[1]

*[1]iBB - Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisboa, Portugal*

DNA nanostructures are often assembled using the 'scaffolded DNA origami' strategy, whereby a long single stranded DNA (ssDNA) molecule (the scaffold) is folded with the assistance of short oligonucleotides (the staples) into the target nanostructure. The design of the ssDNA and staple sequences required to assemble a given nanostructure can be made using different computational algorithms. The scaffolds are then produced biologically, e.g., by isolating the DNA of phages like M13mp18 or by PCR with appropriate templates and primers.

With this project we aim to set up scalable production and purification methods to obtain ssDNA scaffolds from the DNA of the M13mp18 bacteriophage. After E. coli cultures in shake flask or batch bioreactors, the 7,249-nt long, circular, single stranded M13mp18 genome is purified with a process that starts with a centrifugation step that separates host cells from the extracellular bacteriophages. Next, phages will be precipitated and chemically lysed with 200 mM NaOH/1 % SDS to release the ssDNA genome. Following neutralization with acetate, the ssDNA can be purified by ultrafiltration and anion-exchange chromatography. The final product is analysed by HPLC, BCA and real-time PCR, achieving a quality suitable for therapeutic applications. This ssDNA is then used as a scaffold for the folding of an asymmetric DNA-origami structure with the adequate set of staple strands. After folding, the excess of staple strands will be removed by PEG precipitation and the quality of the final DNA-origami is assessed by agarose gel electrophoresis and negative staining transmission electron microscopy.

# Challenges for purification of a pneumococcal recombinant protein

Manuella Cazelato Pires[1], Renan Minin de Mori[1], Marcela Cabette Crivellenti[1], Patricia Zorzete[1], **Viviane Maimoni Gonçalves[1]**

*[1]Instituto Butantan, São Paulo, Brasil*

Streptococcus pneumoniae is a bacterium that can cause severe diseases following a physiological imbalance. Despite the existence of vaccines, their formulation, based on capsular polysaccharides, has low coverage of serotypes, which leads to a selective pressure and serotype replacement that mitigates the benefits of vaccination with time.
The aim of this work is to develop the purification process of an antigen for a new protein based vaccine that offers serotype-independent protection. The toxin pneumolysin (Ply) was genetically detoxified (PdT), cloned with His-tag and a TEV cleavage site (His-TEV-PdT) on pET28a vector, and produced by E. coli BL21(DE3). Cells were disrupted in a high-pressure continuous homogenizer. After centrifugation, the clarified sample was adjusted for immobilized metal-affinity chromatography (IMAC-Ni+2). The elution fraction from IMAC was concentrated and loaded in gel filtration (GF) chromatography. The His-TEV-PdT eluted from GF was hydrolyzed with TEV protease for tag removal, followed by a subtractive IMAC for PdT recovery in the flowthrough. Two main challenges were faced during the purification: a presence of a host-cell protein (HCP) with the same molecular mass as the target (54kDa), and His-TEV-PdT aggregation together with HCP. The 54kDa-protein was mostly removed in the 1st IMAC, but its presence leads to overestimation of PdT in the raw material, resulting in low apparent global yield (9.5%). Aggregates were removed in GF and PdT was recovered after the 2nd IMAC with >90% purity. As PdT has no activity to be measured, further methods for quantification are necessary to calculate the real yield.

# Cell Tolerant Radial Affinity Chromatography (cTRAC)

**Michel Eppink**[1], Marcel Raedts[2]

*[1]Byondis BV, Nijmegen, Netherlands*
*[2]Proxcys BV, Emmen, Netherlands*

Biopharmaceutical proteins (e.g. monoclonal antibodies) are important therapeutic medicines for different human diseases such as oncology and infectious diseases. These complex biomolecules are generated in biotechnological processes using Chinese Hamster Ovary (CHO) cell lines. Upstream Processing (USP) expresses the proteins whereas Downstream Processing (DSP) purifies the proteins. These USP and DSP processes are mostly operated batch wise in clearly defined unit operations. However, the developed processes are labor intensive, take much time, product losses and produce a lot of waste. By integrating the USP bioreactor and the first DSP process step would improve the overall process, less unit operations, less complex, more consistent results obtained, lower overall cost price and the process can be performed in closed operations preventing infections (e.g. bacteria, fungi, yeast).
To integrate the bioreactor directly to the capture column the expressed monoclonal antibody can be directly purified on the capture step consisting of a disposable cell Tolerant Radial Affinity Chromatography (cTRAC) column and the cells efficiently removed as waste in closed disposable bags. This concept is unique in the field of biotechnology processes as normally depth filtration and centrifugation steps are needed to remove the cells and collect the cell free material in a harvesting tank before the first purification step. Overall, this new concept would reduce the process with two unit operations by removing the depth filtration or centrifugation and harvesting collection tank. Results of this concept will be presented showing the performance of this integrated USP-DSP on the product quality of the monoclonal antibody.

# Step Gradient SMB for mAb polishing using salt tolerant anion exchangers

**Egbert Müller[1],** Jonas Wege[1], Carsten-Rene Arlt[1], Ildi Majollari[1]

*[1]Tosoh Bioscience GmbH, Griesheim, Germany*

Protein A chromatography is a widely used method for the purification of monoclonal antibodies (mAbs), Typically, a subsequent polishing step is used to deplete remaining product and process related impurities. The use of simulated moving bed (SMB) technology has shown to be a promising approach for transferring chromatography processes to continuous processes. In this study, we present the implementation of a step gradient SMB system for the polishing of protein A purified mAbs using a salt tolerant anion exchange resin.
The step gradient SMB system was developed on the OctaveTM BIO multi column chromatography instrument based on a four-zone configuration with the use of a step gradient elution profile consisting of two different eluent compositions. The performance of the system was evaluated by measuring the purity, yield, and aggregate content of the purified mAbs. The impact of the operating parameters, including the flow rate, step gradient profile, and buffer composition, on the system performance was also evaluated. The results demonstrated that the step gradient SMB system was able to efficiently remove impurities and aggregates, resulting in highly pure mAbs .
Overall, the results of this study demonstrate the potential of step gradient SMB technology for the polishing of protein A purified mAbs. The implementation of this system provides a cost-effective and efficient approach for the purification of mAbs with high purity and yield, which can significantly reduce the downstream processing cost and time.

# Advantages and Opportunities of Multicolumn Countercurrent Solvent Gradient Purification Accessed by Tuning the Product Internal Recycling Phase

**Mattia Sponchioni**[1]

*[1]Politecnico Di Milano, Milano, Italy*

Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) has recently demonstrated great potential in solving the traditional limitations associated to the polishing of biopharmaceuticals through single-column batch chromatography. Among the advantages of MCSGP, the possibility of alleviating the characteristic tradeoff between yield and purity typical of single-column operations, the consistent product quality reached at steady state, which streamlines the quality control, and the process automation, reducing the requirements for human supervision.

MCSGP can be implemented using two twin-columns, alternatively operated in parallel and in series for a fully automated internal recycling of the impure side-fractions that are discarded in batch chromatography. This allows reaching simultaneously high purity and improved product recovery. The design of this recycling stage critically impacts the performances of MCSGP and is governed by two degrees of freedom, namely the dilution factor and gradient slope, which can be leveraged to improve the versatility of the whole process and access significant advantages. These include a boost in the productivity or, alternatively, the possibility of improving the recovery of products strongly adsorbed to the resin, which is very beneficial in the case of difficult-to-manufacture products.

In this contribution, these advantages are showcased in the MCSGP of PEGylated proteins, demonstrating the flexibility of this process and the possibility to adapt it to meet the specific needs of the different manufacturing scenarios.

# Scalable magnetic bead-based cell separation technology for the depletion of receptor positive cell subpopulations

**Nils Brechmann[1],** Kristofer Eriksson[1], Torbjörn Pettersson[1], Véronique Chotteau[2,3]

*[1]Magicbioprocessing, Uppsala, Sweden*
*[2]AdBIOPRO, VINNOVA Competence Centre for Advanced BioProduction by Continuous Processing, Stockholm, Sweden*
*[3]Cell Technology Group (CETEG), Dept. of Industrial Biotechnology, School of Engineering Sciences in Chemistry, Biotechnology and Health, KTH Royal Institute of Technology, Stockholm, Sweden*

iPSC-based allogenic therapies provide the promising possibility for off-the-shelf cell therapies, which could help to lower production costs and ultimately the therapy costs. Especially, remaining pluripotent cells originating from the therapy's starting material can impact the efficiency of the therapy and more importantly can have significant impact in the patient's health and the depletion of pluripotent cells is crucial. Needing large production scales for commercialization for off-the-shelf therapies, but with the small size of magnetic cell sorting beads used nowadays, being significantly smaller than a cell, the method provides the risk of internalization, non-uniform magnetic labelling and low mobility due to low magnetization of the cell. Therefore, today's magnetic beads are limited in terms of large production potential.
We have previously shown that our large (≈ 100 µm) magnetic bead system MAGicbeads is highly gentle towards cells (Brechmann et al., 2021) and provided excellent scalability at pilot-scale for the purification of mAbs (Brechmann et al., 2019).
We have developed a new method for cell separation based on flexible Protein A/G base conjugated large magnetic agarose beads, providing flexible adjustment of the receptor recognising antibodies, potentially targeting different receptors throughout the manufacturing process. Evaluation of the sorting capabilities of this novel flexible sorting system with a model population of HER2+ cells and hMSCs showed high depletion efficiencies of up to 91 % (Brechmann et al., 2022). With high biocompatibility, high robustness against mechanical stress and minimal unspecific binding, the system further proofed to be also applicable to the isolation of iPSCs.

# Characterising feed and membrane interactions in tangential flow filtration of lentiviral vectors: hints for recovery improvement

**Noor Mujahid[1],** Andrea Rayat[1], Yasu Takeuchi[1,2]

[1]*University College London, London, United Kingdom*

[2] *Medicines and Healthcare products Regulatory Agency, London, United Kingdom*

Cell and Gene Therapies (CGT) that utilise lentiviral vectors (LV) to carry therapeutic genetic material to host cells have been approved for clinical use. However, for this technology to be widely used, major bottlenecks in LV manufacturing need to be overcome, particularly the high cost and low recovery. Membrane processing is commonly employed in the downstream steps, from normal flow filtration (NFF) during clarification and sterile filtration to tangential flow filtration (TFF) during vector concentration or formulation. This presentation will discuss the use of TFF in processing pre-chromatography LV feeds using three LV pseudotypes with VSV-G, RDpro or Cocal-G envelope proteins. We will show the interaction between processing sequence (ultrafiltration/diafiltration), mode of operation (constant flow or constant transmembrane pressure (TMP)) and membrane properties (pore size and chemistry) on yield, productivity, and quality of vectors. We will show results from our high-throughput ultra-scale down experiments which were scaled up using a commercial hollow fibre system. Through these experiments, we have identified that the sequence of TFF unit operations is key to obtaining high recovery, starting with diafiltration first. In addition, we reveal that operating TFF at higher TMP (up to 2 Bar) to increase productivity does not lead to a significant loss in functional titre ($p=0.4681$) which demonstrates that LVs are able to withstand high shear and are more robust than commonly perceived.

# Numerical calculations of membrane structure, virus removal performance, and filtration behaviours of virus filters based on a heterogeneous membrane structural model comprising multiple layers with different pore size distributions

**Hironobu Shirataki[1],** Ranil Wickramasinghe[2]

*[1]Asahi Kasei Medical, Tokyo, Japan*
*[2]University of Arkansas, Fayetteville, USA*

Virus filters are essential in bioprocessing for safe protein production. These filters are designed to reject virus particles but allow the protein product to be recovered in the permeate. Understanding the correlation between virus filter structure and performance is important for efficient process design. Here, an advanced multilayer structure model is applied to virus filtration experimental data in order to analyse virus reduction.
By applying a multilayer structural model comprised of theoretical layers having pore size distributions to published visualization data, we demonstrate in the present study that virus removal performance and filtration behavior can be quantitatively calculated. Virus filter structure can be classified into asymmetric high porosity, symmetric high density and laminated structure types, and all types exhibit sufficient virus removal properties when used in normal processes. Laminated structure filter shows extremely high virus particle removal performance, achieving the most robust virus removal even in filtration with process pauses, although flux decay tends to occur during filtration of solutions containing even very small amounts of aggregate. Conversely, asymmetric high porosity filter may show the lower virus removal in processes involving multiple pauses that exceed practical conditions but shows stable filtration behavior with lower increase in pressure increase and lower flux decay even for the filtration of solutions containing aggregates. Such filtration behavior and virus removal properties can be quantitatively expressed and predicted by numerical calculations in models based on the theoretical multilayer structure.

# Multiattribute monitoring of charge-based heterogeneity of recombinant monoclonal antibodies using 2D HIC-WCX-MS

**Deepika Sarin**[1], Sunil Kumar[1], Anurag Singh Rathore[1]

[1]*Indian Institute Of Technology, Delhi, Delhi, India*

Charged heterogeneity of monoclonal antibody (mAb) products is regarded as a critical quality attribute (CQA) depending on its impact on the safety and efficacy profile of the product. Hence, manufacturers are expected to perform a comprehensive characterization of the charge heterogeneity to ensure that the manufactured product meets its specifications. Further, monitoring is also expected during the product lifecycle to demonstrate consistency in product quality. However, conventional analytical methods for characterization of hydrophobic and charge variants are nonvolatile salt-based and require manual fraction collection and desalting steps before analysis through mass spectrometry can be performed. In the present study, a workflow of a two-dimensional liquid chromatography method using mass spectrometry (MS)-compatible buffers coupled with native mass spectrometry was performed to characterize hydrophobic variants in the first dimension and charge variants in the second dimension without any need for manual fractionation. This novel two-dimensional (2D) hydrophobic interaction chromatography (HIC)-weak cation-exchange chromatography (WCX)-MS workflow identified 10 variants in mAb A, out of which 2 variants are exclusive to the 2D orthogonal method. Similarly, for mAb B, a total of 11 variants are identified, including 5 variants exclusive to the 2D orthogonal workflow. When compared to stand-alone, HIC resolved only 4 variants for both mAbs and WCX resolved 7 variants for mAb A and 6 variants for mAb B. In addition, the proposed method allows direct characterization of hydrophobic/charge variant peaks through native mass spectrometry in a single-run workflow.

# Quantification of concentration of mAb and excipients in a high concentration ternary mixture using ATR-FTIR spectroscopy and chemometrics

**Tushar Savane[1],** Sunil Kumar[2], Anurag Rathore[1,2]

[1]*School of Interdisciplinary Research, IIT Delhi, New Delhi, India*
[2]*Department of Chemical Engineering, IIT Delhi, New Delhi, India*

Monitoring critical quality attributes charge-related heterogeneities essential biopharmaceutical manufacturers.
Size-exclusion chromatography (SEC) preferred analytical technique for quantification of aggregates fragments and used for in the product, whereas weak-cation exchange chromatography (WCX) is widely characterization charge variants of biotherapeutic products, in particular monoclonal antibodies (mAbs). Multi-attribute monitoring (MAM) ability to monitor these attributes two-dimensional chromatography (2D-LC). Typically, approach, second-dimension samples directly analysed through spectrometry, dimension limitations respect coupling spectrometry. of in a single run flow in this only the as the first In the present study, a novel 2D-SEC-MS/WCX-MS workflow has been proposed, in which chromatography of both dimensions (D1 and D2) were directly coupled with mass spectrometry, through which size-related and charge-related variants of monoclonal antibody mAb A were analysed simultaneously in their native form. In comparison to stand-alone SEC and WCX methods, this method enables simultaneous analysis of size and charge variants in a single workflow without manual intervention allowing analysis of low abundant variants. Further, this method has 75% less sample requirement and shorter analysis time (25 minutes vs. 90 minutes) when size and charge variants were analysed individually. The proposed native 2D-LC-MS workflow was used to analyse stressed sample of mAb A, in which D1 analysis revealed the presence of aggregates (8-20%), which were primarily dimers, whereas D2 analysis showed an increment offers the using liquid are mass has with to direct with mass in acidic variants (9-21%).

# Towards comprehensive SAX × RP 2D-LC-MS/MS host cell protein profiling in biopharmaceutical manufacturing

**Markus Mozgovicz[1],** Nico Lingg[2], Sebastiaan Eeltink[1]

*[1]Vrije Universiteit Brussels VUB, Brussels, Belgium*
*[2]University of Natural Resources and Life Sciences, Vienna, Austria*

Biopharmaceutical manufacturing processes often release host cell proteins (HCP) during upstream processing, that may affect the safety, quality, stability and efficacy of the final product. Hence, regulatory authorities require extensive monitoring of HCPs during downstream protein purification. However, current detection methods such as enzyme-linked immunosorbent assay have several limitations due to differences in quantity and affinity of anti-HCP polyclonal antibodies for various HCP species. Therefore, it is crucial to develop orthogonal methods for quantitative HCP analysis.
Here, we provide a bottom-up proteomics workflow for quantitative HCP analysis, employing comprehensive strong anion exchange, reversed phase liquid chromatography tandem mass spectrometry. This study has important implications for understanding the relationship between the LC efficacy, the peptide quantification and bioprocess-related HCP removal strategies, ultimately leading to increased protein identification. For that, the resolving power of each LC dimension was optimized for model peptides towards maximized peak capacity per unit time, whilst maintaining short total analysis times.
Ongoing studies will provide further insight into the impact of enhanced LC performance on monitoring the clearance potential of high risk, or low-intensity HCPs by data independent acquisition mass spectrometry. As proof of concept, we demonstrate HCP identification in bioprocess-related samples of various complexities, provided by different host cell lines.
The ultimate objective of our study is to identify and monitor the clearance of HCPs in a novel DSP workflow for sIgA. Quantitative and qualitative data on these HCPs will offer further inside, that can guide the process development towards targeted removal strategies and overall process efficiency.

