MSS2024

Abstract book

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Portoroz, Slovenia

+ virtual

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MSS is a five-day scientific symposium highlighting the solutions from process development to manufacturing, empowered by innovative analytics for emerging advanced therapies and vaccines. Our mission is to spread knowledge on the evolution and applications of different purification and analytical technologies while creating platforms where technology suppliers and acquirers can interact and establish partnerships. We provide an excellent international forum for researchers and scientists from both commercial and academia to meet and share the latest advances and trends in the biopharma industry.

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Lectures

Enabling CIMmultus DEAE columns at scale for plasmid DNA capture

Justina Martinkiene, Tingting Cui, Greta Csalane Besenyei, Will Bryan-Smith
AstraZeneca, Cambridge, United Kingdom

Increasing demand of highly purified plasmid DNA (pDNA) at gram scale asks to have controllable, robust and scalable workflow to capture pDNA from cell lysates. With the aim to develop a process that meets those criteria, we did screening experiments and tested various resins, columns, and membranes suitable for pDNA capture and found that CIMmultus DEAE monolith columns offers most promising results in terms of high binding capacity for pDNA, high working flow rates and easy to scale up properties. This presentation will explain:

- scientific challenges that were experienced throughout the development process and solutions that were applied to solve or maintain those challenges across the batches
- resin and membrane screen study data and why CIMmultus DEAE monolith column was chosen for further development
- work that has been done to enable monolith columns to work across different size pDNA constructs (4 and 12kB) and deliver high purification yields while maintaining high pDNA purity
- column scale up results where purification scale was increased from 1mL to 800mL
- how introduction of the monolith columns improved pDNA manufacturing process
- outstanding gaps and future work around pDNA capture step robustness and capability to handle pDNA with elevated level of impurities

High-quality, phase-appropriate plasmid manufacturing to accelerate advanced therapies

John Bowen, Judy Jan, Branden Ricketts, Patrick Kritz, Dave Sendecki, Syam Pillai, Brian Tomkowicz, Alexei Saveliev
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Plasmids are circular pieces of double stranded DNA that are critical for many applications in Advanced Therapies such as a critical starting material for gene therapy or mRNA modalities. It can also serve as the final drug product for many DNA-based vaccines. However, many challenges exist to meet both the high quality and large quantity requirements demanded by drug developers. Multiple plasmid grades exist to support innovators in their various stages from discovery, through clinical trial production, and ultimately commercial supply. R&D plasmids are used for preclinical evaluation, but the level of quality can vary greatly from supplier to supplier. Levels of GMP grade plasmids have also evolved and have been defined typically by the plasmid manufacturer, with a mid-tier grade, commonly called "GMP-Like" or trademarked by the organization (appropriate for clinical) and "GMP Grade" suitable for commercial GMP products. To ensure high quality plasmid is supplied, several phase appropriate strategies are employed from a technical, operational, and quality perspective. A "flexible" plasmid manufacturing platform that can accommodate a variety of plasmid sizes and sequence complexities is critical. This flexible platform utilizes platform unit operations while providing flexibility such as multiple fermentation processes, a selection of E. coli strains for plasmid propagation, and a variety of scalable purification strategies to meet different target product profiles. Aside from a flexible platform, providing novel "off the shelf" plasmids for adeno-associated virus and lentivirus production provide an R&D toolkit for Advanced Therapy developers. Finally, the use of Next Generation Sequencing (NGS) has provided great value during fermentation development, especially for difficult to replicate sequences such as plasmids containing Inverted Terminal repeats (ITRs). Collectively, these plasmid technologies provide high quality, phase-appropriate plasmid manufacturing services to innovators to accelerate advanced therapy development.

High-throughput manufacturing of personalized plasmid DNA cancer vaccines

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Personalized medicine is a progressive approach in the treatment of diseases, based on the genetic variability of each single patient, enabled by the ever-increasing knowledge in the field of genetic engineering and novel technologies. 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 antigensis developed.
Here we present our manufacturing platform for individualized plasmid DNA vaccines in compliance with GMP standards. A well-established production process with currently over 100 manufactured patient-specific batches provides a major contribution to a clinical trial with the objective to treat different types of cancer.
The whole manufacturing cycle from tumor biopsy over identification of tumor-specific biomarkers, neoepitope selection and sequence design, synthesis of pDNA starting material to the manufacturing of the individualized drug substance and drug product demands short turnaround times and robust processes. Our parallelized small-scale manufacturing process starts with the generation of a personalized *E. coli* cell bank followed by a fed-batch fermentation, harvest and lysis. Purification is accomplished by a two-step chromatography process using monoliths, followed by formulation via UF/DF and concluded by low-bioburden filling. In-process and batch release testing accompany the whole manufacturing process. Our presentation will give insight on facility design, process optimization strategies (e.g. DoE) and approaches to the continuous monitoring of process performance to ensure a small footprint, rapid turnaround times and cost effective manufacturing of high-quality personalized plasmid DNA.

Sartorius in-line lysis system: Pioneering success in plasmid DNA isolation

Matevž Korenč
Sartorius BIA Separations, Ajdovščina, Slovenia

The isolation of plasmid DNA (pDNA) typically involves a cell lysis step using a strong base (alkaline lysis), followed by neutralization and precipitation to separate the desired pDNA from other cellular components. However, uncontrolled alkaline conditions and mechanical stress can degrade the sensitive pDNA, while highly viscous solutions may lead to heterogeneities or necessitate extensive mixing, risking further degradation. This presentation will introduce a fully automated in-line lysis system that streamlines this process with a closed single-use loop, suitable for process development through to pilot scale in a GMP environment. Featuring advanced mixing control, the system can automatically and continuously process 10–50 kg of resuspended cells per workday, depending on the configuration. Notably, this presentation will highlight tangible successes, including an innovation award from GZS and a compelling success story with Biovectra, who has experienced the benefits of using the beta and GMP version of our product.

Key Learning Objectives:

- Grasp the challenges of alkaline lysis at pilot scale and how the Sartorius BIA Separations in-line lysis system addresses them.
- Discover the tangible successes and industry recognition of the Sartorius BIA Separations in-line lysis system, including an award for innovation and a success story with Biovectra.
- Explore at-line in-process control strategies for managing challenging crude lysis samples with our innovative solution.

Debottlenecking vaccine manufacturing; effective supply of quality pDNA

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Plasmid DNA (pDNA) plays a critical role in the production of next generation biotherapeutics, including cell & gene therapies and vaccines. These state-of-the-art therapies, which are transforming the prevention and treatment of devastating diseases, come in a variety of forms.