# Target Quantitative Analysis of Glycoproteins by Parallel Reaction Monitoring (PRM) LC-MS/MS

**Yehia Mechref**[1]

*[1]Texas Tech University, Lubbock, United States*

MS-targeted analyses are frequently utilized to analyze and validate quantitative changes in biomolecules across diverse omics fields. Targeted studies were commonly conducted using multiple reaction monitoring (MRM) in triple quadrupole mass spectrometers. The development of the Orbitrap® base instruments made it possible to use the parallel reaction monitoring (PRM) technique which simultaneously monitors the full MS/MS of the targeted analytes with higher sensitivity and accuracy than MRM. Regarding the glycoproteomics analysis PRM has not been commonly used to quantify glycopeptides. Additionally, no protocol evaluates the effect of important MS/MS acquisition parameters of glycopeptides and their isoforms. This presentation discusses a comprehensive PRM study of glycopeptides and isomeric glycopeptides using two types of LC-MS instruments, the Orbitrap Fusion Lumos and the Q Exactive HF. Standard glycoproteins and disease-related serum samples were used as a source of glycopeptides for the study. The methodology includes revising the transition selection where the area signals of the glycopeptide diagnostic fragment ions were extracted and evaluated with precision and accuracy. The optimal normalized collision energy (NCE) values for different N- and O- glycopeptide types are defined. Additionally, we discuss in this presentation the precision and accuracy of the PRM quantitation of glycopeptide isomers. The presentation also comprehensively discusses and define a guideline designated to the application of PRM-targeted analysis of glycopeptides and their isomers.

# In-line enzymatic digestion strategies beyond trypsin for the sensitive targeted bottom-up analysis of protein biomarkers by capillary electrophoresis-mass spectrometry

**Estela Giménez[1],** Hiba Salim[1], Fernando Benavente[1]

*[1]Department of Chemical Engineering and Analytical Chemistry. Institute for Research on Nutrition and Food Safety (INSA·UB), University of Barcelona, Barcelona, Spain*

Targeted bottom-up proteomics is generating a great interest for the straightforward, accurate, and sensitive measurement of specific protein biomarkers from surrogate peptide fragments. However, this approach typically requires long and labor-intensive off-line enzymatic digestions and may introduce artifacts associated with the sample processing steps. As an alternative, we present two in-line digestion methods for the bottom-up analysis of proteins by capillary electrophoresis-mass spectrometry (CE-MS). In-line immobilized enzyme microreactor (IMER) and in-line capillary digestion methods, using endoproteinase Glu-C as enzyme, were developed for the analysis of α-synuclein (α-syn). This protein shows a phosphorylated form that has been related to Parkinson's disease [1].

First, IMER-CE-MS was evaluated using home-made and commercial immobilized Glu-C particles. However, this strategy gave poorer results in terms of peptide sequence coverage and sensitivity compared to the off-line digestion, unlike what we previously obtained with trypsin and IMER-CE-MS [2]. In contrast, the in-line capillary digestion, based on a sandwich injection of the protein with enzyme solutions [3], allowed the detection of a peptide covering the potential phosphorylation site of α-syn, minimizing sample handling and total analysis time compared to the off-line digestion. After optimization, the developed method was applied to the analysis of biological samples for the targeted high-throughput detection of α-syn. The presented method could be easily adapted to analyze other protein biomarkers in which a digestion with Glu-C is necessary for an adequate sequence and post-translational modifications coverage.

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# Porous platform ink for fast and high resolution 3D printing of stationary phases for affinity chromatography

**Mariachiara Conti**[1]

*[1]The University of Edinburgh, Edinburgh, United Kingdom*

Through 3D printing, stationary phases with design complexity and customization could be fabricated at no additional costs. 3D printed chromatography stationary phases could take a leading role in improving downstream processing of biological products, but so far it has been challenging to fabricate porous structures with resolution below few hundreds of micrometres due to the lack of material availability and limitations on 3D printing technology.
In this work we combined 3D printing with polymerisation-induced phase separation to create hierarchically porous structures with macroscopic porosity and superimposed nanoscale porosity. We used a newly developed 3D printable platform ink based on glycidyl methacrylate, which serves as a base reactive matrix for subsequent chemical functionalisation.
Ordered porous columns were 3D printed with spatially controlled pore sizes ranging from hundreds of nanometres to few micrometres at a resolution of around 200 µm. In this work, we studied the immobilization of protein A to the 3D printed columns using FT-IR and UV-Vis absorbance. Dynamic binding of IgG to the protein-A columns was investigated at different flow rates and concentrations. The 3D printed columns were evaluated for their viability to replace clarification and capture steps in a real monoclonal antibodies production process. The results of this study informed technoeconomic analysis for the production and integration of 3D printed columns into a real mAbs production plant.
We demonstrate that we are now able to 3D print porous columns with various ordered structures, diameters and a range of active ligands to meet different chromatographic modalities and downstream steps.

# Novel multi-modal salt-tolerant cation-exchange membrane applied for the purification of a single-chain variable fragment produced in *Pichia pastoris*

**Dan Pham[1],** Marina Linova[1], William Smith[2], Jinxin Fan[2], Joseph Lavoie[2], Ruben Carbonell[2], John Woodley[1]

[1]*Technical University Of Denmark, Kongens Lyngby, Denmark*
[2]*North Carolina State University, Raleigh, USA*

A novel salt-tolerant cation-exchange (CEX), nonwoven membrane with a multi-modal ligand, 2-mercaptopyridine-3-carboxylic acid (MPCA), was used to capture a single-chain variable fragment (scFv) from a Pichia pastoris supernatant. Two fed-batch fermentations resulted in scFv titers of 395.0 mg/L and 555.7 mg/L respectively, with a product purity of roughly 83% (mass scFv / mass of total protein). The effects of pH, residence time (RT), scFv loading and scFv concentration on dynamic binding capacities (DBC), scFv yields and % purities were determined. As an example, in one experiment the CEX-MPCA membrane exhibited a DBC of 34.5 mg/mL at pH 5.5 and a residence time of 1 min. In a separate experiment, as the RT varied from 2.0 minutes to 0.2 minutes, the DBC showed a small drop from 38 to 36 mg scFv / mL of membrane volume. In this range of residence times the elution yields remained relatively constant (77% to 70%) and purity of the product varied from 89% to 92%. These results indicate that this type of nonwoven fabric membrane chromatography adsorbent has significant potential to enhance productivity in product capture steps in downstream processing of biologics in a bind-and-elute mode. The binding capacities compete well with those of resins used in column chromatography, while operating at residence times of 5-10-fold lower than those of resin columns at similar pressure drops. However, work still needs to be done to develop membrane devices to make these novel membranes enter commercial application, including flow distribution design and developing membrane packing protocols.

# PEG-aided precipitation for adjusting acidic variant content in monoclonal antibody pools

**Dorota Antos[1],** Tomasz Rumanek[2], Michal Kolodziej[1], Patrycja Zimoch[2], Wojciech Piatkowski[1]

*[1]Rzeszow University of Technology, Rzeszów, Poland*
*[2]Doctoral School of Engineering and Technical Sciences at the Rzeszow University of Technology, Rzeszów, Poland*

Structural heterogeneity of monoclonal antibodies (mAbs) is the most common cause of formation of charge variants, which are classified as acidic (av), main (mv) and basic species (bv). The av and bv species differ in biological activity from the mv species, however av are often reported to have reduced therapeutic potency compared with the other variants. Therefore, reduction in the av content in mAb pools is often prioritized over reduction in the bv content.
To adjust the variant content, ion exchange chromatography (IEX) is used as the gold standard. Nevertheless, similarity of structures and charges of variants is a cause of peak overlap, which results in low yield of the separation. Moreover, the process throughput is limited by binding capacity of chromatographic resins. As an alternative to chromatography, PEG-aided precipitation of variants has been developed. Electrostatic interactions between molecules of differently charged variants trigger opposite tendency of partitioning av and bv variants between the supernatant and precipitate phases, therefore the process can be used for reducing the av content in mAb pools to a manufacturer defined level.
To improve the operation yield, multistage precipitation can be employed, which can be realized in crosscurrent or countercurrent mode. In the crosscurrent process, the precipitate withdrawn from each stage is re-dissolved and subjected to precipitation in a subsequent stage using a fresh portion of the precipitant (i.e., PEG solution). In the countercurrent process, both the precipitate and the precipitant are reused in subsequent stages.

# Understanding and Mitigating Persistence of CHO Host-Cell Proteins in Monoclonal Antibody Bioprocessing

**Abraham Lenhoff**[1]

*[1]University of Delaware, Newark, United States*

Host-cell proteins (HCPs) have long been recognized as a major class of process-related impurities in the manufacture of monoclonal antibodies (mAbs), and typical platform processes are well suited to reducing overall HCP amounts to acceptable levels. However, small amounts of individual HCPs that can be deleterious to the product or the patient have sometimes been found to persist into the final drug product, and understanding the mechanisms for such persistence and mitigating the effects have received appreciable attention over the past decade. This presentation will present evidence regarding two proposed persistence mechanisms, namely HCP-mAb association and the presence of HCP-rich aggregates. Results to be presented include chromatographic and proteomic data as well as biophysical measurements such as protein association and confocal microscopy, and the prospects for enhanced HCP clearance will be discussed.

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# A chromatography system modeling strategy for precise *in silico* process scaling

**Tatjana Trunzer[1],** Lena Enghauser[1], Sabrina Stahlberger[1,2],
Florian Grau[1], Tobias Hahn[1]

[1]*Cytiva, Germany*
[2]*Karlsruhe Institute of Technology (KIT), Germany*

Chromatographic purification processes for biopharmaceutical products are often developed at laboratory scale and need to be scaled up for production or scaled down for efficient process characterization. While the biopharma industry performs this exercise on a regular basis, the basic understanding of flow rate effects on mass transfer and adsorption/desorption phenomena when scaling up or scaling down are limited and can be challenging. Even when applying mechanistic modeling, system and column effects are rarely distinguished, challenging the determination of scale-dependent and scale-independent effects.
We examined the impact of different, individually configured elements in the flow path of chromatography systems to enable in silico scale-up and scale-down. In our extensive study we investigated different flow paths in ÄKTA™ chromatography systems. Besides, we studied different flow rates and compared their effects for traditional packed particle columns and for fiber-based Fibro™ PrismA prototypes.
Our study showed that there is no one-size-fits-all model for chromatography systems. The modeling of flow-through conditions with different system configurations provided detailed insights into the dependency of dispersion and mixing effects on the number and volumes of different flow path items. A more complex model was derived for a fully equipped ÄKTA avant™ system, and reduced models were developed for a simple, basic configured ÄKTA pure™ as well as the larger ÄKTA pilot™ system containing fewer flow path items. Based on these models, we identified a flow rate-dependent tubing and device-specific calibration strategy. This strategy allows to precisely distinguish between scale-dependent mass transfer effects and scale-independent thermodynamics.

# Detective Stories in Chromatography: the Inseparable Pair, the Missing Peak, and the Gang of Three

**Giorgio Carta**[1]

*[1]University of Virginia, Charlottesville, United States*

In this talk, we review three different cases related to the separation of biomolecules that deviate from the norm producing that seem to defy conventional wisdom. The first is the separation of isoleucine and γ-amino butyric acid on cation exchange resins. This separation is affected by the on-column formation of adsorption azeotropes. Unlike azeotropes commonly found in vapor-liquid equilibrium that have a fixed composition at a given pressure, adsorption azeotropes can occur over a range of compositions depending on the operating conditions. The second case involves the HIC of a hydrophobic mAb. While distinct peaks are observed with shallow gradients, the peak disappears altogether when using a steep gradient. The interplay of gradient slope with a U-shaped retention factor curve causes the mAb to become trapped in the column. The third case involves the chromatography of bivalent bispecific antibodies comprising scFv domains tethered to an IgG framework with flexible linkers. These molecules have a flexible structure resulting in multiple conformations. One peak is observed at low flow rates. Two or more distinct peaks appear, however, at high flow rates. So, one could get the impression that we get “better separation” at high flow rate, which, of course, would contradict basic tenets of chromatography. For each of these cases, we demonstrate how we have used the fundamental theory of chromatography to understand these behaviors and find solutions. As the complexity of future biopharmaceuticals increases, the potential for observing such complications is likely to increase requiring that we understand the ensuing dynamics.

# Model based process optimisation of an industrial chromatographic process for separation of lactoferrin from bovine milk

**Lukas Gerstweiler[1],** Paulina Schad[2], Tatjana Trunzer[3], Max Meyr[3], Jagan Billakanti[4]

*[1]The University Of Adelaide, Adelaide, Australia*
*[2]Heidelberg Univeristy, Heidelberg, Germany*
*[3]Cytiva R&D, Karlsruhe, Germany*
*[4]Cytiva Australia, Parramatta, Australia*

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# Digital Twins for High Throughput Chromatographic Process Development

**Marcel Ottens**[1]

*[1]Delft University of Technology, Delft, Netherlands*

With the increase in computational power over the last decades, the use of modeling and simulation in process design for (petro)chemical industry has become common ground. Computational tools like ASPEN are standard in the design and operational analysis of (petro)chemical plants. However, in the bio pharmaceutical field, such modeling and simulation techniques are only recently being investigated for use and (potential) implementation. Being the workhorse of purification in the biopharmaceutical industry, chromatography is a good candidate for this modeling approach. Detailed mechanistic models describing chromatographic separation behavior are available. A bioseparation process normally consists of multiple chromatographic and conditioning steps, hence, an extreme large design space needs to be investigated, and an efficient process design approach is paramount. This presentation will show the implementation of a hybrid bioseparation process design approach using a combination of mechanistic models and high throughput experimentation for process development and optimization of the production of industrial relevant biomolecules.

# Mechanistic modeling of cation exchange chromatography scale-up considering packing inhomogeneities

Jonas Koch[1], Daniel Scheps[2], Matthias Gunne[2],
Oliver Boscheinen[2], **Christian Frech[1]**

[1]*University of Applied Sciences, Mannheim, Germany*
[2]*Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany*

In process development and characterization, scale-up of chromatographic steps is a crucial part and brings a number of challenges. Usually, scale-down models are used during the development of the process step. The scale-up is often based on the concept of linear scale-up, and in addition, constant column properties are assumed. In this work, a mechanistic model describing an anti-Langmuirian to Langmuirian elution behavior of a polypeptide, calibrated with a pre-packed 1 mL column, is applied to demonstrate scalability to larger column volumes of up to 28.2 mL.
Considering individual parameters (ligand density, porosities, and normalized gradient slopes) for each column size, similar elution salt concentrations, peak heights, and shapes can be experimentally demonstrated when scaling.
Scale-up simulations show good agreement between experimental results and predictions at low loading. The deviations at higher loadings can be attributed to packing inhomogeneities. Further scale-up simulations show significantly improved model predictions when radial inhomogeneities in the packing are considered.

# "Flow-through Affinity Chromatography": a transformative approach to remove persistent and high-risk host cell proteins in Biomanufacturing

**Sobhana Alekhya Sripada**[1], Ashton Lavoie[1,2], Taufika Islam Williams[3], Driss Elhanafi[4], Marina Linova[5], John Woodley[5], Stefano Menegatti[1,4,6]

[1]*Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, United States*
[2]*Bridge Bio Gene Therapy, Raleigh, United States*
[3]*Molecular Education Technology and Research Innovation Center, North Carolina State University, Raleigh, United States*
[4]*Biomanufacturing Training and Education Center (BTEC), North Carolina State University, Raleigh, United States*
[5]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, Kongens Lyngby, Denmark*
[6]*NC Viral Vector Initiative in Research and Learning (NC-VVIRAL), Raleigh, United States*

Engineered biotherapeutics today are clinically successful due to excellent cell targeting, therapeutic activity, and safety. However, their success is accompanied by high titer expression and diverse host cell protein (HCPs) removal. Particularly critical HCPs are those with inherent immunogenicity and enzymatic activity – threatening patient safety and product stability while being persistent in downstream bioprocessing. Diversity in expression systems: mammalian (CHO, HEK293) to fungal systems (P. pastoris) is concurrently emerging due to yeasts ability to express complex products at high concentrations using simpler culture media. However, unlike CHO, fungal systems in biomanufacturing are in their infancy along with their bioprocess-relevant information.
To address HCP removal challenges, we introduced "flow-through affinity chromatography" embodied by "Guard™" adsorbents functionalized with peptide ligands designed to target full proteome capture. CHO LigaGuard™ features binding capacities upto 20 mg HCP/mL resin, HCP clearance of ~750- to ~125-fold across 100 CV loading. Proteomic analysis confirmed the removal of high-risk HCPs like cathepsins, histones, GSTs, and lipases. Combining LigaGuard™ with product capture steps afforded mAb yields of >85% and HCPs as low as 8 ppm. For yeast cultures (P. pastoris) producing antibodies and fragments (ScFv), PichiaGuard™ features binding capacities of 25 mg HCP/mL resin, ~2,000- to ~500-fold HCP clearance upto 50 CVs loads while ensuring ≤80% product yield. Proteomics demonstrates superior performance of PichiaGuard™ versus IEX/MMC counterparts in removing challenging HCPs like aspartic proteases, ribosomal subunits etc. thus proving its value in biomanufacturing. We thus show implementation of advanced analytics for separation technology development capable of process intensification.

# CASPON – a platform process for non-platform proteins

**Nico Lingg[1], Daniel Elsner[2],** Cécile Brocard[2], Alois Jungbauer[1]

*[1]Austrian Centre of Industrial Biotechnology, Vienna, Austria*
*[2]Boehringer Ingelheim RCV GmbH &Co KG, Vienna, Austria*

Developing a manufacturing process for microbial biopharmaceuticals is cumbersome. Generally, for a non-mAB NBE that has never been produced before, a manufacturing process must be developed from scratch which is time consuming and costly. Whilst tag-based systems have been used for decades for the production of proteins in academia, they have been largely absent in biopharmaceutical manufacturing due to various limitations.
Here, we present the recently developed CASPONTM technology, which overcomes the limitations of tag-based systems for large-scale GMP manufacturing and reaps the benefits of using a platform for these non-platform proteins. The platform consists of two components: Firstly, a tailored fusion-tag, which increases (soluble) expression levels and contains a hexa-histidine sequence for affinity capture. Secondly, a specifically engineered CASPONTM-enzyme, which is based on human caspase-2 and allows for the removal of the tag after affinity capture generating a native N-terminus of any protein.
The performance of the CASPONTM platform is illustrated on the example of five biopharmaceuticals produced in E.coli, including human growth hormone, granulocyte colony stimulating factor or parathyroid hormone. Yield and impurity concentrations, such as host cell protein, endotoxin and DNA were determined. Furthermore, peptide mapping was conducted for the identification of the co-purified host cell proteins. The presence of the native N-terminal amino acid of the proteins of interest after cleavage was determined by intact mass spectrometry.
Furthermore, the implementation of the technology for large-scale GMP manufacturing is well under way and an outlook for the future and impact of the technology will be given.

# Purification of recombinantly produced Somatostatin-28 comparing hydrochloric acid and polyethylenimine as *E. coli* extraction aids

**Matthias Müller[1],** Martin Gibisch[1], Cecile Brocard[2], Monika Cserjan-Puschmann[1], Rainer Hahn[1]

*[1]University of Natural Resources and Life Sciences. Department of Biotechnology, Institute of Bioprocess Science and Engineering; Muthgasse 18, Vienna, Austria*
*[2]Boehringer Ingelheim RCV GmbH & Co KG, Dr.-Boehringer-Gasse 5-11, Vienna, Austria*

Peptides are used for diagnostics, therapeutics and as antimicrobial agents. The majority of peptides are still produced by chemical synthesis, but recombinant peptide production has recently become an attractive alternative due to the advantages of high titers, less toxic waste and correct folding of tertiary structure. Somatostatin is a peptide hormone that regulates the endocrine system, cell proliferation and inhibits the release of numerous secondary hormones in human body. It is 28 amino acid short and has one disulfide bond, which makes it to an optimal model peptide for a whole downstream purification process. We produced the peptide in the periplasm of E. coli using an affinity fusion technology system based on the permuted human caspase-2 [1]. A show case of two different downstream paths will be presented, both starting either with hydrochloric acid or polyethylenimine as extraction aids. After release of affinity tagged Somatostatin-28 out of E. coli's periplasm, several polishing steps were used, receiving a pure peptide solution in the end. The process was supported by reversed phase and size exclusion HPLC as well as mass spectrometry to monitor the yield and correct disulfide bridge formation. Also, levels of impurities like host cell proteins, DNA and endotoxins were monitored after each downstream unit to elucidate comparison of both purification pathways.

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# A scalable method to purify reflectins from inclusion bodies

Iana Lychko[1], Catia Soares[1], Margarida Dias[1], **Ana Cecilia Roque[1]**

*[1]UCIBIO, Nova School of Science And Technology, Caparica, Portugal*

Structural proteins are an attractive inspiration for functional biobased materials. In nature, cephalopods skin colour modulation is related to the dynamic self-assembly of a family of structural proteins known as reflectins. To fully reach their potential as engineered bio-based materials, reflectins need to be produced by biotechnological means. One of the challenges is associated with establishing and optimizing reflectin purification processes to achieve the highest yield and productivity. Here, we studied purification strategies for two reflectin sequences from different organisms which were recombinantly expressed in a bacterial host at laboratory scale. Reflectins purification was then assessed by two chromatographic and one non-chromatographic methods. Methods were compared considering final purity and yield, productivity, cost and sustainability. The non-chromatographic method based on inclusion bodies washing presented the most promising results (protein purity >90% and purification yields up to 88%). Our results contribute to define bioprocessing strategies to address the vision of biodegradable and sustainable protein-based materials.

# Optofluidic Force Induction (OF2i) a BRAVE new way in time-resolved particle characterization

**Christian Hill**[1,2], Marko Simic[3,2], Christian Neuper[4,2], Ulrich Hohenester[3]

[1]*Medical University of Graz - Biophysics, Graz, Austria*
[2]*BRAVE Analytics GmbH, Graz, Austria*
[3]*University of Graz - Institute of Physics, Graz, Austria*
[4]*Graz Centre for Electron Microscopy, Graz, Austria*

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# Multi-detector Field-Flow Fractionation for quality assessment of nano-sized drug delivery systems

**Roland Drexel**[1], Michaela Mühlbauer[1]

[1]*Postnova Analytics GmbH, Landsberg am Lech, Germany*

The success of mRNA lipid nanoparticles (LNPs) in the battle against Covid-19 has highlighted the potential of nanoparticle drug products. Besides the recent success story of mRNA-LNPs there are several other platforms that are either already well-established or currently under investigation as potent drug delivery systems. [1]
To eventually ensure their safe and efficient use, such delivery systems are described by a set of quality parameters generally summarized as critical quality attributes (CQA). These include e.g. particle size distribution, aggregation behavior, corona formation or payload. There are several analytical tools available to determine these CQA with Field-Flow Fractionation (FFF) among the most promising techniques. [2]
FFF comprises a family of flow-based techniques, where an external force field enables the fractionation of nano-sized sample constituents in suspension. In Asymmetrical Flow FFF (AF4) for example, fractionation by hydrodynamic size is induced by a second flow field while Centrifugal FFF (CF3) fractionates by mass/density. Like in liquid chromatography, FFF can be coupled downstream with multiple detection systems, such as UV/Vis, NTA, MALS and DLS, enabling a comprehensive physico-chemical characterization of nano-sized samples. [3]
We here present applications to determine several CQA of nano-sized drug delivery platforms to underline the potential of multi-detector FFF characterization. In particular, RNA payload determination of LNP-based vaccines, internal structure determination and aggregation behavior of drug delivery systems will be presented and discussed.

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# Novel Approaches for recombinant AAV genome and capsid characterization

**Christoph Gstoettner[1],** Andrei Hutanu[2], Aurelia Raducanu[3], Klaus Richter[4], Markus Haindl[3], Raphael Ruppert[3], Elena Dominguez-Vega[1]

*[1]Leiden University Medical Center, Leiden, Netherlands*
*[2]Hoffman-La Roche, Basel, Switzerland*
*[3]Roche Diagnostics GmbH, Penzberg, Germany*
*[4]Coriolis Pharma GmbH, Planegg, Germany*

Recombinant adeno associated viruses (rAAV) are very promising gene delivery vehicles and are gaining increasing attention to cure genetic diseases. Benefits of rAAVs are the site specific genome integration, their lack of pathogenicity as well as the ability to target specific tissues due to the existence of different serotypes. However, the quality control of these rAAVs are still a major challenge. AAV quality assessment requires monitoring several attributes from the protein capsid and genome. While several analytical technologies, such as analytical ultracentrifugation (AUC), transmission electron microscopy or DNA sequencing have been established, still there are limitations in regard to their accuracy, sample consumption, and user-friendliness. We have developed new chromatographic approaches for the assessment of capsid proteins (VP1, VP2 and VP3) by RP-LC combined with mass spectrometry. We will show that we are able to detect several different proteoforms of these three proteins as well as a clipped variant of VP3. For monitoring of the genome we developed an approach based on ion pairing reverse phase LC (IP-RP-LC) which allowed us to assess the genome integrity. We will show that with our approach we found next to the expected genome also AAVs carrying overfilled species as well as fragments of the genome. Also we were able to assess external DNA arriving from host cells during rAAV production. We confirmed our findings by complementary techniques such as capillary gel electrophoresis or AUC. Furthermore, we will also show that multidimensional approaches offer additional possibilities for automation and reduced sample consumption.

# Process modelling of chromatography of bio-nanoparticles based on linear gradient elution data

**Shuichi Yamamoto**[1]

[1]*Yamaguchi University, Ube, Japan*

Bio-nanoparticles (BNPs) such as viruses, virus-like particles, exosomes, RNAs and DNAs are important as drugs, vaccines or vectors for gene and cell therapy. Although chromatography is needed for purifying BNPs, it is not easy to design the efficient chromatography process due to the large size of BNPs, which lowers the mass transfer rate and the binding capacity significantly compared with proteins.
In this study modelling of ion-exchange chromatography (IEC) of BNPs with monolithic supports and porous particle packed beds was attempted in comparison with IEC of proteins. The model separation system is the separation of empty (E) and full (F) particles of adeno-associated virus (AAV) by IEC. Linear gradient elution (LGE) experiments at various gradient slopes and flow-rates were carried out. The two parameters were obtained by the Yamamoto method (the normalized gradient slope vs. the peak retention salt concentration IR). These data were employed for the numerical simulation of EF particle separations. The IR of AAV particles were much lower than the values for proteins. The number of binding sites of AAV particles were also not so high (<5) compared with the values for proteins.
Optimization of LGE can be carried out by using numerical simulations. Stepwise-elution (SE) and flow-through chromatography (FTC) are preferred for the process chromatography as the operation is simple. We have carried out numerical simulations in order to find the separation protocol where EF particles can be separated efficiently with the reduced solvent consumption and separation time by combining LGE, SE and FTC.

# Advanced Approaches to Gene Therapy Viral Vector Separations

**Rebecca Hochstein[1]**, Alexei Voloshin[1]

*[1]3M, Saint Paul, United States*

Gene therapy is a fast-evolving and promising field. With 3 commercially approved therapies and hundreds in the clinical and pre-clinical stages of development, it is clear that wide deployment of this medical technology is imminent. However, the industry's ability to bring entire pipelines of gene therapies candidates to clinical and commercial space quickly and efficiently hinges on development of scalable and robust manufacturing technology platforms. Currently the manufacturing processes in this space rely on carry-over paradigms and strategies from recombinant proteins and have a number of limitations when it comes to producing drugs based on viral vectors.
Here we present novel approaches to downstream processing of viral vector modalities relevant to gene therapy space, such as adeno-associated virus (AAV). We describe separation and purification of these viral particles using novel functional fibrous media technology. Fiber materials are inherently well suited for large particle separations due to their convective nature and ability to remove diffusional barriers for large particles. This enables us to perform scalable separations over the entire size range of particles, ranging from cells to DNA and host cell proteins (HCPs) all at the same time. Furthermore, we can perform advanced manipulations of the bioprocess stream, such as cell capture and lysis directly on the fiber media without the use of high pressure homogenization or detergent addition, greatly simplifying the recovery and purification process. We believe that these approaches can accelerate industry transition to simple, scalable, and robust manufacturing platforms to bring the benefit of gene therapy to society.

# Development of a robust workflow for purification of a fusogenic oncolytic virus

**Rita Fernandes[1],** Sven Göebel[2], Yvonne Genzel[2], Jennifer Altomonte[3], Cristina Peixoto[1]

*[1]IBET - Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal*
*[2]Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany*
*[3]Department of Internal Medicine II, Klinikum rechts der Isar, Technische Universität München, Munich, Germany*

Oncolytic virotherapy describes the use of oncolytic viruses (OVs) to selectively infect and kill cancerous cells as a treatment against various types of cancer. By utilizing the natural propensity of OVs to exploit various defects in cellular antiviral pathways often present in cancer cells, a direct destruction through oncolysis and an indirect destruction by stimulating the host immune system without harming surrounding healthy cells can be achieved. Actually, the market for OV therapies is expected to evolve in the coming years given the enhanced patient outcomes. However, specific properties of fusogenic OVs, such as the formation of large multinucleated syncytia are hampering their progress in clinics for therapeutic use. Morever,
the manufacturing still presents challenges given the lack of suitable analytics for downstream processing monitoring or characterization of the final product.
In this work, we investigated the development of a GMP-compliant process for a novel chimeric OVs that have shown promising pre-clinical data in both single and combination therapy in various cancer models.Several tools were used for deeper process understanding and monitoring, as Nanoparticle Tracking Analysis, Dynamic Light Scattering and Transmission Electron Microscopy. An innovative approach using Capillary Electrophoresis (CE) with a fluorescence labelling procedure was implemented. This technique enabled the detection of the viral proteins through Sodium dodecyl sulfate-capillary gel electrophoresis (CE-SDS) method coupled to a laser-induced fluorescence detector. The evaluation of the viral vector construction quality was also demonstrated.
Overall, the work performed enabled a fast implementation of GMP-compliant process for a chimeric OV with extensive analytical characterization.

# Functionalized non-woven fibers for purification of large labile enveloped viruses

**Patricia Pereira Aguilar[1,2],** Alois Jungbauer[1,2], Nico Lingg[1,2]

*[1]Austrian Centre of Industrial Biotechnology (acib), Vienna, Austria*
*[2]University of Natural Resources and Life Sciences Vienna (BOKU), Institute of Bioprocess Science and Engineering (IBSE), Vienna, Austria*

Enveloped viruses and virus-like particles have shown their efficacy not only as vaccines, but also as gene and cancer therapy agents. Bioprocessing of this type of bionanoparticles remains challenging, especially in regards to downstream processing, as critical impurities are often co-purified due to their similarities with the product. Host cell derived bionanoparticles, such as extracellular vesicles, and chromatin have a similar size and overall net charge to the product. Moreover, due to the large size of these bionanoparticles (30-600 nm in diameter), conventional chromatography resins have limited available surface area for binding (beads' outer surface only). Functionalized non-woven fibers offer an optimal solution to overcome the low available surface area of conventional resins due to their open structure. Moreover, due to their convective flow properties, non-woven fibers allow for fast purification without mass transfer limitations, increasing process productivity. Here, we present purification of recombinant measles virus in both flow-through and bind/elute modes, using non-woven fibers with anion-exchange functional groups. In both cases high purity and high process yield were achieved. The separation of different particle populations is demonstrated by immune assay, high resolution cryo-electron microscopy and light scattering methods. The non-woven fiber chromatography approach is particularly attractive, as the technology is readily scalable from laboratory to commercial manufacturing scale. Additionally, the fiber matrix can be both sterilized and sanitized using standard industry methods that makes it suitable for bioburden reduced and for aseptic processing, particularly important for large viruses and virus like-particles which cannot be sterilized by 0.22µm filtration.

# Novel affinity ligands for Adenoassociated virus (AAV) and Lentivirus (LV) purification

Eduardo Barbieri[1], Gina Mollica[1], Brandyn Moore[1], Sobhana Sripada[1], Ryan Kilgore[1], Shrirarjun Shastry[1,2], Casee Loudermilk[1], Zachary Whitacre[1], Katie Kilgour[1], Elena Wuestenhagen[3], Annika Aldinger[3], Heiner Graalfs[3], Oliver Rammo[3], Michael Schulte[3], Michael Daniele[4,5], **Stefano Menegatti[1,2,3,5]**

[1]*Chemical and Biomolecular Engineering - NC State University, Raleigh, United States*
[2]*Biomanufacturing Training and Education Center - NC State University, Raleigh, United States*
[3]*Merck KGaA, Darmstadt, Germany*
[4]*Biomedical Engineering - NC State University, Raleigh, United States*
[5]*NC Viral Vector Initiative in Research and Learning (NC-VVIRAL) - NC State University, Raleigh, United States*

With the development of in vivo gene therapies and ex vivo cell therapies, adeno-associated viruses (AAV) and lentiviruses (LVs) have become the key tools in modern medicine. However, the biochemical lability of AAVs and LVs poses critical challenges on downstream processes, leading to high costs and poor transduction activity. Current AAV purification relies on costly affinity resins that mandate harsh elution conditions, while LV purification is based on tangential flow filtration, anion exchange or size exclusion chromatography, both yielding modest recovery and transduction activity, and long process times. Seeking to improve current downstream processes, we sought to develop novel affinity ligands for AAV and LV purification. To this end, we implemented our ligand development device that utilizes multiple fluorescence tagging to identify ligands with bespoke AAV and LV binding strength and selectivity as well as elution conditions. Selected ligands were conjugated to polystyrene-based and polyvinylether-based chromatographic resins and the resulting adsorbents demonstrated excellent AAV binding capacity (> E14 vp per mL of resin) and LV binding capacity (5 E8 – 3 E9 TU per mL of resin) and afford product yield ranging between 40% and 70% upon elution under near-physiological conditions (pH 6 - 7.4). These adsorbents were utilized to purify AAVs and LV from HEK293 harvests achieving high recovery (up to 70%) and reduction of host cell proteins (220-to-770-fold). Notably, these adsorbents demonstrated high resistance to caustic cleaning-in-place conditions (0.5 M NaOH, 30 minutes), affording up to 50 reuses with no observable loss in product yield and quality.

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# FLASH PRESENTATION ABSTRACTS

# Design of affinity separations for the manufacture of *in vitro* transcribed mRNA

**Julian Grinsted**[1], Emir Bouleghlimat[2], Hans Johansson[2], Marco Marques[1], Daniel Bracewell[1]

[1]*Department of Biochemical Engineering, University College London, London, United Kingdom*
[2]*Purolite Ltd., Llantrisant, United Kingdom*

mRNA has recently emerged as a drug substance with applications as both a therapeutic and vaccine. The manufacture of mRNA uses a cell-free process known as in vitro transcription (IVT). IVT is versatile and allows manufacturing facilities to be rapidly repurposed. However, generation of product and process-related impurities, such as dsRNA, during IVT necessitates downstream separation processes such as chromatography. Affinity chromatography is a valuable tool for removal of bulk impurities from mRNA, being well suited as a primary-capture step. The technique uses immobilised Oligo deoxythymidine (oligo-dT) ligand chemistries to form base pairs with the mRNA's poly(A) tail. This captures the mRNA, whilst impurities flow through the column. mRNA can then be eluted by simply reducing the buffer salt concentration. Whilst oligo-(dT) is a specific and simple separation mode, substantial improvements in binding kinetics and capacity are desirable.
We therefore aimed to characterise and enhance the performance of beaded oligo-(dT) separations for mRNA purification. This involved investigating the impact of bead and ligand structure on capacity and kinetics. Jetted divinyl benzene beads functionalised with oligo-(dT) were screened. Ligand and spacer length were varied between resins. 18-25nt oligo-(dT) mounted on 1-40kDa dextran spacers was challenged with >200nt poly(A) to determine binding capacity and breakthrough steepness. We observed a greater than fourfold change in dynamic binding capacity between resins. Additionally, confocal microscopy was used to measure intrusion of poly(A) into beads. Our findings provide a platform for beaded affinity resin development and contribute to growing literature on mRNA purification.