However, all these applications depend on, in their manufacture or mode of action, scalable production of DNA. Current pDNA upstream processes typically have volumetric yields in the hundreds of milligrams per litre, which may satisfy the pDNA demand for a few biotherapeutics but not all the diverse biotherapeutics being approved. To satisfy high yield and high purity plasmid DNA in its correct topology for these applications (ccc) is a harder challenge than in typical protein processing as the level of impurities make up nearly 97% of biomass (proteins, endotoxins, other DNA, RNA).

To address these challenges, our group strategy is to explore the establishment of an integrated, versatile, pDNA manufacturing platform which may include next- generation continuous pDNA recovery process and novel cell programming as well as purification strategies. By adopting this strategy, we hope to generate synergies that will help us to achieve the three key themes in pDNA production: ease, speed and cost.

Biotechnological platform to obtain a minicircle DNA vaccine targeting cervical cancer

Ângela Sousa
CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal.

Cervical cancer (CC) ranks as the fourth most commonly diagnosed cancer among women globally and is the foremost in low-income countries. Approximately 99% of cases are attributed to Human Papillomavirus (HPV) infection, presenting a significant public health challenge. Nucleic acid vaccines have emerged as a groundbreaking technology, offering an effective, safe, and prompt response to pandemics, such as coronavirus disease 19. DNA vaccines offer greater stability and the ability to encode multiple antigens compared to messenger RNA vaccines. They also boast lower-cost manufacturing, enhanced thermostability, and easier distribution than conventional vaccines. Additionally, they induce robust preventive and therapeutic immune responses by presenting antigen genetic information to antigen-presenting cells (APCs), making them a promising candidate for cervical cancer treatment. The innovative minicircle DNA (mcDNA) vector overcomes some limitations of plasmid DNA (pDNA) through an advanced in vivo recombination process, which excises prokaryotic sequences once plasmid DNA amplification is complete.

This study proposes the implementation of a biotechnological platform to obtain the first immunotherapeutic multigenic mcDNA vaccine, which encodes mutant HPV16 E6/E7 antigens (E6/E7mut). Following the construction of the parental plasmid (PP-E6/E7mut) and transformation into Escherichia coli ZYCY10P3S2T, optimization of its recombination into mcDNA-E6/E7mut was achieved through design of experiments. Subsequently, mcDNA biosynthesis was scaled up in a bioreactor. After alkaline lysis, the crude lysate was directly treated with diatomaceous earth and filtered to eliminate precipitated impurities, centrifugation steps and the need of organic solvents or high salt concentrations. The mcDNA was purified using the cadaverine monolithic column, in compliance with regulatory agency requirements. Finally, ternary delivery systems based on mcDNA/PEI/R8-mannose were developed and characterized to efficiently complex, protect and deliver mcDNA into APCs for the

proper expression of E6/E7mut antigens. This work presents a rapid, straightforward, universal, cost-effective, and appealing technology to the biopharmaceutical industry.

Acknowledgements:

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Optimization of scalable lentiviral plasmid production for GMP manufacturing

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Lentiviral vectors hold significant potential for gene therapies, necessitating efficient and scalable plasmid production. This study aimed to optimize a GMP-compliant E. coli lentiviral plasmid manufacturing process for use in a HEK 293 CMC pipeline. We systematically evaluated various chromatographic purification methods following alkaline lysis, focusing on critical design parameters including plasmid loss, reproducibility of COA parameters, culture size, buffer volume, iteration time, and process ease for our team. Our study compared diverse chromatographic systems and vendors. Results demonstrated optimal column design, CIP considerations, scale up path, and technical support from column manufacturer. These findings facilitate scalable, GMP-grade lentiviral plasmid production, supporting advancements in gene therapy applications.

Novel, synthetic DNA templates for the production of mRNA

Carmen Mañas Torres
4basebio, Cambridge, United Kingdom

The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA for use in gene therapy and vaccines. In addition to worldwide lack of capacity and long lead times, complex sequences such as long homopolymeric sequences including long polyA tails are difficult to propagate in bacteria.
4basebio has developed a proprietary, scalable, fully enzymatic synthesis process for the production of linear DNA constructs via our Trueprime™ amplification technology. The process yields DNA at 1g/L, several orders of magnitude higher than plasmid fermentation yields, allowing a small footprint using benchtop equipment.
The process is size and sequence independent, allowing for large scale production of linear DNA with high yield and purity in less than a week. Unlike plasmid DNA, 4basebio DNA eliminates contamination from endotoxins or host proteins, and excludes bacterial sequences such as antibiotic resistance genes. Complex sequences including ITRs and homopolymeric sequences are easily produced without risk of deletion or recombination.
Currently, we make 4 types of DNA, each with unique application-specific benefits. For mRNA production, opDNA™ can be used directly in IVT processes, without the need for enzymatic linearisation. Using opDNA™, we were able to achieve significantly higher mRNA yields as compared to linearised plasmid, with equivalent capping efficiency and dsRNA impurities. Proinflammatory cytokine/chemokine levels in isolated primary, human PBMCs are comparable to mRNA produced from linearised plasmid, with equivalent protein expression both in vitro and in vivo.
We have demonstrated that opDNA™ templates can be used for the production of IVT mRNA. Moreover, the technology could overcome the difficulties associated with complex polyA tails for mRNA constructs, which are inherently difficult to synthesise via bacterial propagation systems. The combination of 4bb mRNA and Hermes™ non-viral delivery platform can greatly accelerate the therapeutic development of gene therapy and vaccine programmes.

Purification of linearized template plasmid DNA decreases double-stranded RNA formation during IVT reaction

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Certest Biotec, Zaragoza, Spain

Following the COVID-19 pandemic, messenger RNA (mRNA) has transformed conventional vaccine production, leading to a surge in RNA-based therapeutics and novel scientific understandings. A key research area focuses on the formation of double-stranded RNA (dsRNA) during in vitro transcription (IVT), a significant impurity triggering cellular immune responses. Hence, there's growing emphasis on refining purification processes to eliminate dsRNA. While efforts traditionally concentrated on post-IVT mRNA purification via chromatographic methods, the impact of linearized plasmid quality remains underexplored. Plasmid production entails intricate steps like bacterial culture growth, harvesting, and filtration, often resulting in inconsistent batches with limited control over dsRNA by-products. This study delves into how purifying linearized plasmids affects dsRNA formation. Various techniques, including resin filtration and chromatographic separations, were explored. Optimizing a chromatographic method using monolithic columns with C4 chemistry proved effective, yielding homogeneous linearized plasmids, and reducing dsRNA levels in mRNA batches during IVT. This underscores that dsRNA formation is influenced not just by RNA polymerase and IVT conditions, but also by linearized template quality. Plasmid impurities could contribute to dsRNA production by providing additional templates that anneal with mRNA molecules. Therefore, the quality of plasmid purification is crucial in dsRNA generation during transcription. Further research is required to fully grasp the mechanisms and implications of plasmid-derived dsRNA, potentially reshaping mRNA vaccine development.