# Increased Virus-Like Particle Recovery with Disassembly Prior to Purification

**Nick Samuelson[1],** Michael Winters[1], Murphy Poplyk[1], Randy Soukup[1]

*[1]MSD, West Point, United States*

Human papillomavirus (HPV) vaccines are comprised of virus-like particles (VLPs) from the HPV L1 capsid protein. L1 proteins are recombinantly expressed in Saccharomyces cerevisiae where they self-assemble into heterogeneous, irregularly shaped VLPs. Current purification approaches utilize cation-exchange and hydroxyapatite chromatography in bind-and-elute mode to purify VLPs from the clarified cell lysate. The purified, heterogenous VLPs are subsequently disassembled to VLP subunits, such as L1 monomers and capsomeres, using reducing agents and re-assembled via diafiltration in tangential flow filtration steps to form stable and immunogenic VLPs. An alternative approach was developed in which disassembly was performed prior to purification and the VLP subunits were purified through chromatography under reducing conditions. Due to differences in binding characteristics between the L1 subunits and VLPs, optimization of mobile phase and chromatography operations were required, such as the use of hydroxyapatite in a flowthrough mode of operation. The purification process with disassembled L1 protein led to a significant increase in L1 yield with comparable purity to the current approach. The purified, disassembled material was re-assembled via TFF buffer exchange and had comparable product attributes to the VLP purification process.

# Process Intensification in the Purification of an Oligonucleotide Sequence by MCSGP with UV-Based Dynamic Control

**Ismaele Fioretti[1],** Lars Aumann[2], Thomas Müller-Späth[2], Mattia Sponchioni[1], Massimo Morbidelli[1]

[1]*Politecnico di Milano, Milan, Italy*
[2]*ChromaCon - A YMC company, Zurich, Switzerland*

Oligonucleotides (ONs) are shooting up in the biopharmaceutical industry as a promising class of biotherapeutics, due to their ability to regulate the gene expression. The complexity in the purification of the product from molecularly similar impurities is preventing the ON large scale manufacturing at affordable costs. Multicolumn countercurrent solvent gradient purification (MCSGP) has been demonstrated a valuable process to break up the purity-yield tradeoff typical of batch operations and to increase the productivity at comparable purities. However, the optimization of the process parameters characterizing the MCSGP of ONs can be labor-intensive, the resulting set points could be poorly robust, and fluctuations arising at different levels when conducting a continuous process for long time may lead to deviations of the product quality from the specification.
In this work, we demonstrate how these limitations can be overcome by applying a UV-based dynamic control strategy, i.e. AutoPeak®, to the design and conduction of the MCSGP for a 20mer single-stranded DNA sequence. AutoPeak® automatically regulates the extension of the recycling and collection windows of the MCSGP cycle by cycle leveraging the UV signal recorded on-line. By application of this dynamic controller, the cyclic steady state can be reached faster and maintained more easily compared to the equivalent, non-regulated process. In addition, considering a 96% purity specification, it was possible in few runs to improve the starting batch yield of only 10% up to >95% in the continuous operation. The performance improved as well in terms of productivity and buffer reduction, by more than 2-fold.

# Automated two-column chromatography for the purification of Oligonucleotides and Peptides

**Thomas Müller-Späth[1],** Giulio Lievore[1], Richard Weldon[1], Sebastian Vogg[1], Lars Aumann[1]

[1]*Chromacon AG, Zurich, Switzerland*

Oligonucleotides and peptides have gained growing importance as therapeutics in recent years due to their unique properties and potential for treating a wide range of diseases.
The increased demand requires adequate production technologies, predominantly in downstream processing.
Twin-column chromatography (MCSGP) is a powerful technique for the purification of oligonucleotides and peptides. It utilizes the concept of internal recycling of impure side fractions eluting from a first column, and re-adsorbing them on a second column with the aid of inline dilution. MCSGP therefore improves yield, while eliminating the need for side-fraction handling and re-chromatography, thereby providing a sustainable manufacturing alternative. In recent years the technology has been scaled up for production under GMP.
While MCSGP uses two columns of the same type and dimensions, process intensification can already be achieved by improving "traditional" single column chromatography through process integration and automation. By operating two chromatography steps in series and avoiding an intermediate hold step, higher process throughput can be achieved.
The optimization of abovementioned two-column techniques can be accelerated significantly by use of mechanistic modelling.
In this presentation we show latest results of the application of two-column continuous chromatography technologies to oligonucleotides and peptides.
Besides a brief introduction to the process concepts, process modeling results are shown to complete the case studies.

# Optimizing chromatography for maximum efficiency: an innovative approach to optimize productivity, resin utilization, and buffer consumption

**Touraj Eslami[1,2,3],** Nico Lingg[1,2], Alois Jungbauer[1,2]

[1]*acib Gmbh, Graz, Austria*
[2]*University of Natural Resources and Life Sciences, Wien, Austria*
[3]*evon GmbH, St. Ruprecht an der Raab, Austria*

In preparative and industrial chromatography, balancing productivity, column utilization, and buffer consumption are crucial for the overall economy of the process. The dynamic binding capacity governs the process economy and is influenced by the aging of chromatography columns. This study presents a new online optimization approach for capture chromatography that employs a residence time gradient during loading to improve the trade-off between productivity and resin utilization. The extended Kalman filter serves as a soft sensor for product concentration, and the model predictive controller, using the pore diffusion model as a simple mechanistic model, performs online optimization to maximize productivity and resin utilization while accounting for varying feed concentrations. The proposed optimization approach was experimentally validated and demonstrated to be effective in saving up to 43% of the buffer while simultaneously improving productivity and resin utilization. The outcome of this approach is comparable to a multi-column continuous counter-current loading process but with less hardware complexity. It offers a stable and efficient solution for a long-term operation that takes resin aging into consideration. In addition, this approach surpasses the limits of traditional chromatography by demonstrating the possibility of increasing productivity beyond the feasible range while enhancing resin utilization and buffer consumption.

# Enhancing chromatography by use of electrochemically modulated membranes

**Dennis Röcker[1],** Paula Fraga-García[1], Sebastian Schwaminger[1,2], Sonja Berensmeier[1]

*[1]Technical University of Munich, Garching, Germany*
*[2]Medical University of Graz, Graz, Austria*

Ion-exchange chromatography is one of the most popular methods for the separation of biomolecules. However, it requires large amounts of highly saline elution media to separate the desired target species, resulting in strong waste streams and cost-intensive processes. As a unique opportunity for more cost-efficient and sustainable processing, we present results of an electrochemically modulated membrane chromatography process using gold-coated membranes developed and supplied by i3 Membrane.
We have designed and constructed a laterally fed device, acting not only as chromatographic capsule, but also as an electrochemical cell. Within this device, the membrane electrodes function as flow-through working electrode. Hence, by applying an electrical potential, the conductive membranes can be modulated in their surface charge and consequently their binding behavior towards charged molecules. In potential-controlled flow-through experiments, we have established a proof of principle for the binding and elution of maleic acid as charged model analyte. While a positive applied potential increases the binding capacity of the membranes towards the negatively charged analyte, a potential switch results in strong electrostatic repulsion and triggers the elution of the bound target. Thus, by modulating the applied potential in adsorption and desorption phase we can tailor the elution efficiency and peak symmetry. However, numerous questions concerning the response of the novel electrodes under potential remain open. Consequently, we currently work on the investigation of the complex interplay between material properties, fluid dynamics and the response of the membrane stationary phase under potential.

# Peptide ligands: a bespoke affinity platform for next-generation biotherapeutics and gene-editing products

**Ryan Kilgore[1],** Wenning Chu[1], Brandyn Moore[1], Sobhana Sripada[1], Eduardo Barbieri[1], Shriarjun Shastry[1,2], Yuxuan Wu[1], Dipendra Bhandari[3], David Fischler[3], Michael Crapanzano[3], Ruben Carbonell[1,2], Stefano Menegatti[1,2,3]

*[1]Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, United States*
*[2]Biomanufacturing Training and Education Center (BTEC), North Carolina State University, Raleigh, United States*
*[3]LigaTrap Technologies, Raleigh, United States*

Next-generation biotherapeutics – including engineered antibody fragments, gene-editing nucleases, and gene-delivery vectors – form a diverse and exciting family of products that give us a glimpse into the future of medicine. Despite their promise, these products face critical manufacturing challenges due to immature purification processes. Addressing this issue, our team has established a toolbox for the rapid development of affinity peptide ligands with bespoke biorecognition activity. The centerpiece of this technology is a library screening device that yields ligands with high selectivity and capacity, and the ability to release the target at the desired elution conditions. We demonstrated this technology using three products of increasing complexity. First, we present peptide ligands for the purification of therapeutic monoclonal Fab kappa and lambda, affording high yields and purities under mild elution (~95% and 100-fold removal of HCPs at pH > 4): these results, along with high dynamic binding capacity (DBC10% ~16 mg/mL), place these ligands on par with commercial affinity technologies. Second, we present a set of ligands with high capacity for CRISPR-Cas ribonucleoproteins (Cas9 and Cas12a), affording yield ~60-80% and high clearance of E. coli contaminants. Recently, we introduced peptide ligands for the universal purification of exosomes from different sources (human plasma and HEK293, COLO-1, U87, MM1, and PC3 cell lines), achieving yields ~80% and >100-fold removal of HCPs upon mild elution (moderate conductivity and physiological-to-basic pH). Collectively, these results demonstrate the renewed promise of peptides as affinity ligands for the affordable and flexible downstream bioprocessing of latest-generation, high-value biotherapeutics.

# Implementation of polyHIPE monoliths for preparative and analytical separation of bacteriophages and their genomic DNA

**Staš Vrh[1,2],** Rok Mravljak[1], Aleš Podgornik[1,3]

[1]*Faculty for Chemistry And Chemical Technology, University Of Ljubljana, Ljubljana, Slovenia*
[2]*JAFRAL, Ljubljana, Slovenia*
[3]*COBIK, Ajdovščina, Slovenia*

Bacteriophages are viruses that specifically infect bacteria, and offer a promising alternative to conventional antibiotic treatment. Phage-based control of pathogens is already established in the agricultural and food sectors and their potential applications are expanding. With the increasing prevalence of antibiotic resistance and the development of new applications, the demand for bacteriophage production is expected to increase in the future. Downstream processes of phage production generally use methods with low efficiency and poor scalability, which hinders their large-scale production. The development of monolithic chromatographic supports has allowed for the introduction of chromatography into the downstream processing and analysis of bacteriophages. PolyHIPE (Polymerized High Internal Phase Emulsion) are a new class of monolithic chromatographic supports that have not yet been tested for phage separation. Therefore we investigated the suitability of differently functionalized PolyHIPE chromatographic columns for phage and their genomic DNA separation and optimized the chromatographic method. PolyHIPE chromatographic columns that were functionalized with quaternary amines were utilized to achieve the separation of T4 and T7 phage as well as their genomic DNA from phage lysate. The pressure drop remained stable, even after several consecutive cycles of binding and elution. The optimized chromatographic method was then used to analyse the composition of phage lysate and to monitor its changing during phage cultivation. Our findings suggest that employed chromatographic method is appropriate for the in-process control of phage cultivation and that PolyHIPE can offer a promising alternative to conventional chromatographic supports used for the large-scale bacteriophage production.

# Purification and characterization of recombinant secretory immunoglobulin A from CHO cell culture supernatant

**David Scheich[1],** Diogo Ferreira-Faria[2], Maria Drdić[1], Andres Männik[3], Rainer Hahn[1], Alois Jungbauer[1,4], Nico Lingg[1,4]

[1]*University of Natural Resources and Life Sciences Vienna, Vienna, Austria*
[2]*Universidade de Lisboa, Lisbon, Portugal*
[3]*Icosagen Cell Factory, Tartu maakond, Estonia*
[4]*Austrian Centre of Industrial Biotechnology , Vienna, Austria*

Secretory immunoglobulin A (sIgA) acts as a first line of defense in the mucosal immune system. Isolation of sIgA from whey using established unit operations in protein purification has already been demonstrated. While recombinant sIgA would have countless therapeutic applications, its implementation into clinical practice has been hindered by its low manufacturability. Unlike IgG, no platform process is available for this promising future biopharmaceutical.
Here we present an overview of the current status of sIgA platform DSP. An initial affinity purification step with CaptureSelect IgA-CH1 was utilized to recover sIgA from the clarified cell culture supernatant. At approximately 460kDa, the high molecular weight of sIgA limits pore access for the agarose-based CaptureSelect resin, resulting in a highly mass transfer limited process. Monomeric and dimeric IgA molecules co-eluted as the main impurities of the capture step. Ion-exchange chromatography (IEX) resins were screened for applicability in the polishing step. Macroporous resins (POROS 50, Uno Sphere) enables the traditional bind and elute chromatography mode for large molecules. Particles with small pores (Sepharose-based) restrict access for sIgA, commendable to flow-through purification. Elution of bind-elute IEX was optimized based on the Yamamoto model. Purified sIgA fractions contain a mixture of molecules with different glycosylation patterns, which influence the antibody effector functions. We established in-vitro assays for the characterization of the effector functions using surface plasmon resonance.

# Cationic flocculants assisted clarification

**Anna-Carina Frank[1],** Daniel Komuczki[1], Clara Hofmann[2], Natalie Deiringer[2], Felicitas Guth[2], Elisabeth Grünstein[2], Rainer Hahn[1], Peter Satzer[1]

*[1]University of Natural Resources and Life Sciences, Institute of Bioprocess Science and Engineering, Vienna, Austria*
*[2]BASF SE, Ludwigshafen, Germany*

Intensification of cell culture-based unit operations such as fed-batch processes of monoclonal antibodies using Chinese hamster ovary cells leads to higher cell densities and high product titers. The high concentration of biomass significantly increases the burden on clarification steps such as depth filtration. In particular, depth filtration becomes less economically and technically feasible as substantially more filter area is required. It is known that specific cationic flocculants facilitate the separation of cells and the removal of host cell derived impurities. Although they have been extensively used before, a study on the correlation between these cationic flocculants and their removal potential of industrially relevant critical host cell proteins (HCP) has not yet been conducted. We therefore established fingerprints of various flocculants on their impurity removal potential by analysis of residual HCP profiles using proteomics.

# Chromatography-based purification of enveloped virus-like particles displaying different influenza surface antigens for an immunologic study in mice

**Alexander Zollner[1],** Leticia Guzman Ruiz[1], Reingard Grabherr[1], Florian Krammer[2], Alois Jungbauer[1,3], Patricia Pereira Aguilar[3,1]

*[1]University of Natural Resources And Life Sciences, Vienna, Vienna, Austria*
*[2]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, USA*
*[3]Austrian Centre of Industrial Biotechnology (acib), Vienna, Austria*

Enveloped virus-like particles (eVLPs) are interesting candidates for next-generation, quickly adaptable vaccines against seasonal viruses like influenza. The Baculovirus expression vector system (BEVS) can be easily used to produce eVLPs based on the HIV-1 gag protein displaying different Influenza surface antigens. Still, purification processes remain challenging due to a wide range of co-produced impurities, such as host cell-derived proteins, dsDNA and bionanoparticles (e.g., extracellular vesicles and exosomes) as well as baculovirus particles. We established a platform downstream process for eVLPs independent of the displayed surface antigen. The process is based on two chromatographic unit operations: core-shell beads flow-through chromatography (using Capto Core 700) to remove small impurities like host cell-derived proteins and dsDNA followed by Heparin affinity chromatography (Capto Heparin) to separate different particle populations. Additionally, an endonuclease digestion step using M-SAN was applied to lower the dsDNA content to a level acceptable for medical application. Final formulation and buffer exchange was achieved via ultracentrifugation. Via this process, we purified five different eVLP constructs displaying different/combinations of Influenza surface antigens (Hemagglutinin and Neuraminidase) and a naked VLP control sample. The purified and concentrated eVLPs were used in an immunologic study with mice to investigate immune responses and evaluate the influence of the different antigens or combinations in the immune response to be used as future influenza vaccine candidates.

# Baculovirus working stock: the production and purification of an intermediate product for large scale VLP production in insect cells

**Lena Achleitner[1,2],** Patricia Aguilar[1,2], Nico Lingg[1,2], Peter Satzer[1,2], Alois Jungbauer[1,2]

[1]*acib – Austrian Centre of Industrial Biotechnology, Vienna, Austria*
[2]*Department of Biotechnology, Institute of Bioprocess Engineering, University of Natural Resources and Life Sciences, Vienna, Austria*

Large quantities of baculovirus are required for efficient virus-like particle (VLP) production using the baculovirus expression vector system. These so-called baculovirus working stocks are generally only clarified before being used for cell infection for VLP production. This results in the transfer of various impurities from the baculovirus production into the VLP production. Here we present our results on a baculovirus production process with low levels of impurities and a chromatographic purification of baculovirus using non-woven fibres in a bind-elute mode. Functionalised fibres have the advantage of providing large accessible surface area for adsorption of large molecules such as baculoviruses. Moreover, these fibres have the additional advantage over chromatography beads of being easily steam-sterilisable. The latter is of utmost importance, as sterile baculoviruses are needed for infecting cells for VLP production and sterile filtration cannot be performed due to drastic titer losses. A bind-elute method can remove host cell proteins in the flowthrough and concentrate the baculovirus in the elution. This enables a stable VLP production as concentration differences of impurities and titer fluctuations can be prevented and the baculovirus stock has constant quality. We investigate the effect of varying ligand chemistry in linear and step gradient elution mode on infectious recoveries. Furthermore, the influence of baculovirus stock quality on VLP production was investigated.

# Uncovering the black-box of data-driven models in biotechnological process modeling

**Matthias Medl[1],** Theresa Scharl[1], Friedrich Leisch[1], Astrid Dürauer[2]

*[1]University of Natural Resources and Life Sciences, Institute of Statistics, Vienna, Austria*
*[2]University of Natural Resources and Life Sciences, Institute of Bioprocess Science and Engineering, Vienna, Austria*

The viability of data-driven models for the monitoring of critical process parameters of biotechnological processes has been evaluated and proven multiple times. However, one common concern with data-driven models is that they are seen as black-boxes which do not provide process understanding and that their inner workings are often poorly understood. This lack of understanding is problematic as (i) it can be difficult to predict how the model will behave when it encounters data different from training data, (ii) scientists lose out on the opportunity to deepen their process knowledge and (iii) it is unclear whether the model learns real or spurious correlations. In this study we show how model explainability methods can be employed to gain understanding on the inner workings of highly complex data driven models – in this case deep learning ensembles – and thus how the aforementioned problems can be overcome. Therefore, deep learning ensembles were first trained to predict critical process parameters during protein A capture chromatography in real time and then both the permutation and the occlusion feature importance were computed for the models. The feature importances were then used to verify whether the models' learnings agreed with first principles knowledge. Furthermore, we present a workflow on how to investigate the influence of sensor fouling and failure on the model predictions. We believe that the presented methodologies or similar ones play an essential role in the safe deployment of data-driven models in biopharmaceutical manufacturing.

# Selective antibody capture using low-cost magnetic particles in an automated high-gradient magnetic separator

**Ines Zimmermann[1],** Marko Tesanovic[1], Friederike Eilts[1], Sonja Berensmeier[1]

[1]*Technical University of Munich, Garching, Germany*

Packed-bed chromatography is a main bottleneck in biopharmaceutical DSP regarding throughput, scalability, and cost-efficiency. A promising alternative is magnetic separation using non-porous, superparamagnetic particles as the adsorbent matrix, characterized by low mass transfer limitations, fast adsorption kinetics, and easy scalability. Therefore, we synthesized magnetic nanoparticles (MNPs) via a simple co-precipitation reaction and immobilized innovative Protein A-derived affinity ligands on their surface using covalent and non-covalent mechanisms. One type of functionalization was performed site-oriented on the bare MNPs with a designed MNP-binding tag, which binds by simple contacting without elaborate chemical modifications.

Based on systematic small-scale characterization, an automated pilot-scale high-gradient magnetic separation (HGMS) process was developed and performed. The process included (1) MNP functionalization, (2) adsorption of antibodies, (3) washing of MNPs, (4) elution of antibodies, and (5) MNP recovery for following process cycles.

In a process with 9.7 g MNP@ligand, around 4.4 L (0.47 g/L IgG) of CHO cell culture supernatant were purified with an overall antibody yield of 85 % and purities of 97 %, indicating a successful application. Online sensors (UV absorbance, pH, temperature, conductivity, flow) enabled process control as a unique process analytical technology (PAT) feature of our HGMS system. Model-based process optimization is currently done in our group as part of the development of a digital HGMS twin (work by M. Tesanovic).

Our work shows that the functionalization of cheap MNPs with state-of-the-art affinity ligands in combination with a PAT-equipped and cGMP-compliant HGMS device could contribute to overcoming current bottlenecks in DSP.

# Mild purification of antibody fragments from human and mouse origin

**Malin Jönsson[1],** Julia Scheffel[1], Sara Kanje[1], Magnus Wolf-Watz[2], Sophia Hober[1]

*[1]KTH Royal Institute of Technology, Stockholm, Sweden*
*[2]Umeå University, Umeå, Sweden*

Monoclonal antibodies of human or mouse origin are used as detection reagents, therapeutics and for diagnostics of a variety of diseases, and novel antibodies are continuously being developed. This ever-expanding monoclonal antibody market is today restricted by manufacturability requirements such as the harsh acidic conditions applied during protein purification. Although the gold standard for antibody purification – utilizing Protein A or Protein G affinity chromatography – results in great product yields after a selective capture of the antibody, the sequential highly acidic elution step poses a threat to the product quality. Antibodies and Fc-fusion proteins risk forming aggregates, as a consequence of the low pH required, compromising the potency and safety as well as limiting the development of new therapeutics to those that can withstand these acidic conditions. Here, we present a mild purification strategy based on our development of novel calcium-dependent ligands enabling elution of antibodies or antibody fragments from human and mouse origin at neutral pH. Through a semi-rational design combined with directed evolution, we have been able to engineer calcium-dependency into domains derived both from Protein A and Protein G rendering the inherent binding to Fab or Fc regulated by the presence or absence of calcium. Further, the molecular mechanism underlying the calcium switch has been investigated by analysing protein structure and dynamics with NMR spectroscopy. The removal of calcium breaks the protein-protein interaction, eluting the antibody or antibody fragment without the need for applying acidic conditions, enabling future development of safer and more functional antibody therapies.

# Precipitation of monoclonal antibodies with polyethylene glycol and zinc chloride: process performance and rheological behavior

Ana Carolina Moreno Pássaro[1], Amanda C. Barcellos Leite[1], Caio N. Almeida[1], Luis A. Peroni[2], Iara R.A Pereira Bresolin[1], **Igor Bresolin[1]**

[1]*Federal University of São Paulo (Unifesp), Diadema, Brazil*
[2]*Rheabiotech Anticorpo Ltda, Campinas, Brazil*

Monoclonal antibodies (mAbs) have become fundamental in several areas of study, such as clinical and diagnostics, being able to recognize analytes in complex mixtures and contribute to the development of detection devices, such as rapid serological tests. These tests utilize anti-human IgG mAbs that work as capture reagents during the detection of polyclonal IgG in human blood. Therefore, we aimed to study the precipitation technique combining polyethylene glycol (PEG) and ZnCl2 as a non-chromatographic capture alternative for non-therapeutic mAbs that still require high purity levels, evaluating the resulting rheological conditions as well. Experiments were carried out using PEG6000 (0 to 16% m/v) and ZnCl2 (0 to 3 mM) as precipitating agents to purify anti-human IgG mice IgG1 mAbs from cell culture supernatant produced by hybridomas, determining its solubility curves and the most promising conditions for batch precipitation and precipitate resolubilization. A viscosity analysis for the resolubilized antibodies and precipitation supernatant was also performed. Antibodies concentration was evaluated through HPLC analytical protein A affinity chromatography, whereas precipitates' purity was estimated through HPLC size exclusion chromatography. Experiments resulted in a precipitate with 100% yield and 59% purity, obtained by precipitation with 12% PEG6000 and 3 mM ZnCl2. Viscosity analysis indicated a Newtonian behavior for PEG-only solutions, whereas the addition of ZnCl2 led to a shear thinning behavior for both supernatant and resolubilized precipitate (dynamic viscosity of 2.41 mPa s). Considering the results, the use of pre-concentration methods is recommended in order to further decrease the required PEG concentration and increase precipitate's purity.

# 3D Printed Stationary Phases: The Future of Chromatography?

**Daria Omralinov**[1], Jan Gläsel[1], Bastian J.M. Etzold[1]

*[1]TU Darmstadt, Darmstadt, Germany*

Looking at today's demand for highly customized devices and materials in separation science, the need for simple, quick and cost-effective manufacturing techniques becomes evident. The state-of-the-art in chromatography are spherical beads, produced for example by suspension polymerization and used as randomly organized chromatographic beds. Computer simulations have shown that the use of ordered structures can improve the performance of chromatographic operations. Three-dimensional (3D) printing of chromatography materials would thus enable a new advantageous way in production of precisely ordered and custom tailored chromatographic stationary phases. Furthermore, 3D printing would allow to print reproducible chromatography columns as single-piece devices including the packing, the internal flow distributors as well as the external fluid connectors.
However, to realize the vision of 3D printed chromatography columns, several challenges need to be overcome. The achievable resolution has to be improved, selective chemical surface functionalization must be realized, a secondary porosity might be necessary and sufficient mechanical and chemical stability is needed.
The aim of this work is to overcome current limitations for 3D printing employing photopolymerization approaches by identifying a specialized photo resin formulation suitable for chromatography applications. Here, a methacrylate-based photo resin with a modifiable functional hydroxyl-group was developed. This allowed the printing of colorless and hydrophilic chromatography columns with ordered open-cell monolithic structures down to 150 µm channels. In addition, the 3D-printed polymers were successfully post-modified using various functionalization methods to generate ion-exchange properties. The first application tests showed successful binding and elution of model proteins.

# Production of bioactive recombinant monoclonal antibody fragment in periplasm of *E. coli* expression system

**Preeti Saroha[1],** Anurag Rathore[1]

[1]*Department of Chemical Engineering, IIT Delhi, New Delhi, India*

The microbial expression system is the most widely studied host for the production of biotherapeutic products such as antibody fragments, single chain variable fragments and nanobodies. However, recombinant biotherapeutic proteins are often expressed as insoluble proteins, thereby limiting the utility of E.coli as expression system. To overcome this limitation, various strategies have been developed such as changes at DNA level codon optimization, fusion with soluble tags and variations in process parameters includes temperature, and inducer concentration. However, there is no one size fits all strategy. The most commonly used approach involves induction at low temperature, as reducing the temperature during cultivation has been reported to increase bioactive protein production in E.coli. In this study, we examine the impact of various process parameters such as temperature and inducer concentration, as well as, high plasmid copy number vector for achieving enhanced soluble expression of TNFα inhibitor Fab. An interaction amongst these parameters has been observed and their optimization has been demonstrated to result in expression of 30 ±3 mg/L antibody fragment using E.coli. This case study illustrates how process optimization can contribute towards making biotherapeutics affordable.

# Single-pass diafiltration using a double-membrane module

Tomas Kurak[1], Shahla Shahriari[1], **Milan Polakovic[1]**

[1]*Slovak University of Technology in Bratislava, Bratislava, Slovakia*

Single-pass tangential flow filtration (SPTFF) is a process combining continuous diafiltration and concentration in a series of membranes without retentate recirculation. The disadvantage of current SPTFF systems is the higher consumption of diafiltration buffer compared to traditional batch diafiltration. In this work, a novel SPTFF membrane module was used which allows simultaneous concentration of retained molecules (proteins) and reduction of unretained molecules (salts) or buffer exchange. The membrane cassette consists of three channels separated by two membranes. The membranes separate the feed-retentate channel and either the diafiltration buffer channel or the permeate channel. The diafiltration buffer is pumped to a closed channel and passes along the entire length of the membrane to the feed-retentate channel. The degree of salt reduction depends on the buffer/feed ratio (diavolumes). Protein concentration can be controlled by the feed/retentate ratio. The permeate containing the removed salts passes through the membrane to the permeate channel equipped only with an outlet port.
The effect of diavolumes and concentration factor on the degree of salt reduction was investigated experimentally using a model feed solution containing BSA as the model protein and NaCl as the model low-molecular-weight impurity. A 99% salt reduction was achieved with 5 diavolumes similarly as in batch diafiltration. With a simultaneous 5-fold concentration, the consumption of diafiltration buffer dropped to only 3 diavolumes. A developed mathematical model of the membrane module demonstrated good prediction potential for process design, optimization and scale-up.
Acknowledgements: The support of the grants VEGA 1/0515/22 and APVV-21-0321 is acknowledged.

# Determination of immobilized proteins via pH transition method

**Ales Podgornik**[1], Rok Ambrozic[1], Rok Mravljak[1]

[1]*Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia, Ljubljana, Slovenia*

Immobilized proteins are one of the most important affinity ligands. Despite that, there is no simple method that allows direct, non-invasive detection. Presented method is based on the pH transition, forming during change of solution ionic strength. The method utilizes the ionic character of the immobilized protein, while implementing biologically compatible buffers. Proteins glucose oxidase, horseradish peroxidase, bovine serum albumin, lysozyme and protein A were immobilized in different amounts on a porous polymeric matrix and their pH transition was measured using lactate buffer of various concentrations and pH values. A linear correlation was found between the amount of immobilized protein and the amplitude of the pH transition, allowing the detection down to 2 nanomoles of immobilized protein. By changing the buffer concentration and pH, the sensitivity of the method can be tailored. Results were described by a mathematical model based solely on protein amino acid sequence, buffer pKa value(s) and amount of immobilized protein. Since the proposed method is non-invasive, it can be routinely applied during optimization of immobilization protocol, for quality control, but also as an in-process monitoring tool.

# A Peroxidase from Inclusion Bodies as valuable Tool in Breast Cancer Treatment

**Oliver Spadiut[1]**

*[1]TU Wien, Wien, Austria*

The omnipresent enzyme Horseradish peroxidase (HRP) is still produced from hairy root cultures in a time-consuming and inefficient way. I will present a novel and scalable recombinant HRP production and purification process in Escherichia coli that yields a highly pure, active and homogeneous single isoenzyme. We successfully developed a multi-step inclusion body process giving a final yield of 960 mg active HRP/L culture medium with a purity of ≥ 99 %. Our preparation of recombinant, unglycosylated HRP from E. coli is a viable alternative to the enzyme from plant and highly interesting for therapeutic applications.

# Accelerating Virus-Like Particle Downstream Process Development Using Asymmetric Flow Field-Flow Fractionation (AF4)

**Leo Jakob[1,2],** Catarina Maia[2], Florian Steiner[2], Alexander Zollner[2], Alois Jungbauer[1,2], Patricia Pereira Aguilar[1,2]

[1]*acib GmbH, Wien, Austria*
[2]*Institute of Bioprocess Science and Engineering, BOKU, Wien, Austria*

In the process development of viral or virus-like particle products, a range of analytical techniques must be employed to determine specific product titer, particle concentration, and contaminant concentration. Compared to recombinant protein production, the analytical workload is substantial, representing a major bottleneck in the development of these processes. Asymmetric flow field-flow fractionation (AF4) presents a promising analytical method that could enhance the current analytical portfolio, potentially replacing more time-consuming techniques or automating labor-intensive ones. Equipped with UV, multi-angle light scattering, and quasi-elastic light scattering detectors, a single separation run provides information about small component impurities, particle shape, particle radii, and size distribution. As previously demonstrated for various bionanoparticles, we successfully separated the small components fraction (proteins, DNA, etc.) from the particle fraction. This separation facilitates protein and DNA quantification of selected fractions and characterization of bionanoparticles. Furthermore, light scattering data shows a very good correlation with particle concentrations determined by nanoparticle tracking analysis, a technique that is time-consuming and requires operator supervision. For at-line analytics of an HIV-1 gag VLP purification run, labor hours were reduced by more than 10-fold when employing AF4 for particle concentration determination compared to nanoparticle tracking analysis measurements. In the course of the analysis, size distribution and shape ratios of the particles were determined along with the analysis of small components such as proteins and DNA. Ultimately, asymmetric flow field-flow fractionation streamlines downstream process development by combining particle concentration determination and monitoring of small impurities in a single, readily automatable technique.

# Anion exchange chromatography for extracellular vesicles purification

**Ricardo Silva**[1], Sara Rosa, Cláudia Silva, Ana Azevedo, Ana Fernandes-Platzgummer

[1]*iBB - Institute for Bioengineering and Biosciences, Lisboa, Portugal*

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# Downstream Process Development for intact Virus-Like Particles (VLPs) from yeast expression system *Pichia pastoris*

Pragya Prakash[1], **Rashmi Sharma[1],** Anurag Singh Rathore[1]

[1]*Indian Institute of Technology Delhi, New Delhi, India*

Virus-Like Particles (VLPs) have emerged as a potential alternative in the field of biotherapeutics. Till date, VLPs have been expressed in both prokaryotic (bacterial) as well as eukaryotic (mammalian, yeast, insect, plant) expression systems (ESs). Most ESs produce different VLP components which are later assembled into complete VLPs. The present work establishes a novel single step chromatographic method for the purification of intact VLPs from P. pastoris. A purification strategy has been proposed wherein high relative yield of ~50% and overall yield of ~11% was achieved. The yield achieved is considerably higher as compared to existing methods. The purified capsomeres are then reassembled and formulated for a substantial reduction in HCPs (ppm) & HCD (ppm) content, within and around the intact drug molecule. Also, several perspective w.r.t to product loss in downstream processing have been discussed with emphasis on truncated VLP as prime CQA. The purified VLPs were characterized using SDS-PAGE, Western Blot, Size-Exclusion Chromatography (SEC) and Peptide Sequencing techniques. The molecular weight of VLP monomer and pentamer were observed as 56 kDa and 280 kDa respectively in SEC. The single step process obtained will ensure cost reduction as well as product loss mitigation thus paving way towards robust, cheaper, effective and economically feasible VLP platform technology. Additionally, it will propel the exploration of yeast ESs for biotherapeutics synthesis, development and usage.

Keywords: Biotherapeutics, chromatographic, downstream, Pichia pastoris, purification, VLP

# Downstream processing of non-viral protein nanocages for biotechnological and biomedical applications: development of chromatography-based purification strategies

**Jorge João**[1,2], Sara Sousa Rosa[1,2], Pedro M R Paulo[3], Ana M Azevedo[1,2], Duarte Miguel F Prazeres[1,2]

*[1]iBB – Institute for Bioengineering and Biosciences and Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal*
*[2]Associate Laboratory i4HB – Institute for Health and Bioeconomy at Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal*
*[3]Centro de Química Estrutural, Institute of Molecular Sciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal*

Non-viral protein nanocages (NVPN) are highly ordered nanometer scale architectures, which are typically formed by homo- or hetero-self-assembly of multiple monomers into symmetric structures with different dimensions and morphologies. Due to their very attractive intrinsic characteristics, several applications have been implemented such as drug delivery, vaccine development, bioimaging, biomineralization, nanomaterial synthesis and biocatalysis. In particular, biotechnological and biomedical applications have been extensively investigated, showing that protein nanocages can be a promising and an interesting tool. The ability to generate large amounts of pure and well-folded protein assemblies is crucial to transform nanocages into valuable bioproducts, whereby more efficient biomanufacturing processes are needed. The main objective of this work was the development of scalable and cost-effective downstream processes for NVPN based on chromatography. Two models of NVPN, one natural, the small heat shock protein from Methanococcus jannaschi (MjsHSP nanocages with 24 monomers, 12 nm and 396 kDa), and the other artificial, the trp RNA-binding attenuation protein (TRAP) nanocages (with 24 monomers, 22 nm and 2.2 MDa), were used. Both nanocages were produced in Escherichia coli and in an alternative expressing host (Vibrio natriegens). Different approaches of chromatography (anion/cation exchange, size exclusion and multimodal) as well as traditional and novel chromatographic supports with distinct properties were evaluated. The purified NVPN were analysed by PAGE methods and characterized by dynamic light scattering, transmission electron microscopy, fluorescence correlation spectroscopy, and atomic force microscopy. The obtained results demonstrated that a downstream processing strategy based on chromatography could be an efficient platform to obtain pure NVPN.