End-to-end solutions for IVT mRNA therapeutic development

Daniel Meng, Charles Bai
VectorBuilder, Guangzhou, China

Decades of fundamental research into mRNA have led to the development of successful vaccines to combat the SARS-CoV2 pandemic. Instant efficacy and exceptional scalability make IVT mRNA a promising therapeutic compound, especially when compared with viral vectors. Currently, there are hundreds ongoing clinical studies that have expanded IVT mRNA applications from vaccines to fields such as oncology, protein replacement for genetic disorders. VectorBuilder offers a full range of CRO and CDMO services for IVT mRNA that facilitate the transition from research stage to clinical trials. We offer extensive technical assistance to enhance vector performance, focusing on improving mRNA potency and cost-effective template production during scale-up. Custom mRNA production will be applied considering the features of the mRNA, the desired specifications, and process development outcomes. Furthermore, VectorBuilder has developed a range of purification processes, including LC, RP-HPLC, and Oligo dT chromatography, to remove undesired byproducts and material residues. We employ a well-established analysis panel to assess all critical attributes of mRNA, including integrity, impurity levels, and capping efficiency, for both in-process tests and final release. Our commitment to quality and expertise in mRNA technologies makes VectorBuilder a trusted partner in advancing mRNA therapeutics.

Towards an efficient purification process for saRNA: exciting challenges and progress

Justine Jakpou
Ziphius Vaccines, Merelbeke, Belgium

In the post-COVID world, mRNA-based vaccines are among the most interesting candidates to establish a technology platform, owing to their proven ability to respond fast to global health crises. Ziphius aims to develop a self-amplifying RNA (saRNA) platform and leverage its use to create vaccines for the prevention of infectious diseases and gene supplementation therapies for rare genetic disorders. Compared to conventional mRNA, saRNA has the advantage of having self-replicating features, as this molecule encodes a viral RNA replicase in addition to the sequence of the protein(s) of interest. Consequently, a significantly high amount of a shorter subgenomic RNA encoding the protein(s) of interest is produced, leading to increased protein expression levels at low doses.
In the realm of monolithic chromatography columns, a great tool arsenal exists for the purification of RNA. The diversity of available column chemistries enables multiple-step orthogonal purifications, ensuring high purity levels in the final drug substances. While this toolbox is widely described for mRNA, its translation to saRNA introduces a set of new challenges. These molecules are much larger than conventional mRNAs, resulting in a higher charge density, considerable heterogeneity, and enhanced sensitivity to degradation, along with diverse process-related impurities. Directly applying conventional mRNA processes is not an instant recipe for success, given the distinctive physicochemical properties of saRNA. Nonetheless, at Ziphius, a variety of strategies were adopted to surmount these challenges, some of which proved highly effective in paving our way toward a robust and efficient purification process.

The many struggles and success of the establishing a pDNA manufacturing platform

Ahmet Hilmi Tekin
BioNTech Manufacturing Marburg, Marburg, Germany

Emerging diseases and the urge for rapid and convenient vaccination led to the necessity of alternatives to conventional vaccines. DNA vaccines carry many advantages; therefore the demand of plasmid DNA as starting material is expected to rise significantly over the next years. The development of a new process in a pharmaceutical environment without a tech transfer is filled with many obstacles. Additionally, to the unknown plethora of parameters to choose as well as the mysteries behind the steps that want to be uncovered and characterized in a process, there is the uncertainty on how to decide which way to go or which path to follow.

In order to carry on the development and continuation of more mRNA-based vaccinations for diseases that could not be treated by known conventional methods as of today as well as the rapid reaction of future pandemics that might occur, the need for relatively little amounts of DNA for clinical studies and trials led to the idea of implementing a small-scale pDNA production site in order to fulfill the needs.

At the BioNTech Marburg site we established a small-scale plasmid DNA manufacturing facility where we are able to cover the process from bacterial transformation for generation of suitable cellbanks to start our fed-batch fermentation step with to fill & finish of our final product. The whole process consists of known production steps that need to be adapted to meet the needs of our process and product like the previously mentioned fed-batch fermentation of E. coli, harvest and lysis. The purification of the DNA is attained via multiple concentrations, buffer exchange steps and chromatography. All this is done in an environment which our Marburg site has already decades of experience with as a pharmaceutical production site and that leads to exciting and demanding challenges that we need to master to achieve our goal: good manufacturing practice.

In order to meet our own high expectations, we quickly came to the realization that we need to characterize and learn as much from our process as possible. One way to attain this was the PATfix pDNA analysis platform. The platform enables us to gain a quick insight into every step of our manufacturing process. The PATfix platform since has become a highly valued tool for our process development and optimization efforts.

An Integrated Perspective on mRNA Vaccine Process Development

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In the past years, mRNA vaccines have emerged as a promising technology for a panoply of applications, from prophylactic and cancer treatments to metabolic and genetic diseases. This is mostly due to the precision and perceived safety as well as flexible manufacture. The later is the most notable: mRNA is produced in a cell-free system, *in vitro* transcription reaction, IVT, where DNA template is transcribed into mRNA in the presence of RNA polymerase and nucleotides[1]. The highly defined nature of this cell-free reaction makes the synthesis process highly prone to optimisation. We have demonstrated that the IVT reaction could produce mRNA in quantities beyond 5 $g_{mRNA}.L^{-1}$ in 4 hours. Using AI approaches to automate the experiment design, we were able to produce 12 $g_{mRNA}.L^{-1}$ of reaction in just 2 hours[2]. The mRNA produced has since then been increased beyond 15 $g_{mRNA}.L^{-1}$ using molecular biology approaches. Notably, the AI based approach proved to be cost- and time-effective, as it only required a total of 60 reactions to achieve optimal reaction conditions from a total of 12 parameters studied.