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POSTER
ABSTRACTS

# Effect of conditions on the separation of proteins on a multimodal cation exchange adsorbent

**Monika Antosova[1],** Jana Krazel Adamikova[2], Milan Polakovic[1]

*[1]Slovak University of Technology, FCHFT, Bratislava, Slovakia*
*[2]Henkel Slovensko, Bratislava, Slovakia*

A primary requirement for downstream processes used in producing therapeutic proteins is the effective impurity removal of all impurities. Multimodal adsorbents contain ligands that provide multiple interactions with solutes, resulting in unique selectivity, enhanced product yield, increased productivity, and cost reduction. The mechanism of protein binding to multimodal ligands is complex and influenced by several factors, including the type of ligand, protein, and conditions such as pH, salt type, and concentration. This study aimed to investigate the effect of adsorption conditions on the interactions between proteins and multimodal ligands since understanding the adsorption mechanism offers the possibility to modulate the selectivity of protein separation.
Our findings suggest that a combination of ionic and hydrophobic interactions applied in the binding of proteins with a positive or zero net charge in the presence of NaCl. The glycosylation of proteins significantly influenced their adsorption properties. Hydrophilic glycosylated ovalbumin had minimal retention, while fetuin, which contained hydrophobic sialic acid, was better adsorbed. The influence of salts with different kosmotropic/chaotropic characters on protein adsorption was also investigated. Our results showed that in the presence of kosmotropic salts, proteins were bound by both ionic and hydrophobic interactions at high adsorbed amounts. In contrast, the presence of chaotropic salt significantly suppressed the adsorption. The study of pH effects on protein adsorption showed that increasing the buffer pH was an effective way to achieve high elution recoveries.

*This work was supported by grants from the Slovak Research and Development Agency (APVV-20-0312) and Scientific Grant Agency (VEGA 1/0515/22).*

# Using $Ca^{2+}$ - dependent fusion protein with affinity precipitation for advanced antibody purification

**Jonghwan Lee[1,4],** Jinho Bang[1,2], Heesun Park[1,3,] Sanguine Byun[4], Sunghyun Kim[1]

*[1]Korea Institute of Ceramic Engineering And Technology (KICET), Cheongju-si, South Korea*
*[2]Department of Industrial Pharmacy, Chungbuk national University, Cheongju-si, South Korea*
*[3]Life science and biotechnology Division of life science, Korea University, Seoul, South Korea*
*[4]Department of Biotechnology, Yonsei University, Seoul, South Korea*

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# Screening of hydroxyapatite binding peptides for protein purification-tag

**Jinho Bang[1,2],** Heesun Park[1,3], Jonghwan Lee[1,4], Sunghyun Kim[1]

*[1]Korea Institute of Ceramic Engineering And Technology (KICET), Cheongju-si, South Korea*
*[2]Department of Industrial Pharmacy, Chungbuk national University, Cheongju-si, South Korea*
*[3]Life science and biotechnology Division of life science, Korea University, Seoul, South Korea*
*[4]Department of Biotechnology, Yonsei University, Seoul, South Korea*

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# High-capacity purification of therapeutic mRNA with OligodT immobilized Fibro prototype chromatography media

**Emily Berckman[1],** Susana Brito dos Santos[2], Susanna Lindman[3], Peter Lundback[3], Peter Guterstam[2], Sheng-Ching Wang[1], Andrew Swartz[1]

*[1]MSD, West Point, United States*
*[2]Cytiva Sycamore House, Stevenage, United Kingdom*
*[3]Cytiva Bjorkgatan 30, Uppsala, Sweden*

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# Development of Peptide Glucosyl-transferase Inhibitors with Comprehensive Coverage Across Clostridioides difficile Toxin B Sub-Types

**Carly Catella[1],** Sudeep Sarma[1], Carol Hall[1], Nathan Crook[1], Stefano Menegatti[1,2]

*[1]MSD, West Point, United States*
*[2]Cytiva Sycamore House, Stevenage, United Kingdom*
*[3]Cytiva Bjorkgatan 30, Uppsala, Sweden*

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This work was supported by grants from the Slovak Research and Development Agency (APVV-20-0312) and Scientific Grant Agency (VEGA 1/0515/22).

# Development of an affinity purification step for the implementation in a continuous process setup

**Christian Fiedler**[1]

*[1]TAKEDA Pharmaceuticals, Wien, Austria*

To establish a continuous purification process for rADAMTS13; a complex highly glycosylated enzyme, an affinity capture step was required. The affinity capture step must comply with the requirements of high flowrates during the unit operation especially during the loading phase. A further focus was to apply mild wash and elution conditions to obtain rADAMTS13 with high purity and to maintain enzymatic activity. An affinity resin according to these objectives was co-developed with a vendor. Eventually, the development was successful, and an affinity step was established that fulfills all prerequisites for future implementation in a continuous process.
Keywords: rADAMTS13, Affinity, continuous, enzymatic activity

# Anion exchange membrane chromatography as capture step in plasmid DNA purification: Beneficial effect of salts on binding and elution

**Duc M. Do**[1], Eric Volk[1], Alisa Strobel[2], Oliver Rammo[2], Christian Frech[1]

[1]*University of Applied Sciences, Mannheim, Germany*
[2]*Merck Liefe Science KGaA, Darmstadt, Germany*

Plasmid DNA (pDNA) plays an important role as an alternative delivery system to viral vectors in gene therapy and DNA vaccination.
The major challenge in downstream processing of pDNA from bacterial fermentations is the removal of chromosomal DNA (cDNA), high molecular weight RNA, and ineffective plasmid variants. These contaminants are particularly difficult to separate from the desired supercoiled pDNA due to their similar physical and chemical properties.
The focus of this work is on the use of anion exchange membrane chromatography as the first separation step, specifically how different salts, salt concentrations, and combinations can modulate the binding and elution behavior of pDNA. For the study, an 8kbp pDNA was captured from a clarified E. coli cell lysate using a Natrix Q chromatography membrane. The results show that it is possible to modulate binding and control how much of the contaminants are bound to the membrane by adjusting the Cl- concentration in the loading material. At a NaCl concentration of 160 mM in the lysate, pDNA binds to the membrane without the RNA and cDNA, resulting in a highly pure final eluate.
At lower NaCl concentrations, increased binding capacity is observed, with some RNA and cDNA impurities binding to the membrane along with the pDNA. However, elution with a combination of different salts allowed very efficient separation of pDNA. The addition of a prewash step further enhances the separation process, allowing effective removal of additional contaminants such as hcps and endotoxins and elution of pDNA in high purity.

# Recombinant Nuclease Cas9 for Therapeutic Genome-Editing – the Manufacturer´s Point of View

Elijahu Babaev[1], Ernst Böhm[1], **Linda Gombos[1],** Hauke Hüttmann[1], Angela Neubauer[1], Gottfried Stegfellner[1], Hans Huber[1]

[1]*Biomay, Wien, Austria*

Genome editing with the CRISPR/Cas method has revolutionized molecular biology and precision medicine. Recombinant nuclease Cas9 is an essential part of therapeutic correction of monogenic disorders, such as sickle cell disease and beta thalassemia, for which the submissions of biologics license applications (BLA) have been completed. In this case study, we present the results of a manufacturing campaign to supply the recombinant nuclease Cas9 under the industrial requirements of Good Manufacturing Practice (GMP).
Cas9 was expressed by using IPTG-induced E. coli (pET/T7). A manufacturing process was developed, essentially consisting of fed-batch cultivation, mechanical cell disruption, three-step chromatography, formulation by UF/DF and final filling.
Therapeutic nucleases for genome editing have to fulfill defined quality standards with respect of activity, homogeneity and purity. A set of analytical assays for in-process control and final quality control was developed and validated, and corresponding specifications were defined. In addition, extended biochemical characterization of Cas9 was performed. Particular emphasis was put on the determination of Cas9 activity (in-vitro potency assay) and homogeneity, aggregates and truncated variants (HP-SEC).
Long-term stability was sufficiently demonstrated by multi-year stability studies. By performing forced degradation studies, the impact of extreme physico-chemical conditions on potency was evaluated.
Exemplary data will be shown on critical quality attributes (CQA) and down-scaled process characterization using statistical design of experiments (DOE). Finally, process validation was performed by conducting consecutive and full-scale process performance qualification (PPQ) runs.
In this comprehensive overview, the concepts, difficulties and results of late-stage clinical / pre-marketing GMP manufacturing will be discussed.

# Identification of Resolution Limits and Recycling Solutions for the Characterization of Monoclonal Antibodies by Size Exclusion Chromatography.

**Fabrice Gritti**[1]

*[1]Waters Corporation, MILFORD, United States*

Minor modifications in the sequence of monoclonal antibodies (mAbs) or the presence of undesirable fragment impurities can compromise the efficacy of these new therapeutic molecules. Therefore, it is crucial to monitor the critical quality attributes (CQAs) of manufactured mAbs in real-time. Size-exclusion chromatography (SEC) is a key analytical method to quantify the presence of aggregated species and low-abundance fragments. However, a major challenge arises from poor resolution between the mAb peak and the closest fragment impurities.

In this presentation, the flow reversal method is used to identify the physical phenomenon responsible for the resolution limit of analytical SEC columns packed with 1.7 um 200 Å BEH Particles. The technique involves reversing the flow direction to reveal flow velocity biases across distances ranging from 50 µm to 2.3 mm (column inner radius). The results demonstrate that long-range velocity biases over a scale length of at least 500 µm are the sole cause of the tail of mAb peaks and the subsequent poor separation of fragment impurities from the mAb. The results are confirmed by the simulation of mass transfer of mAbs in packed beds.

To alleviate such SEC resolution limits, alternate pumping recycling liquid chromatography (APRCL) is used to accurately quantify F(ab')2 and Fab/c impurity fragments. By applying up to 10 cycles, these two fragments were found to be present at 0.88 ± 0.03% and 0.11 ± 0.02%, respectively, for an analysis time of less than 45 minutes. This quantitative information is critical during the whole development/production process of mAb drugs.

# Process Intensification using connected process for purification of mAbs: PD to Scale Up for Robust, cost effective, and agile manufacturing

**Sanket Jadhav[1],** Abijar Bhori[2], Himanshu Gadgil[2], Priyanka Gupta[3]

*[1]Sartorius Stedim Netherlands B.V., Leiden, Netherlands*
*[2]Enzene Biosciences, Pune, India*
*[3]Sartorius Stedim North America, Inc., Saratoga, United States*

Connected processing is an intensification strategy where multiple operations are connected and processed in parallel enabling the manufacturer to achieve higher productivity, while keeping time and cost low. Work presented here showcases optimized intensified platform at small-scale for DSP of mAbs and then further scale up to connected process over Resolute® BioSC pilot to purify harvest at 200L scale.
Upstream material with titer of 4g/L titer of mAbs (10L fed batch) was used to develop small-scale DSP using capture, virus inactivation, Sartobind Q, and CMM HyperCel in series. The choice of the consumables and process parameters enabled seamless transition from batch to connected process. During scale up, these 4 steps were run in a connected mode using Resolute® BioSC modular system and corresponding cost modelling was performed.
Capture on protein A using twin columns gave step recovery>94% and purity>97% by SE-HPLC. Implementation of Sartobind Q membrane chromatography in a flow through mode enabled higher flux and capacities as well as reduction in HCP. CMM HyperCel showed optimal removal of impurities (HCP, HCD) and recoveries resulting in robust process (purity>98.5%, recovery>95%). Furthermore, connected processing led to >40% reduction in DSP time against intensified batch process boosting the productivity. Resolute® BioSC chromatography system eased automation of 4 critical steps at large scale and considerably reduced the manufacturing footprint.
In summary, this work establishes an intensification process platform with seamless scale up in a connected mode to increase productivity, cost efficiency, while sustaining the quality and consistency required for a DSP process.

# Improving Efficiency in Monoclonal Antibody Purification: An Experimental Evaluation of Membranes for Single-Pass Tangential Flow Filtration

**Johann Kaiser[1,2],** Janus Krarup[2], Manuel Pinelo[1], Ulrich Krühne[1]

[1]*Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), Lyngby, Denmark*
[2]*CMC API Development, Novo Nordisk A/S, Bagsværd, Denmark*

The purification of monoclonal antibodies (mAbs) typically involves multiple chromatography and ultrafiltration (UF) steps, with the latter used for product concentration and buffer exchange. Single-pass tangential flow filtration (SPTFF) has emerged in biopharmaceutical manufacturing during the past years as a UF technology to concentrate intermediates or the product and to reduce in-process volumes in a single pass. Compared to conventional TFF, the complexity of SPTFF with its multi-stage design makes it challenging to understand the impact of design aspects such as membrane cassette configuration and membrane selection. Membrane selection is crucial, with molecular weight cut-off (MWCO) and membrane material being significant factors.
This study evaluated membrane cassettes made of different materials and with different MWCOs for mAb concentration through an experimental approach. The impact of various design aspects, including material selection, MWCO, operating conditions, and cleanability on filtration performance were examined. The results showed that differences in flux were primarily influenced by membrane material and product properties, such as viscosity, rather than the MWCO. Specifically, regenerated cellulose (RC) membranes were shown to be generally superior to polyethersulfone (PES) membranes in terms of cleanability and consistency of permeate fluxes over time.
Overall, this study provides valuable insights into the design considerations for SPTFF and highlights the importance of membrane selection for the effective concentration of mAbs. The findings can assist in selecting appropriate membranes for mAb concentration using single-pass filtration and improve the efficiency of the filtration process, potentially leading to more cost-effective and sustainable protein purification.

# Prediction of adsorption model parameters for cation exchange chromatography of proteins using molecular dynamics simulation and a self-developed coarse-grained modeling method

**Benjamin Kiss[1],** Csaba Magyar[2], András Szilágyi[2], Áron Zsigmond[1], Anna Grád[1], István Mazsaroff[1], Zsolt Lőrincz[1]

*[1]TargetEx Ltd., Budapest, Hungary*
*[2]HUN-REN Research Center of Natural Sciences , Budapest, Hungary*

The prediction of protein adsorption parameters a priori is a fundamental and long-standing objective in the field of chromatography. Nowadays, thanks to predictive AI algorithms, protein structural information is more easily accessible than ever before. Several methodologies have emerged to predict these parameters utilizing protein structural information, but a general method has not yet been developed. To this end, we compared the predictive capabilities of molecular dynamics (MD) simulation and a self-developed coarse-grained modeling approach in the context of cation exchange chromatography in linear gradient elution mode.
Exploring the interactions between the stationary phase surface and the proteins to calculate the parameters of the steric mass action (SMA) model, we conducted lab experiments using a linear gradient elution mode on an SP Sepharose FF column and high-throughput batch experiments for isotherm determination. Experiments were carried out with varying gradient slopes and pH conditions to comprehensively assess the chromatographic behavior. Our analysis focuses on two proteins: lysozyme and an anti-Taq polymerase monoclonal antibody. For the in silico modeling, we used MD simulations in solvent and a faster course-grained model focusing on the electrostatic interactions to predict the binding mode of the protein to the stationary phase surface. We analyzed and compared the SMA model parameters estimated from the two in silico approaches and our laboratory experiments.
The results of this study contribute to the ongoing efforts to advance a priori chromatography modeling, paving the way for more accurate and cost- and resource-efficient protein separation process development.

# Influence of chromatographic conditions on the adsorption of therapeutic antibodies and aggregates on multimodal adsorbents

**Tomáš Kurák[1],** Veronika Rupčíková[1], Tomáš Molnár[1], Milan Polakovič[1]

*[1]Institute of Chemical and Environmental Engineering, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Bratislava, Slovakia*

Multimodal chromatography is an important downstream processing technique because it allows for highly specific and efficient separation of target molecules from complex mixtures. This technique uses a combination of different modes of interaction between the stationary phase and the target molecule, such as hydrophobic, ion-exchange, and hydrogen bonding. This opens room for seeking optimal separation conditions to achieve highly selective purification. However, interactions on such adsorbents are very complex; making it problematic to predict the effect of pH, buffer type, or ionic strength on adsorption interactions.

The aim of the work is to investigate the use of multimodal adsorbents to purify antibodies from aggregates. For this purpose, the performance of three commercially available beads (Capto-MMC, Capto-adhere, and Toyopearl-MX-Trp-650M) was compared with prototypes of beads and chromatographic membranes using BMEA ligands. Both membrane and bead prototypes were prepared using matrix materials with three different porosities. The influence of pH, salt time, and ionic strength on the binding capacities of antibodies and aggregates was investigated using static batch experiments. Based on the results from static adsorption, suitable conditions for dynamic experiments were chosen. In addition to investigating the influence of different mobile phase composition, we also investigated the influence of the residence time and loading capacity on the dynamic binding capacities as well as the purity and yield of the separated antibodies.

Acknowledgement: This contribution was created with the support of the Slovak Grant Agency for Science VEGA 1/0515/22 and with the support of the Slovak Research and Development Agency APVV- 20-0312.

# Mixed-mode size-exclusion silica resin for polishing human antibodies in flow-through mode

**Jacob Lebarre[1],** Stefano Menegatti[1], Ruben Carbonell[1]

[1]*North Carolina State University, Raleigh, United States*

The polishing step in the downstream processing of therapeutic antibodies is tasked with clearing residual impurities (process and product-related) from Protein A eluates. Chromatographic product polishing in flow-through mode has gained significant traction in the industry, being conducive to continuous processing, and thus to lower production time and costs. To date, the work on flow-through chromatography has focused on the capture of process-related impurities, chiefly host cell proteins and DNA; conversely, lesser effort has been dedicated to the removal of product-related impurities, such as antibody fragments and aggregates. The inherent similarity between monomeric and fragmented antibodies and the subtle differences in frag-mentation patterns, in fact, has led to bind-&-gradient-elution as the mainstream method for clearing product-related impurities. With the aim of converting antibody polishing fully to flow-through mode, this study introduces mixed-mode size-exclusion silica (SEMM-silica) resins as a new chromatographic adsorbent for the capture of antibody fragments and aggregates. The pore diameter features a narrow distribution and is selected to exclude mono-dispersed monomeric antibodies while allowing their fragments to access the pores, where they are captured by the mixed-mode ligands. Optimization of the design and operation conditions of the SEMM-silica resin – namely, pore size (10 nm) and ligand composition (quaternary amine and alkyl chain) as well as the linear velocity (100 cm/h), ionic strength (5.7 mS/cm), and pH (7) of the mobile phase – resulting into a final antibody yield up to 80% and monomeric purity above 97%.