With ever increasing mRNA titres the production of product-related impurities will also increase alongside. It is desired that these impurities are removed to avoid translation inhibition, and potentially for the induction of uncontrolled immune-inflammatory reactions. We have therefore explored different chromatography modalities to achieve high-quality mRNA products. Using an AI based approach, binding conditions of traditional affinity chromatography were optimised, and the capacity increased at least 4 times. Furthermore, one-step purification process was evaluated to potentially reduces significantly overall manufacturing costs. With this in mind, multimodal chromatography was used to separate of mRNA from dsRNA.

We have demonstrated that the established mRNA manufacturing process is prone to optimisation. Higher titres and new purification strategies are necessary to establish sustainable, flexible and cost-effective manufacturing process, ultimately making the vaccines affordable to all!

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[2] Rosa, S. S., Nunes, D., Antunes, L., Prazeres, D. M., Marques, M. P., & Azevedo, A. M. (2022). Maximizing mRNA vaccine production with Bayesian optimization. *Biotechnology and Bioengineering, 119*(11), 3127-3139.

Chromatography in mRNA production: from analytics to purification

Rok Sekirnik
Sartorius BIA Separations, Ajdovščina, Slovenia

The COVID-19 pandemic triggered an unprecedented surge in development of mRNA-based vaccines and other therapeutics, such as protein replacement therapy and cancer. mRNA is produced by a cell-free process based on in vitro transcription (IVT) reaction, a RNA-polymerase-catalyzed polycondensation of NTPs into a nascent mRNA chain guided by DNA template. We developed an mRNA production workflow adaptable to production from mg to multi-g scale, based on rapid at line high pressure liquid chromatography (HPLC) monitoring of consumption of nucleoside triphosphates (NTPs) with concomitant production of mRNA, with a sub 3 min read out, allowing for adjustment of IVT reaction parameters with minimal time lag. IVT was converted from batch to fed batch with automated feeding, resulting in doubling the reaction yield compared to batch IVT protocol, reaching 10 mg/ml for multiple constructs, thus decreasing the per-gram cost by up to 50%. To understand yield and cost drivers of IVT reaction, a design of experiment study of factors led to yield increase to 25 g/L in batch mode. A purification train including affinity chromatography selective for polyadenylated mRNA (Oligo dT) coupled with reverse-phase chromatography was optimized to remove IVT components (NTPs, DNA, T7), and IVT by-products, in particular dsRNA, a major immunogenic impurity which activates dsRNA-dependent enzymes and leads to inhibition of protein synthesis. Elimination of dsRNA improves translation and minimizes the activation of innate immune response. In context of clinical mRNA applications, such as neoantigen mRNA vaccines, which require as many as nine administrations, minimization of innate immune response may be critical to clinical success.

Bioproduction of messenger RNA: key benefits and challenges

Chantal Pichon
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Messenger RNA (mRNA) has emerged as a promising biopharmaceutical for a vast array of therapeutic applications. In vitro transcription (IVT) is currently the available technology for mRNA production. mRNA has a vast array of therapeutics applications, including immunotherapies, replacement of defective proteins in genetic and chronic diseases, gene editing, regenerative medicine and cell reprogramming. However, it requires a complex supply chain and costly purification process. Despite the advantage of being cell free, finding an alternative way for a cost-effective production is of interest when multiple dosing is required. There are different strategies that have been proposed to investigate whether mRNA biomanufacturing is achievable and could be a more profitable alternative. In this lecture, I will outline the different options that could be proposed for that. For each strategy, there are clear assets but also major challenges to tackle. As a showcase, I will present our Yscript project consisting in the generation of a specific mRNA bioproduction process in yeasts integrating innovative extraction and purification processes, a complete shift of paradigm compared to IVT production. The long-term vision is to significantly advance the current state of knowledge in mRNA manufacturing in order to promote the well-being and health of citizens, while reducing the economic burdens and social disparities associated with biopharmaceutical therapies.

Optimization of mRNA production and purification: characterization and controlling of post-transcriptional polyadenylation and its effect on mRNA quality in vitro

Pooja Tiwari[1], Janja Skok[2], Tina Vodopivec Seravalli[2], Sergeja Lebar[2], Ana Ferjančič Budihna[2], Polona Megušar[2], Matija Povh[2], Nina Mencin[2], Rok Sekirnik [2]

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[2]Sartorius BIA Separations, Ajdovščina, Slovenia

Recent years have witnessed an unprecedented increase in development of mRNA-based vaccines and other mRNA-based therapeutics. While mRNA therapeutics show tremendous potential, there are still technical obstacles to overcome, two of the most significant being mRNA's intrinsic instability and its immunogenicity. In order to address these challenges, several different approaches can be used, including optimization of 5'cap structure and 3'poly(A) tail length as well as optimization of purification process.

Even though DNA encoded poly(A) tail is commonly used, long (>120 nt) stretches of adenines in DNA can lead to cloning difficulties and plasmid instability during fermentation, making enzymatic post-transcriptional addition an interesting alternative. Very limited information is published on post-transcriptional polyadenylation, therefore our main focus was on improving the understanding of in vitro polyadenylation process and investigating the effects of poly(A) tail length on mRNA functional efficacy in vitro.

We developed robust mRNA synthesis and purification process based on post-transcriptional capping and polyadenylation followed by two-step chromatography purification, enhancing scalability and final product quality compared to traditional precipitation methods.

With the use of rapid HPLC analytics we were able to monitor the consumption of ATP and control the length of added poly(A) tail near real-time. With better understanding of poly(A) polymerase activity and reaction kinetics we established a correlation model of polyA tail length as a function of ATP consumption kinetics. We successfully synthesized and purified mRNAs of various discrete poly(A) sizes

ranging from 100 nt to 500 nt. Poly(A) tails were additionally characterized by RNase T1 digestion of mRNA and Oligo dT purification of poly(A) tails followed by a novel CIMac SDVB analytical method, able to separate oligonucleotides from 20 to 6000 nt. Finally, the effect of poly(A) tail length on immunogenicity, stability, and translational efficiency of mRNA molecule was tested in cellular models.

Innovating and digitalising mRNA vaccine and therapeutics production processes

Zoltán Kis
University of Sheffield, Sheffield, United Kingdom

The RNA platform technology has emerged as one of the most promising and strategic technologies for rapid global vaccination, infectious disease control, biotherapeutics development, and preparedness for future healthcare challenges. Since this is a platform technology, the manufacturing infrastructure, the product and process analytical technologies, the product-process interactions, and learnings can be re-used or transferred from existing products to new products. To reach the full potential of this disease agnostic RNA platform, we are developing a set of synergistic technologies consisting of physical production processes (enzymatic RNA synthesis, downstream purification, and lipid nanoparticle formulation), analytical technologies, computer models and software. These technologies are co-developed under a patient-centric Quality by Digital Design (QbDD) framework. In this QbDD framework, first-principle or data-driven relationships are established between the critical process parameters (CPPs) of the production process and the critical quality attributes (CQAs) of the RNA vaccine and therapeutic product. The obtained models can be used for defining the design space and for advanced automation using model-predictive control. By combining the QbDD framework with the RNA platform, vaccines and therapeutics can be developed and mass-produced faster against a wide range of diseases. However, to accelerate the development and manufacturing of RNA products, regulatory approval of the digital tools used for process and product quality control is required. A form of “pre-qualification” or “pre-approval” could expedite development and regulatory approval by re-using and computationally processing disease agnostic-prior knowledge, based on the platform nature of both the RNA vaccine manufacturing process and of the QbDD framework.

Manufacturing of mRNA for preclinical and clinical studies

Tjaša Legen, Maximilian Buff, Lena Wicke, Andreas Kuhn
BioNTech SE, Mainz, Germany

Advances in messenger RNA (mRNA)-based therapeutics have ignited optimism in the fight against a broad spectrum of illnesses and infectious diseases. mRNA therapeutics are often produced via a simple and highly adaptable in vitro transcription process, which can accelerate the development of novel drugs and their subsequent transfer to the commercial market. However, scaling up mRNA production and purification, while minimizing impurities and by products, remains challenging. Thus, manufacturing of mRNA for preclinical and clinical studies requires well designed and robust processes with well characterized analytical strategies. Our aim is to develop a highly adaptable and simplified process for a broad range of therapeutics and drugs manufactured for even the smallest groups of patients. This presentation will discuss process development and transfer of mRNA production to the manufacturing scale.

Engineering a robust and scalable mRNA manufacturing process

Bill Grier
Omega Therapeutics, Cambridge, Massachusetts, USA

In the aftermath of the Covid-19 pandemic, the rapid development and successful deployment of mRNA-based vaccines has led to an unprecedented level of interest and investment into RNA platforms. While the overall process for producing mRNA is relatively straightforward, there are still many challenges to overcome in order to scale-up and reliably manufacture high-quality mRNAs for therapeutic applications. We have developed an alternative platform process to fit within established manufacturing footprints that is capable of increased product yield, higher integrity, and improved impurity clearance. Here, we will discuss the Quality by Design approach and tech transfer considerations that were used in the development of this updated mRNA manufacturing strategy.

Next generation lipid nanoparticles for RNA vaccines and therapeutics

Dan Peer
NeoVac, Oxford, United Kingdom

I will detail the Lipid Nanoparticles (LNPs) 2.0 platform for cell specific delivery of RNA payloads. I will show vaccine applications in infectious diseases (e.g. COVID) as well as the first bacterial mRNA vaccine. In addition, I will detail the ability of our platform to entrap very large payloads including for DMD and multiple transcripts of mRNA for cancer therapeutics.

LipidBrick®: An innovative cationic lipid library for efficient and tunable mRNA-LNPs

Guillaume Freund
Polyplus, Illkirch, France

Lipid nanoparticles (LNP) have demonstrated high efficiency delivering RNA therapeutics in vivo. However, the properties of such nanoparticles obtained with conventional ionizable lipids are often hard to modulate; particularly their biodistribution profile. As a result, ionizable LNPs often predominantly end up targeting the liver. One of the current challenges in the field consists of adjusting the particle chemical composition to the targeted application. One of the current challenges in the field consists of adjusting the chemical composition of the LNPs to the intended application. Here, we characterized a library of 10 innovative imidazolium-based cationic lipids as key component of cationic LNP (cLNP). We disclose their chemical structures and demonstrate their efficacy in generating LNPs through characterization of their hydrodynamic diameters, Zeta potentials, and mRNA encapsulation efficiencies. The resulting particles display high transfection efficiencies and have little to no impact on cell viability in vitro, on HEK293 and CaCo-2 cell lines. In vivo, the biodistribution of the cLNP highly depends on the cationic lipid chemical structures, targeting mainly lungs and spleen. Among this library, we have also identified one cationic lipid as a potent additive in Moderna's Spikevax formulation for extrahepatic biodistribution.

Engineered EVs for mRNA cargo loading and delivery

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Messenger RNA (mRNA) has emerged as an attractive therapeutic molecule for a range of clinical applications. For in vivo functionality, mRNA therapeutics require encapsulation into effective, stable, and safe delivery systems to protect the cargo from degradation and reduce immunogenicity. Here, a bioengineering platform for efficient mRNA loading and functional mRNA delivery was developed using extracellular vesicles (EVs) as naturally derived nanoparticles. Engineered EVs carrying the highly specific PUFe RNA-binding domain fused to CD63 were produced in cells stably expressing the target mRNA with compatible binding sites. Using this system, the target mRNA was actively loaded into the produced EVs during EV biogenesis with loading efficiencies exceeding those previously reported for other EV-based approaches. In combination with the expression of an mRNA-stabilizing protein, PABPc, and a fusogenic endosomal escape moiety, VSVg, functional extrahepatic mRNA delivery via EVs in vivo was achieved at mRNA doses substantially lower than currently used for lipid nanoparticles. Our technology overcomes major limitations currently associated with EV-based nucleic acid delivery systems and could enable new applications for mRNA therapeutics.

Thermodynamic analysis of chromatography media and mRNA properties in oligo dT affinity chromatography

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The application of messenger RNA (mRNA) as a versatile modality for generating functional proteins to combat infectious diseases has generated significant attention in the biopharmaceutical industry. Just as with other biopharmaceutical products, in vitro transcribed mRNA presents a distinctive impurity profile, necessitating the isolation of the full-length product (FLP) during downstream processes. Oligo dT (deoxythymidine) has been demonstrated to be an effective tool for isolating mRNA molecules containing a long chain of adenine nucleotides on the 3' end referred to as the polyA tail. This feature of mRNA plays an important role in regulating in vivo translation and stability. There are many different commercial chromatography medias for purifying polyA containing mRNA. Monolith, membrane, and bead chromatography matrices are available with the dT ligand that differ in backbone chemistry, porosity, and dT homopolymer length. A methodology combining experimental and computational approaches for characterizing the impacts of mRNA attributes (sequence length, polyA length) on the different stationary phases is presented. The optimal column model was transferred to a pilot scale chromatography system to further validate the reliability of this purification method. By employing a holistic approach encompassing bench scale chromatography and thermodynamic analysis, the presented methodology offers a powerful toolset for enhancing the isolation of intact, functional mRNA molecules, thereby advancing the field of biopharmaceutical production.