# Development of perfusion processes for *Pichia pastoris*: Opportunities for integrated purification of biopharmaceuticals

**Marina Y. Linova**[1], Satish K. Kodiripaka[1], Sobhana A. Sripada[2], Stefano Menegatti[2], John M. Woodley[1]

[1]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, Kgs. Lyngby, Denmark*
[2]*Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, USA*

Process intensification and continuous manufacturing are seen as drivers of next-generation biomanufacturing. Enabling technologies, process integration, cost analysis, and sustainability studies have mainly been focused on mammalian production platforms. Simultaneously, there has been a growing interest in alternative host production, especially for the yeast Pichia pastoris. The achievements in genetic engineering and synthetic biology suggest that yeast biomanufacturing could expand to a broader range of products and production volumes [1,2].
To address the combined advancement in those fields, we demonstrate the development of P. pastoris perfusion processes and evaluate their potential as an alternative strategy to fed-batch. Even though perfusion processes have been well-studied for mammalian cell cultures, such studies are limited for yeast cultures. There is currently insufficient information about the implications of process parameters, configurations, and feed media composition for P. pastoris processes. The present work investigates the impact of perfusion on cell viability, volumetric productivity, and cell-specific productivity. Two cell retention methods, membrane-based and acoustic-based, are compared. Our results show a stable protein production and a viable yeast culture. Furthermore, we present a case study of HCPs removal from the perfusate using a peptide-based adsorbent (PichiaGuard) in a flow-through mode, showing the possibilities of integrated downstream processing and evaluating product purity.

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# Preparation and characterization of multimodal chromatography resins for antibody purification: A comparative study with Capto Adhere

**Tomáš Molnár[1],** Veronika Rupčíková[1], Tomáš Kurák[1], Simona Kotuličová[1], Petra Pažitná[2], Peter Šafař[2], Milan Polakovič[1]

*[1]Department Of Chemical And Biochemical Engineering, Bratislava, Slovakia*
*[2]Department of Organic Chemistry, Bratislava, Slovakia*

Isolation of antibodies is a complex and economically demanding process. Multimodal chromatography can replace conventional chromatography methods because MMC functional groups can bind proteins through multiple interactions. These interactions can reduce the number of purification steps, shorten purification time, and increase protein yields. The use of multimodal adsorbents can also improve the quality of products by removing protein aggregates.
The objective of this work was to prepare and characterize MMC resins based on 2%, 4%, and 6% agarose matrices with BMEA (N-Benzyl-N-Methylethanolamine) ligands. The influence of pH and conductivity was examined and compared with the commercial MMC resin Capto Adhere. The static binding capacities of our adsorbent corresponded to the static binding capacity of the commercial adsorbent. When no salt was used, the polyclonal antibody's static binding capacity using 2% agarose matrix remained relatively constant. A similar trend was observed for the 4% agarose matrix. The highest static binding capacity for the 6% agarose matrix was detected using citrate buffer with pH 6 without the presence of salt.
The dynamic binding capacities of our adsorbent and commercial product were nearly identical under the same conditions. Compared to the commercial adsorbent, the dynamic binding capacity of our resin was 35.7 mg/ml at pH 8, while for Capto Adhere, the value was 40 mg/ml. The dynamic binding capacity was 20.6 mg/ml for our resin and 18.6 mg/ml for Capto Adhere at pH 6.

*Acknowledgements: This work was supported by grants from the Slovak Research and Development Agency (Grant number: APVV-21-0321 and APVV-20-0312).*

# Use of Immobilized Recombinant FcGamma III Receptor for Fractionation and Characterization of Antibody Preparations

**Egbert Müller[1],** Petra Grbcic, Marko Filipovic, Djuro Josic

[1]*Tosoh Bioscience GmbH, Griesheim, Germany*

Glycosylation of antibodies is one of main sources of their heterogeneity of therapeutic immunoglobulin preparations based on monoclonal antibodies as well as intravenous immunoglobulin G (IVIG) concentrates. Glycosylation of monoclonal antibody can significantly impact their therapeutic mechanism of action, and the monitoring of glycosylation of therapeutic antibody concentrates is an critical part of development and quality control of these important biopharmaceuticals [1.2]. Abovementioned IVIG concentrates are one of most important products of fractionation of human plasma [3]. In this report we present a method for determination of differences of glycosylation of therapeutic monoclonal antibody preparations, IVIG concentrates and human immunoglobulins G by use of affinity chromatography based on specific interaction with immobilized recombinant FcGamma III receptor [4,5]. Application of this method is the screening and process control in the manufacturing of therapeutic antibody preparations. Investigation of glycan composition in human IVIG concentrates and immunoglobulin G samples of patients gives additional information about variation of glycosylation patterns in human immunoglobulins G and their interaction with the FcGamma III receptor.

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# Accelerating chromatographic isolation and concentration of impurities with the twin-column continuous technique N-Rich

**Thomas Müller-Späth[1],** Giulio Lievore[1], Richard Weldon[1], Sebastian Vogg[1], Lars Aumann[1]

*[1]Chromacon AG, Zurich, Switzerland*

Despite the increase of precision and control in synthesis and upstream procedures, many impurities and side-products are collaterally created during the manufacturing of pharmaceutical compounds. A mandatory aspect of pre-clinical drug development is the isolation of thresholds-exceeding impurities to facilitate their characterization, including structural elucidation, and biochemical, toxicological, and immunogenic studies. Liquid chromatography is the most widely employed and accepted technique across industry, regulatory agencies, and researchers to accomplish this activity.
Even when optimized, chromatographic methods have technical limitations often leading to a trade-off between process productivity and fraction purity. While achieving high purity, analytical-scale isolations (UPLC) allow to collect only tiny amount of material per run, thereby requiring extensive processing times. Contrary the use of semi-preparative batch chromatography often delivers appropriate quantities of material but with highly unresolved profiles and thus insufficient purity. The N-Rich process, an automated counter-current continuous twin-column technique, alleviates the described trade-off in a cyclic fashion: the desired impurities are recycled and selectively enriched, whilst interfering substances are depleted, leading to massive time savings [1].
In this study we demonstrate the process concept and its performances in the enrichment and purification of impurities of two important classes of therapeutics: peptides and oligonucleotides. Starting from the traditional isolation approach (UPLC and semi-preparative batch), we describe the development of the continuous methods and present a detailed performance comparison among the techniques.

*Literature:*

*[1] R. Weldon et al., Enrichment and purification of peptide impurities using twin-column continuous chromatography. J. Chromatogr. A 2022, 1667, 462894.*

# Filtrations in mRNA Purification Processes. Studies of Tangential Flow Filtration and Sterilizing Grade Filtration.

**Marc Noverraz[1],** Grishma Patel[3], Rok Sekirnik[2], Janja Skok[2], Ulrike Pfaffenbichler[4], Yvonne Volz[5]

*[1]Sartorius Stedim Switzerland AG, Tagelswangen, Switzerland*
*[2]Sartorius BIA Separations d.o.o., Ajdovščina, Slovenia*
*[3]Sartorius Stedim North America, Inc., Bohemia, USA*
*[4]Sartorius Stedim Austria GmbH, Vienna, Austria*
*[5]Sartorius Stedim Biotech GmbH, Göttingen, Germany*

The manufacturing process of biopharmaceuticals typically ends with two filtration unit operations: a tangential flow filtration and a final dead-end filtration.
The tangential flow filtration is used to adjust the concentration and buffer composition of the drug substance and the final dead-end filtration, often sterilizing grade, is used to remove any remaining particles and reach an adequate bioburden status. Those manufacturing steps are indispensable for the good storage, transport, and successful completion of the subsequent drug product manufacturing.
As they intervene after the proper purification steps, it is crucial that they do not impact the quality of the purified drug intermediate by adding impurities by external contamination or by degrading the target molecule while keeping losses to a minimum. This is of particular importance for mRNA, which by its biochemical nature, large size, and extremely high production cost is very sensitive to those aspects.
With TFF, there is a risk of degradation of the mRNA due to the shear forces generated by the pump and the TFF cassette channel screen, and a risk of mRNA loss by transmission through the membrane, or by irreversible adsorption on the membrane. TFF cassettes with different molecular weight cut-offs and inter-membrane channel geometries on a mRNA concentration and diafiltration process have been studied.
With dead-end filtration, the main risk is the loss of mRNA by adsorption on the filtration membrane. The performance of two sterilizing grade membrane filters have been studied for the filtration of mRNA in different buffers.

# Maximize flexibility and throughput. Scalable and efficient purification of synthetic peptides

**Marius Segl[1],** Johannes Menke[1], Yannick Krauke[1],
Ulrike Krop[1], Giorgia Greco[1]

[1]*Knauer Wissenschaftliche Geräte Gmbh, Hegauer Weg 38, Germany*

Peptides are playing an increasingly important role as therapeutics. Most peptides today are synthesised using solid phase peptide synthesis. After cleavage from the resin, the product contains not only the target peptide but also synthesis-related impurities. HPLC is the method of choice to analyse the peptides and isolate pure products from a crude mixture. The aim is to obtain a product of the highest purity and yield without losing sample during the purification process. At the same time, multiple samples often need to be purified automatically to increase throughput. The use of a liquid handler allows automatic injection of multiple samples and fraction collection with maximum flexibility. Especially for high-value samples, it is crucial to use an injection system with minimal sample loss.
Here we present a workflow from method development to scale-up and purification of crude peptides using a preparative HPLC system with a liquid handler. Angiotensin I was purified with high purity and yield from a crude sample matrix. The preparative liquid handler allows the injection of large sample volumes and quantities without sample loss during injection for high throughput purification.

# Extreme proteins require extreme purifications: a scalable and effective bioprocess for nanocompartment production.

**Ferdinando Sereno[1],** Andrea Rayat[1], Stefanie Frank[1]

*[1]University College London - Department of Biochemical Engineering, London, United Kingdom*

Encapsulins are 24-42 nm hollow protein nanoparticles ubiquitously expressed in bacteria and archaea. They feature remarkable temperature, pH and protease resistance and an exceptionally flexible engineering potential that has led to a growing body of research focusing on applications such as vaccine candidates, drug delivery systems (DDS), nanoreactors and nanomaterials. These proof-of-concept studies lack, however, a consistent and scalable production process, that we sought to develop.
We designed a standardised bioprocess for the three most-well studied Family 1 encapsulin production by investigating the three main sections in protein production: upstream, harvest and downstream. Concerning the former, we evaluated the role of autoinduction-based media over IPTG induction and growth temperature to improve solubility and yield of the target protein. Next, cell disruption methods were compared to measure the recovery of soluble proteins, with homogenisation proving a superior, scalable choice. Ammonium sulphate precipitation was found to aid host cell DNA removal, while insoluble protein was isolated via inclusion body recovery. Lastly, the downstream pipeline was designed in a three-step process: heat-precipitation of non-thermostable host cell proteins, anion-exchange chromatography and multimodal chromatography for polishing.
To conclude, the bioprocess we devised allows for the production of pure encapsulins, devoid of both host-cell proteins and DNA, with yields superior to the values currently reported in the literature. These findings will be beneficial for the encapsulin research community and provides a workflow that could be applicable to other protein nanoparticles and virus-like particles. Finally, the process has been designed with scalability in mind for future applications.

# Viral clearance in end-to-end integrated process for mAb purification: Total flow-through integrated polishing on two columns connected to virus filtration

**Hironobu Shirataki[1]**

[1]*Asahi Kasei Medical, Tokyo, Japan*

There are few reports of the adoption of continuous processes in bioproduction, particularly the implementation of end-to-end continuous or integrated processes, due to difficulties such as feed adjustment, production batch demarcation and incorporating virus filtration. Here, we propose an end-to-end integrated process for a monoclonal antibody (mAb) with three integrated process segments: upstream production processes with pool-less direct connection, pooled low pH virus inactivation with pH control and a total flow-through integrated polishing process in which two columns were directly connected with a virus filter. The pooled virus inactivation step demarcates the batch, and high impurities reduction and mAb recovery were achieved for batches conducted in succession. Viral clearance tests also confirmed robust virus reduction for the flow-through two column chromatography and the virus filtration steps. Additionally, viral clearance tests with two different hollow fiber virus filters operated at flux ranging from 1.5 to 40 LMH confirmed robust virus reduction over these ranges. Complete clearance with LRV ≥ 4 was achieved even with a process pause at the lowest flux. The end-to-end integrated process proposed in this study is amenable to production processes, and the investigated virus filters have excellent applicability to continuous processes conducted at constant flux.

# Advances in Downstream PAT for Biologics, Vaccines and Gene Vectors

**Daniel Some**[1]

[1]*Wyatt Technology, Santa Barbara, USA*

One of the key challenges during process development and production of biologics and complex drugs is the timely determination of essential product attributes. Rapid feedback of product-relevant characteristics simplifies and accelerates process development and scale-up, then helps ensure product yield and quality during full-scale manufacturing. However, attributes of biologics are generally determined offline, creating a bottleneck for process development and production.

Multi-angle light scattering (MALS) is a versatile technology most commonly found in analytical laboratories for characterizing biophysical properties such as protein or polynucleotide molar mass, size and conformation; size and concentration of virions; and the genomic payload of viral and non-viral gene vectors. Real-time multi-angle light scattering (RT-MALS) is a novel use of MALS which brings many of those capabilities to preparative systems and process development laboratories. RT-MALS determines key product attributes of biologics, in-line or on-line with downstream and fill-finish processes, and can be used to monitor aggregation, distinguish product from impurities, identify process endpoints and more.

This presentation will review the principles, capabilities and limitations of RT-MALS, and then present examples covering downstream processing of viral vectors and proteins. These examples illustrate the potential for RT-MALS to speed up process development while gaining early process knowledge, and eventually to monitor unit operations to increase yield, ensure quality and consistency, and for some products, provide real-time release testing of certain critical product attributes.

# Development of Novel Protein L Resin with Selective Binding to Kappa 1 Light Chain

**Toru Tanaka[1],** Takatoshi Hasemi[1], Akihiro Iwase[1], Kazuki Aida[1]

[1]*Tosoh Corporation, Ayase, Japan*

Bispecific antibodies (BsAbs), which can bind and associate two target molecules, are being put into practical use as next-generation therapeutic antibodies. Typically, BsAb is designed of two types of antibodies with different properties, such as κ-type and λ-type antibodies, to separate and analyze mispaired antibodies that are generated in manufacturing. However, the λ-type antibody has low physicochemical stability, which limits its use in pharmaceutical applications.

In this presentation, we will report on a novel affinity resin that has been improved to bind only to κ1-type antibodies through amino acid substitutions in protein L ligand. It was found that the developed Protein L resin was able to adsorb κ1-type antibodies in the same manner as usual Protein L resin, but did not adsorb other κ-type antibodies. This unique protein L resin might be used for separation of highly stable BsAbs, which is composed of κ1-type and κ3-type antibodies.

# Purifying challenging entities: capture of bispecific antibodies and removal of product-related impurities

**Jakob Liderfelt**[1]

[1]*Cytiva, Uppsala, Sweden*

As the diversity of antibody variants in the biotherapeutics pipeline increases, so does the need for chromatography resins that can capture and purify them. As an alternative to protein A, protein L can be used to capture antibody Fabs, bispecific antibodies, and other molecules containing kappa variants of the variable light (VL) chain. Many of these variants, especially asymmetric bispecific antibodies, are prone to aggregation or to forming product-related impurities such as homodimers and half-antibodies during cell culture. The similarities between these impurities present extra challenges in the downstream process, especially post-capture. Removing product-related impurities at the capture step – using differences in avidity – can help simplify the polishing steps.

# The influence of unspecific viral adsorption on pharmaceutical container surfaces in vaccine process development

**Marco Kress**[1,2], Jürgen Heindl-Wruss[1], Robert Schlegl[1], Alois Jungbauer[2]

*[1]VALNEVA Austria GmbH, Campus Vienna Biocenter 3, 1030 – Vienna, Austria*
*[2]University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 – Vienna, Austria*

Unspecific surface adsorption of biomolecules is a common observation and has been intensively described for proteins and peptides. However, literature referring specific to viral particle adsorption phenomena is limited and rather focused on viral transmission pathways, leaving the potential impact of viral particle adsorption on vaccine development and manufacturing out of scope.
Since development takes place in small-scale environment less favorable volume to surface ratios are present compared to large-scale manufacturing. Depending on the process step we might expect viral protein concentrations in the range of 1 to 100 µg viral protein/mL, that is much less compared to working with proteins. In this early stage of development, proper selection of suitable container and understanding of particle/surface interaction is usually very limited and could result in significant losses of viral particles from process streams. Especially process steps operating with already purified virus material leaves the virus particles as only agent left for potential adsorption reactions. This can lead to unexpected losses affecting analytical results, mass balances and even wrong conclusions in process development activities.
In this study we screened standard pharmaceutical container (e.g. PETG bottles, single-use bags, sample tubes) in view of viral surface losses over time to assess the impact of unspecific adsorption. Active and formalin-inactivated Japanese encephalitis virus (JEV) and active Chikungunya virus (CHIKV) were used as model viruses. Container surface characterization was done by electron microscopy and contact angle measurements.

# Mechanistic modeling case study: The early model catches the leanest process

**Dominik Voltmer[1],** Felix Wittkopp[1], Dominik Hertweck[1]

*[1]Roche, Penzberg, Germany*

Over the past few years, mechanistic modeling has seen a spike in use cases for both early and late stage applications. The potential of these applications has been widely recognized, but often cannot be fully realized because their insights frequently come after decisions have already been made.
In this case study, the aim was to tap into some of this unused potential by applying mechanistic models even earlier in order to impact and accelerate process development and clone selection.
Early stage modeling for the purification of monoclonal antibodies is typically done for the polishing step on an ion-exchanger with a single clone, stable impurity pattern and limited options to influence the input conditions for process development. For this case study, we began modeling previous to the clone selection point and therefore with material from a pool of clones and a more challenging dataset with a wider range of impurities.
This approach represents a significant novelty in the early stage modeling workflow and is, to our best knowledge, a first in class applied technology in a standard portfolio workflow.
The transition from bleeding-edge to cutting-edge technology is shown in this case study by applying the standard modeling work packages like purity, yield and robustness predictions with equal success, despite the more challenging dataset. In addition, being able to predict the impact of changes in impurity patterns and react to stability data in time to influence clone selection are invaluable and show the potent benefits of starting the modeling process early.