All-scale continuous mRNA-LNP manufacturing

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Industrial batch-wise mRNA production was implemented in the context of the pandemic, using manufacturing solutions already available and developed for conventional vaccines. This way to proceed goes with numerous drawbacks and does not take full advantage of the benefits of this new modality.
Dillico proposes to revolutionize the way mRNA pharmaceuticals are developed and manufactured with an innovative integrated and digitized solution. All-ScaleFlow™ technology, based on a continuous manufacturing approach, enables the production of formulated mRNAs, from very small quantities for pre-clinical phases to commercial scale, with the same equipment.
During this talk we will present how All-ScaleFlow™ technology solves the substantial drawbacks of the currently implemented batch production mode and enables to remove the scale-up activity that has hitherto been extremely costly and time-consuming.
Experimental results show that this innovation leads to significant gains: superior quality of mRNA products (real-time control, confined processes, elimination of hold times, low shear stress), great flexibility in terms of capacity and a reduction of raw materials consumption through high yield.

From first concepts to production and broad application of CIM monoliths for separation of macromolecules and nanoparticles and for continuous enzymatic conversion

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First Czechoslovak/Russian patents about production of "porous methacrylate membranes for chromatography", followed by corresponding USA patents were published 1987 and 1989, followed by first publication about application of these membranes for very fast separation of standard proteins [1]. Interestingly, parallel paper about use of stocked up membranes packed in cartridges was published in the same year [2]. In early 90-ies, followed further development of methacrylate monolithic supports especi ally towards their application for separation of proteins from complex biological samples and for fast "in flow" enzymatic conversion [3,4], and their experimental use in our laboratory [3,4]. Experiments that were performed this time gave first hints about tremendous advantage of monoliths – almost complete absence of diffusion as a limited factor in separation of large molecules. Further technical solutions like sample distribution and construction of separation cartridge enabled the first commercial use of monolithic columns, that were named CIM columns [5]. Construction of cylindric columns and their use in radial flow was further important step for use of CIM columns in preparative chromatography [6, 7]. Further giant step in the right direction was demonstration of use of "Short monolithic columns for purification of proteins, DNA and viruses" [8] followed by broad use of CIM monoliths for analytical and preparative separation of nanoparticles, namely viruses, plasmids, large nucleic acid molecules, lipoproteins and large protein multimers and extracellular vesicles [9,10]. These applications are now in the focus of interest,

but the use of CIM monoliths for fast enzymatic conversion [11] and isolation of biologically active biopolymers, with the focus of human plasma fractionation [12] and removal of pathological macromolecules from biological fluids will be presented.

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Tailoring surface of chromatographic monoliths to achieve higher separation performance for large biomolecules

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Improvements in preparative chromatography-based processes are usually monitored through the parameters, such as selectivity, capacity, durability and product recovery. The focus of my presentation is on strategies to improve the recovery for expensive large biomolecules (pDNA, mRNA), where only 1% improvement of recovery for a compound priced at 100 k€/g converts into 1000 €/g of pure profit. To improve such parameters, researchers are not only dedicated to the development of innovative chromatographic methods on existing chromatographic supports, but they are also paying attention to the tailoring of the stationary phase surface by designing and coupling novel ligands.

The first example covers the grafting of the monolith surface with linear charged polymethacrylate brushes and studying the effect of the graft layer thickness and density on the dynamic binding capacity (DBC) and recovery for different types of biomolecules. DBC for model protein and pDNA increased with graft length as well as graft density and up to 10 times higher capacities could be achieved compared to nongrafted columns regardless the model type. However, the elution volume and recovery were found to be molecular structure dependent. There was a negative trend of decreasing the elution recovery for pDNA molecules with the thickness/density of the grafted layer. After graft optimization, the optimal anion exchanging (AEX) column with 1 mL bed volume was evaluated for pDNA capture from neutralized E.coli lysate. With a capacity of 13.5 mg pDNA per mL support, ≥95% elution recovery, and complete RNA clearance, the pDNA was successfully purified at loading flow rate of at least 15 CV per min.

The second example deals with a mRNA purification case study on the AEX chromatographic monolith, focusing on the recovery

improvement by introducing surface ligands enabling multimodal interactions with nucleic acids. Our newly developed and patented material enabled baseline separation of mRNA from plasmid DNA with both NaCl gradient and ascending pH gradient approach, while mRNA elution is achieved in pH range of 5-7. Concentrated elution of RNA with close to 100% recoveries and uncompromised integrity was demonstrated by purification of a model mRNA from in vitro transcription reaction mixture.

Addressing materials and resolution challenges in the 3D printing of chromatography columns

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Additive manufacturing has recently transformed the chromatography landscape thanks to its capability to fabricate porous stationary phases with perfectly ordered structure. Industrial implementation of 3D printed stationary phases must now accelerate, with the goal to offer regular morphologies with improved separation performance which can easily integrate into the production line.
This presentation will delve into the recent strides taken to tackle the two primary challenges inherent in creating chromatography columns using 3D printing technology: i) the identification of materials compatible with both 3D printing processes and chromatographic operations, and ii) the rapid, high-resolution, and large-scale printing of such materials.
The initial segment of this work will outline formulations for the direct 3D printing of chromatography columns in a single step. These materials are rooted in methacrylate chemistry, rendering them compatible with Digital Light Processing (DLP) printing technology. Columns with anion and cation exchange modalities are obtained by incorporating functional monomers in the ink formulation, obviating the need for subsequent functionalization steps. A practical application will be showcased, detailing the capture and purification of c-phycocyanin, a protein of significant industrial relevance.
The second part of the talk will focus on multiscale control of 3D printed matrices, from mm to µm to nm, to rapidly achieve tuneable stationary phases for bioseparations. A different material formulation, employing epoxy chemistry to facilitate straightforward functionalization, will be introduced. The material's development, characterization, chemical derivatization, and subsequent evaluation for capturing and separating model proteins will be outlined. Impressively, these formulations offer an unprecedented level of control over morphology at sub-millimeter scales (achieving features as small as 50 µm for linear structures and 200 µm for complex geometries) and feature tunable porosity at sub-micrometer scales.

Notably, these structures can be swiftly 3D printed in as little as one hour, enabling the creation of intricate large-scale models (up to 100 mL columns). The integration of anion and cation exchange ligands onto 3D-printed gyroid structures was accomplished, successfully demonstrating i) the separation of model proteins under dynamic conditions, and ii) the capture of proteins from a clarified cell harvest. These experiments exhibited dynamic binding capacities ranging from 5 to 16 mg mL–1 and yielded up to 86% purity in a single run.

These findings serve as a robust foundation for propelling the implementation and utilization of 3D-printed chromatography stationary phases to the forefront of practical applications.

Efficient and sustainable platform for preparation of a high-quality immunoglobulin G as an urgent treatment option during emerging virus outbreaks

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Current core plasma fractionation technology largely relies on a well-established backbone process encompassing cryoprecipitation and cold ethanol precipitation. Over the years its complexity has remarkably increased due to the implementation of additional steps, not only to improve product's purity, enhance its recovery and assure viral inactivation or removal, but also to isolate new clinically useful plasma proteins from the existing fractions. Consequently, the highest possible IgG extraction efficacy might not be achieved in such complex and lengthy fractionation protocols.
However, in specific situations, especially when immunoglobulins are the sole plasma products, current registered technologies are not appropriate for their preparation, but more efficacious, faster and streamlined ones are required. During COVID-19 epidemics, very simple technological platform for the purification of immunoglobulins G from human convalescent plasma, enriched with SARS-CoV-2-specific antibodies1, was developed on the laboratory scale. It consisted of caprylic acid precipitation of the majority of albumin, followed by 100 kDa diafiltration of the IgG-enriched supernatant for the removal of precipitating agent and low Mw proteins, and final AEX chromatography polishing in the flow-through mode which appeared very effective in depletion of unwanted immunoglobulins of other classes from the IgG fraction, as well as aggregates. Overall IgG yield of 75%, with removal of 95% of IgA and 100% of IgM, was achieved.

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Selective capture and separation of blood plasma derived therapeutic proteins on a new monolithic media CIMmultus PrimaT

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Human plasma contains thousands of various proteins in a concentration ranging from a trace to a relatively high amount (picogram to gram quantities). Each protein has a specific function in the homeostasis of the human body and a lack or a deficiency of a plasma protein might be life-threatening. During a disease process, several plasma proteins are observed to severely decrease due to consumption. A replacement therapy with purified proteins extracted from pooled human plasma is beneficial in normalizing the level and improving the disorders. Due to the limited availability and supply of human plasma, an efficient industrial extraction and purification process of human plasma derived proteins is highly desirable. Chromatographic methods have been proven to be useful in industrial-scale manufacturing of plasma derived protein products. Implementation and combination of ion-exchange, affinity and size-exclusion chromatography have allowed the development of therapeutic products with improved purity and safety for treating congenital or acquired plasma protein deficiencies in patients. In this presentation, the excellent binding selectivity of a new mixed mode monolithic media CIMmultus PrimaT in the isolation and separation of a group of plasma proteins will be highlighted. Not only monolithic media offers advantage over particle based supports on processing time, but the relatively high selectivity will allow to further improve purity and safety of plasma derived products.

Purification and glycosylation analysis of plasma proteins using 96-well monolithic plates

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Glycosylation is the process of attaching sugar molecules (glycans) to proteins during and after their synthesis. This intricate procedure influences the structure, proper folding, and function of glycoproteins. Glycans play a crucial role in facilitating protein interactions on cell surfaces, ensuring proper cell signaling and recognition. Glycosylation is a result of an interplay of genetic and environmental factors. Irregular glycosylation patterns have been linked to various diseases, including congenital glycosylation disorders, infectious diseases, autoimmune conditions, inflammatory diseases, and cancer. These patterns hold significant potential as diagnostic and prognostic biomarkers for diseases. To explore glycosylation patterns in large-scale human populations and clinical studies, extensive efforts have been directed toward developing efficient methods for glycan analysis across numerous samples, known as high-throughput methods. Chromatographic monoliths have emerged as valuable tools for purifying individual plasma glycoproteins, enhancing sample preparation throughput due to their unique chromatographic properties. Monolithic supports in a 96-well plate format have been successfully employed for the high-throughput purification of plasma glycoproteins. Subsequent glycosylation analysis has been performed in population studies, genome-wide association studies, and biomarker discovery. Drawing on a decade of experience with monolithic plates, we will present insights and findings from our studies in the realm of high-throughput glycosylation analysis.

Immobilized enzyme reactors as a cost-effective solution to enhance (bio)assay throughput

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Low-volume immobilized enzyme reactors (IMERs) are a cost-effective way to increase bioassay throughput. Over the years, different materials have been suggested as support for IMER preparation. However, short bed, high-performance monolithic columns such as CIMac™ and CIMic™ Analytical columns are among the best supports for IMER preparation, especially when integration into a downstream separative system is required and large molecules need to be analyzed. In recent years, the therapeutic field has seen remarkable progress and has moved towards larger and more complex drug structures. In this context, monolith columns with flow-through micrometer pores represent an advantageous option due to their large pore size, low limitations to mass transfer, high stability, and low backpressure.

This presentation focuses on our recent advances in the IMER field using short-bed convective interaction media (CIM™) columns, with a specific focus on IMERs that are functionalized with enzymes used in omic fields. Specifically, the presentation will primarily focus on trypsin-IMERs used for on-line protein digestion and subsequent MS-based peptide analysis, and PNGase F-IMERs for N-glycan release from glycoproteins. Both enzymes are commonly used in solution for large (glyco)protein characterization as well as within biotherapeutics quality control. The definition of the glycosylation profile is a critical aspect of biological and biotechnological drug production because it can affect drug distribution, stability, localization, and ultimately activity. On the other hand, glycoanalysis is also a challenging analytical field. To overcome the limitations of in-solution assays, tailored IMERs have been developed and optimized to achieve the best stability and performance under the best operational conditions. Moreover, short digestion/deglycosylation time (within minutes) has significantly reduced the time required for (glyco)protein processing. Lastly, insertion into specific analytical LC-MS platforms has allowed for performing in-line enzymatic reactions followed by the direct analysis of the released products.