# Rapid development of caustic stable AAV affinity chromatography resins for AAV5 and AAV6

**Carsten Voss**[1], Warren Kett[2]

*[1]Repligen GmbH, Ravensburg, Germany*
*[2]Repligen Corporation, Lebanon, USA*

Over the past few years, mechanistic modeling has seen a spike in use cases for both early and late stage applications. The potential of these applications has been widely recognized, but often cannot be fully realized because their insights frequently come after decisions have already been made.
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# Depth filtration for early recovery of soluble expressed microbials

**Maria Weinberger[1],** Lars Demmel[1]

[1]*Boehringer Ingelheim RCV, Vienna, Austria*

After many years of working with inclusion body derived proteins from microbials, soluble expressed microbial proteins become more and more of interest. The main advantage of soluble expression is the avoidance of a buffer intense and low yield refolding step. However, it poses other challenges to downstream processing, such as clarification and removal of process related impurities.
Depth filtration is a widely used method for clarifying cell debris and other particulate matter. There is a wide range of depth filters made from different materials and with different retention characteristics available on the market. Depth filter media with integrated functional primary components are also becoming available, which additionally facilitates the depletion of process related impurities such as host cell proteins (HCPs) and endotoxins. Currently, the choice of depth filter media for clarification is based on empirical knowledge. Due to the sheer number of available options, it is impossible in process development work to test all eligible depth filter media to find the optimum. A common approach is to test two to three different media based on previous experiences and only investigate further if issues arise.
In this work, the relationship between properties of the particulate rich feed suspensions (such as particle load and turbidity) and the filtration behavior (such as loading capacity, pressure increase, endotoxin depletion) will be investigated. This data shall be the basis for the data driven choice of depth filter media in the future, which requires less experimental effort during process development.

# FcRn Immobilised HPLC Affinity Column for Antibody Evaluation

**Tatsuya Yumoto[1],** Naruaki Inoue[1], Ryuma Ikeura[1], Yosuke Terao[1]

[1]*Tosoh Co., Ltd., Ayase-shi City, Japan*

IgG-based drugs are one of the most successful protein therapeutics, in part due to their remarkably long serum half-life. This merit of IgG arises from its interaction with the neonatal Fc receptor, FcRn, which is the homeostatic receptor responsible for IgG recycling and transcytosis. In accord with such features of FcRn, it has been recently reported that the binding affinity between FcRn and antibodies can be an indicator of the serum half-life or the quality of the antibody drugs.

We have successfully developed a novel column for affinity chromatography utilizing recombinant human FcRn. The major characteristic of this column is that it enables high-resolution separation of antibodies in an FcRn affinity-dependent manner. In turn, this column is available for quantitatively determining affinities for FcRn or abundance of a wide variety of antibody drugs, including Fc-engineered antibodies, antibody-drug conjugates or Fc fusion proteins.

In addition to the properties mentioned above, our column is adaptable to evaluate oxidised antibodies. Oxidation is the most critical chemical degradation process of antibodies that can lead to changes in physiological features, such as in vivo serum half-life, efficacy, and stability. To our interest, multiple peaks were detected when the oxidised antibodies were analyzed with our column. This result strongly suggests that our column is useful for the analysis of oxidation state or abundance of antibodies with different oxidation numbers.

Thus, the FcRn immobilised column allows accurate and quantitative evaluation of each antibody's affinity for FcRn, which holds the great promise for development of next-generation antibody therapeutics.

# Challenges in parameter estimation for two-component protein adsorption using batch and small-scale column adsorption

**Jürgen Beck[1],** Johannes Schmölder[2], Eric von Lieres[2], Rainer Hahn[1]

[1]*University Of Natural Resources and Life Sciences, Vienna, Austria*
[2]*Forschungszentrum Jülich, IBG-1: Biotechnology, Jülich, Germany*

Frequently, inverse fitting of multiple relevant experimental chromatography runs - mainly of the elution step - are used to fit parameters to obtain suitable predictions in a defined solution space. The number of chromatographic parameters selected, is crucial for the significance of the result. If the number of parameters is too small, the model may not be able to predict the experimental data accurately. If the number is too large, despite achieving a high quality of fit, it may result in model parameters lacking physical significance. In this study we demonstrate the difficulty of obtaining meaningful parameters for a mechanistic model for a competitive two-component protein system studying resins which differ in their morphology and mass transfer properties. We show experimental data obtained by batch uptake kinetics and breakthrough experiments which were used to study different modelling strategies. We compared a simple approach, prediction of the two-component column adsorption based on single component isotherms and batch uptake curves, to inverse fitting of the column adsorption profiles. Obtaining accurate parameters from small-scale experiments is not trivial and may lead to significant deviations from the true value of a parameter when performing an inverse fit. We discuss which error sources to consider prior to setting up a model and the limits of fitting to data. Furthermore, we show that complex material properties can have a significant influence on protein separation, which cannot be captured using simple models.

# Morphine Production in Genetically Engineered Poppy Cell Culture

**Janos Bindics[1,2],** Johannes Buyel[1,2]

[1]*acib GmbH, Graz, Austria*
[2]*University of Natural Resources and Life Sciences, Vienna (BOKU) , Wien, Austria*

The opiate alkaloid morphine is classified as an essential medicine by the World Health Organization due to its prominent role in pain management. In contrast, roughly half of the human population has limited to nonexistent access to morphine-provided pain management and as a result patients in need of long-term pain relief experience considerable suffering.
Inequities in global morphine accessibility arise from current morphine production technology, which entirely relies on open-field cultivated opium poppy (Papaver somniferum) plant as the sole source of morphine. Industrial poppy cultivation is affected by environmental factors and due to worldwide concentrated production, transportation and political stability affect the resilience of global opiate supply. Establishing means of farming-independent morphine production is expected to mitigate current production and transportation associated risks, however neither chemical synthesis nor genetically modified microorganisms could provide an alternative morphine production approach.
Therefore, we aim to develop a novel, innovative opium poppy cell culture-based morphine production platform. We base our approach on three lines of development: i) elucidating the mostly unknown regulation of morphine biosynthesis in poppy plants to activate morphine production, ii) developing an opium poppy cell line and optimized media to ensure robust production, and iii) developing a sustainable, solvent-free downstream processing technology. We will transform current agriculture-based morphine production into a cost-effective biotechnology process, which is compatible with available fermentation equipment. Ultimately, this can result in improved global supply chain stability and reduced production costs rendering morphine equally available to patients in low- and middle-income countries around the globe.

# Polyethyleneimine efficiently extracts recombinant cytoplasmatic green fluorescent protein produced in Escherichia coli with high purity.

**Alexander Jurjevec[1],** Rainer Hahn[1]

[1]*University of Natural Resources and Life Sciences, Vienna, Austria*

We used a polycationic polymer polyethyleneimine (PEI) to develop a method to extract recombinant proteins produced in the Escherichia coli (E. coli) cytosol. Compared to high pressure homogenization, commonly used to disrupt E. coli cells, our extraction method leads to higher purity of extracts. Upon addition of PEI to the cells, flocculation occurs and the recombinant protein gradually diffuses out of the PEI/cell network. While several aspects such as the E. coli strain, the cell or PEI concentration as well as the protein titer and the pH of the buffer seem to influence the extraction rate, our results show that the PEI molecule (molecular weight and structure) must be chosen appropriately for protein extraction. The method works well with resuspended cells but can also be applied directly to fermentation broths at higher PEI concentration. This extraction approach allows for effective reduction of DNA, endotoxins, and host cell proteins levels by 2 to 4 orders of magnitude, and drastically facilitate the subsequent downstream processing steps such as centrifugation and filtration.

# Mapping time-dependent disulfide bond formation during *in-vitro* refolding of recombinant peptibody: A Fc-fusion protein

**Rupali Kumthekar[1],** Sunil Rana[1], Santosh Ughade[1], Rahul Bhambure[1]

[1]*CSIR-National Chemical Laboratory, Pune City, India*

Disulfide bonds are commonly found in covalent interactions, which play a vital role in establishing the three-dimensional structure of proteins and maintaining their biological activity. This investigation is focused on time dependant mapping of intra and inter-disulfide bonds during in-vitro refolding of recombinant peptibody using LC-ESI-MS/MS. The selected recombinant peptibody is a homodimeric, aglycosylated Fc-fusion protein expressed in E. coli. Using the CID-based fragmentation approach, the amino acid sequence in the disulfide bond containing peptides was confirmed at MS/MS level. During in-vitro refolding of peptibody, a peptide with an inter-chain disulfide bond is observed post 4 h, with low area intensity, and it reached a maximum in 96 h. However, maximum area intensity was reached for the intra-chain disulfide bond in 72 h. Since cysteine residues responsible for intra-chain disulfide bonds are present in the complex part of the Fc domain, the formation of intra-chain disulfide bonds may be an overall rate-limiting step in recombinant Romiplostim refolding. Proper folding and contributions by non-covalent interactions will be required to form intra-chain disulfide bonds. Monitoring the time-based formation of such non-covalent interactions was carried out using intrinsic fluorescence during protein refolding. Observations from intrinsic fluorescence show that 4–6 h is the most crucial time for domain formation. Observations from this study will help characterize structural integrity and quality during process and product development for consistent product quality.

# Fluorescently Labeled Antibody as an Inert Tracer for Characterization of Residence Time Distribution in Counter Current Protein A Affinity Chromatography

**Narges Lali[1,2], Alois Jungbauer[1,2]**

[1]*acib - Austrian Centre of Industrial Biotechnology, Vienna, Austria*
[2]*University of Natural Resources and Life Sciences, Vienna, Austria*

The biopharmaceutical industry has been transitioning from batch to continuous processing. In continuous biomanufacturing, traceability of materials is essential for compliance with regulations. The FDA's recent guidelines on continuous manufacturing require a fundamental understanding of the mass flow of the integrated continuous manufacturing process, which can be achieved through the use of residence time distribution (RTD). The RTD explains how materials move through a process and it can be obtained experimentally by injecting an inert tracer and measuring the broadening of the pulse in the outlet. Finding an inert tracer that is identical to the protein of interest yet detectable is challenging. Fluorescently labeled antibodies are widely used for image acquisition and investigation of the transport mechanism. However, the labeled antibody may have a different higher affinity towards the resin, which can be attributed to slight differences in hydrophobicity and charge caused by the fluorescent dye.
The difference between labeled and intact antibodies was characterized by investigating the binding site of the dye on the antibody subclasses 1 and 2 using mass spectrometry. The dye binding site is important and can indicate if it affects the protein A binding or not. Mab Select Sure resin was used in this study, and it is a common belief that the Z-domain derived from staphylococcal protein A exclusively binds to the Fc-domain of the antibody. Meanwhile, another publication has shown that there is a constant exchange between bound and free antibodies.

# Asymmetric field flow fractionation and Taylor Dispersion Analysis for separation and characterization of Adeno-Associated Viruses for gene therapy

**Sabrina Leigheb[1],** Catarina Maia[2], Verena Buchacher[1], Maral Rahimzadeh[1], Nikolaus Virgolini[1], Patricia Pereia Aguilar[3], Astrid Dürauer[1]

[1]*University of Natural Resources and Life Sciences, Department of Biotechnology, Institute of Bioprocess Science and Engineering , Wien, Austria*
[2]*Instituto Superior Técnico, Lisbon, Portugal*
[3]*Austrian Centre of Industrial Biotechnology (ACIB), Wien, Austria*

The most investigated viral vectors for gene therapy are adeno-associated viruses (AAVs). Since 2012 three AAV-based therapeutics have been approved. However, challenges still exist in achieving high yields and consistent quality for clinical applications. Therefore, the development of advanced analytical techniques for the in-depth characterization of AAVs is essential to establish knowledge-based strategies for their more efficient separation and quantification.

The major challenge in the downstream processing of AAVs is the discrimination between functional particles, which correctly incorporated the GoI, and non-functional ones. Thus, harvested recombinant AAVs always consist of a mixed population of empty, filled, partially filled and over-filled DNA capsids. We have established asymmetric Field Flow-Field Fractionation (AF4) and Taylor Dispersion Analysis (TDA) as complementary methods to electron microscopy, which currently the gold standard for the characterization of filled and empty AAVs. Particles separated by AF4 are analysed by Static and Dynamic Light Scattering, and Multi Angle Light Scattering which allows the in-depth characterization of the AAVs. TDA is a hydrodynamic-based technique used to determine the diffusion coefficient and hydrodynamic radius of particles in solution. Preliminary tests have been performed on non-biological standard material in order to develop a method for AAV characterization. Nanoparticle Tracking Analysis has been used as reference method for the size distribution of the capsids. In combination with ddPCR for genome quantification these methods provide a more accurate determination of AAV genome copy numbers.

In contrast to currently established methods, AF4 and TDA are non-destructive, fast and scalable methods for in-depth AAV vectors characterization.

# Optimization of the in-vitro refolding of biotherapeutic Fab Ranibizumab

**Rashmi Sharma**[1]

[1]*Indian Institiute Of Technology, Delhi, India*

Recombinant biotherapeutics expressed as inclusion bodies require solubilization and subsequent refolding to attain a functionally active state. But the refolding step often acts as a bottleneck due to a low throughput, cumbersome and expensive framework, especially in the case of complex, multi-domain proteins such as antibody fragment (Fab) molecules. Fab Ranibizumab, with two domains and five disulfide bonds, currently presents a daunting task to be refold. In this paper, the in-vitro refolding of this Fab over 24 h was investigated, using two different methodologies. The first protocol employed the traditional two-step DoE for screening and optimization. The entire refolding process was considered as a single process step. The second approach derived inferences from the data of analytical tools like intrinsic fluorescence, zeta potential and RP-HPLC to highlight a possible time-based molecular behavior during refolding. This led to the identification of a breakpoint at the 8th hour of the process, proposing initial occurrences of the native tertiary conformation. Based on this observation, segmented DoEs were conducted to optimize the two time zones of the refolding process (0–8 h and 8–24 h). This unconventional segment-based optimization approach led to a 55 % increment over the standard conventional optimization methodology, performed for the same Fab, Ranibizumab. It prevailed with an effective yield of 32 % over the conventional strategy with 20.6 %.

# Efficient identification of optimal process conditions with Gaussian processes

Matthias Medl[1], **Martina Winter[1],** Irfan Erdem[1], Astrid Dürauer[1], Theresa Scharl[1]

*[1]University of Natural Resources and Life Sciences (BOKU) Vienna, Vienna, Austria*

The identification of optimal process parameters is one of the central challenges in the design of biopharmaceutical production processes. The established method to overcome this challenge is Design of Experiments (DoE), where multiple process parameters are varied simultaneously according to a predefined design plan. Based on the resulting data the optimal process parameters are predicted by response surface models.
Parameter optimization is crucial not only in biomanufacturing but also in very different fields such as searching oil drilling sites, finding ideal brain stimulation settings with transcranial alternating current stimulation or optimization of hyperparameters in machine learning.
In these fields a different parameter optimization strategy has led to great success: Gaussian process optimization. Gaussian process optimization is a non-linear Bayesian method, where the experimental conditions are not predefined in a design plan, but determined dynamically by means of an iteratively updated acquisition function. In practice, the Gaussian process model first suggests process conditions for an experiment, then the experiment is performed in the lab, and the results are re-entered into the model. It then predicts the next process conditions. This is repeated until satisfactory process conditions are found. In this study we have compared the two optimization strategies for process parameter optimization – DoE using a central composite design and Gaussian process optimization – of a capture step for basic human fibroblast factor 2 based on cation exchange chromatography. The two methods were compared for their efficiency relating the experimental effort to the productivity and impurity depletion of the determined conditions.

# Evaluating novel monoclonal antibody (mAb) purification techniques

**Hannah Davison**[1,2], Andrea Rayat[1], Jon Coffman[2], Gary Lye[1]

*[1]University College London, London, United Kingdom,*
*[2]AstraZeneca, Gaithersburg, United States*

In recent years, the high demand for mAbs has caused a drive to improve cell culture processing by improving product titres and developing novel processes such as cultures. For highly utilised bioreactors, this has led to a bottleneck in the purification of monoclonal antibodies, particularly in the primary recovery or capture stage. Conventional methods of bind and elute process chromatography using protein A resin are becoming limited, as they are inherently batch and struggles to cope with required rate of production while keeping facility footprints low. There is therefore a need to develop alternative methods of mAb purification. Using membrane-based methods is of interest as these have been shown to be successful in further downstream processing and has the potential for continuous processing with modifications on design or operational methods.

This poster will summarise the work done in a double stranded project. It firstly covers a theoretical and experimental evaluation of membrane dialysis model for selective and efficient primary recovery of proteins. Using the model, its application to mAbs purification is compared to Protein A chromatography in terms of yield, purification factor and buffer consumption, with results suggesting this technology could have real potential. Additionally, results on the initial investigation of Protein A-mAbs complexes as a basis for a novel purification operation will be presented. These results will aid characterisation and eventual optimisation of the process.

This work is in collaboration with a sponsor company and is part of the UCL Engineering Doctorate programme in Biochemical Engineering and Bioprocess Leadership.

# Inline-tandem purification of viruses from cell lysate by agarose-based chromatography

**Mikael Andersson Schönn**[1]

*[1]Bio-Works, Uppsala, Sweden*

An efficient chromatography-based virus purification method has been developed and validated for the non-pathogenic infectious virus PRD1. Compared to the conventional method that consists of relatively time-consuming and labour-intensive precipitation and density gradient ultracentrifugation steps, the method developed here is performed in a single flow using tandem-coupled anion exchange and size exclusion chromatography (AIEX-SEC) columns. This inline approach minimizes target loss, streamlines time consumption, increases productivity and enables seamless scaling.

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