Novel monolithic capillary columns for multimodal (reversed-phase, hydrophilic interactions, chiral and achiral) capillary electrochromatography

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In this talk, the previously developed poly(carboxyethyl acrylate-co-ethylene glycol dimethacrylate) precursor monolith (referred to as carboxy monolith) was further exploited in the preparation of stationary phases for capillary electrochromatography (CEC) bearing ligands that can accommodate chiral separations as well as achiral separation via reversed-phase chromatography (RPC) and hydrophilic interactions chromatography (HILIC). The carboxy monolith precursor was subjected to post polymerization functionalization with either (S)-(-)-1-(2-naphthyl) ethylamine (NEA) or (-) norepinephrine (NEN) chiral selectors at room temperature in the presence of N, N´-dicyclohexylcarbodiimide (DCC) in chloroform in the case of NEA or 1-ethyl-3[3-dimethylaminopropyl] carbodiimide (EDAC) in water in the case of NEN. The DCC, which is an organic soluble carbodiimide, and the EDAC a water soluble carbodiimide, permit the linkage for the amine functionality of the ligands NEA or NEN to the carboxy group of the monolithic surface forming a stable amide linkage. The NEA column thus obtained allowed not only the enantiomeric separations in the RPC mode via its chiral site but also the separation of nonpolar species via its achiral functionality offering both hydrophobic and ϖ-ϖ interactions for aromatic compounds such toluene derivatives and polyaromatic hydrocarbons. On the other hand, the NEN ligand allowed the enantiomeric separations of racemic mixtures in the HILIC mode as well as the separations of relatively polar compounds such as nucleosides and nucleotides via its polar functionalities. The dual sites of the NEA and NEN present convenient columns for the separations of a wide range of slightly polar and nonpolar chiral and achiral solutes in the RPC and HILIC modes.

Analytical toolbox for reliable characterization of extracellular vesicles

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Robust and well-established orthogonal techniques for biophysical characterization of individual EVs are required to utilize them as therapeutic and diagnostic tools. We have established capabilities to reliably characterize EV samples using three different orthogonal techniques; 1) Nanoparticle tracking analysis, a scattering and fluorescence-based technique to determine the size and concentration of nanoparticles. 2) Flow Cytometry, a fluorescence-based technique with similar principles as flow cytometry with critical enhancements to enable the effective detection of smaller particles. 3) Analytical HPLC, a semi-quantitative technology to analyze the EV samples using three different detectors: Multi angle light scattering, UV and fluorescence.
We propose performing fundamental studies using standard samples and models for both EVs and protein contaminations in the background. We comprehensively, evaluated the 3 technologies in our analytical toolbox, and compared the results with other gold standard techniques such as Cryo-EM. The relevant ratio of EVs compared to other non-EV components of the EV samples is a critical factor in reliably analyzing a crude EV samples. Here, we provide tips and guidelines to the researchers in the field to identify the limitations of any analytical/single particle analysis technology to ensure reliable characterization.

Where does the cell and gene field need to go to thrive? Critical considerations as you move your programs forward.

Andrew Knudten
Advanced Medicine Partners (AMP), Durham, North Carolina, United States

The gene and cell therapy world today is vastly different than the world we were living in a few short years ago. Of course, some fundamental aspects remain unchanged – paramount among them, there remains a massive potential to have a positive impact on patients, families, caregivers, and our overall health care system. However, some critical aspects of our industry have changed significantly. Just a few years back, suboptimal product quality standards were accepted in order to deliver transformative products to patients that desperately needed them. This risk/benefit profile was warranted, and these products have positively impacted and saved countless lives. Consequently, the field has also observed a number of patient safety issues that are directly related to suboptimal product quality in programs currently in the clinic moving towards commercialization. These programs will require solutions before they proceed any further and new programs need a better path from the start.

Recent advancements in technology allow us to make step changes in the quality of gene and cell therapy products while also delivering products under the timelines that patients are hoping for. These technological advancements have helped to shift the risk/benefit calculation for both regulators and patients. We no longer need to accept suboptimal product quality in order to deliver these gene and cell therapies to patients. As we look to provide solutions for more common diseases, the importance of product quality and safety will be even more critical.

As a collective industry, we have dramatically improved our approach to analytical testing, starting materials, nucleic acids, and cell lines, and developed elegant manufacturing processes that prioritize driving down product impurities and producing functional full capsids. Regulators are also willing to work with product developers to implement next generation processes for products at all stages of development. Because of these improvements, product developers should only be satisfied with a CMC approach that delivers what patients deserve and regulators expect.

Key parameters to improve your AAV production

Marine Guise
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Adeno-associated virus (AAV) vectors have emerged as the most effective delivery vector for gene therapy due to their efficiency to target different organs using specific serotypes and their low immunogenicity. During the AAV production process, many steps (incl USP/DSP) require process optimization and provide opportunities for innovative solutions to optimize yields for process economics. Those optimizations must be scalable from small to large scale to reach higher titers and higher viral particle quality (full viral particle). Thus, over the past few decades, extensive efforts to optimize AAV vector production processes have been made. However, further optimizations are still needed to increase production rate and vector yield in a minimal cell culture volume. Among all raw materials and transfection conditions, we show that the transfection reagent, cell line, plasmid design and plasmid ratio can drastically impact the quality of AAV vector. Also process parameters such as pH, temperature and gazing strategies are important and can be game changer on the road to high titers. This presentation will review our technological advancements, to improve AAV productivity and quality, and to propose novel strategies to build upon this research.

New approaches for viral vector genome integrity evaluation

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In the production of viral vectors for gene therapy, the emphasis is on pure, safe, and effective products. The absence of impurities and the presence of a complete vector genome play a crucial role. Currently the focus is still on analysing the ratio of full and empty viral vector capsids, although this does not provide information on the actual capsid content. This brings us to one of the emerging attributes of viral vectors, namely the integrity of the viral genome. The recombinant viral genome contains all the information required for the therapeutic effect of the manufactured construct (e.g., promoter, enhancer, gene of interest, polyA tail). If the viral genome is not complete, no therapeutic effect or a much lower therapeutic effect can be expected; moreover, such fragments may generate neoantigens in cells leading to unexpected immune responses. The integrity of the viral vector genome can be assessed by several methods, each of which has its advantages and disadvantages. We have addressed the problem of AAV vector genome integrity by developing an advanced dPCR multiplex approach. We have shown that different conditions in upstream processes can lead to up to fourfold differences in the amount of full-length vector genome. We are also developing other new approaches that do not involve PCR amplification. Existing methods such as dPCR multiplex and long read sequencing will be presented and discussed in parallel with the approaches under development. The goal of all methods is to provide an accurate quantitative result on genome integrity. Their application in sample testing could better guide process development with the goal of obtaining as many full-length vector genomes as possible.

Development of capture and polishing chromatography steps using Sartorius CIM monolithic chromatography technology for a novel AAV serotype

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Chromatography techniques for the purification of common Adeno-Associated Virus (AAV) such as AAV1, AAV2, and AAV9 are generally well understood and require product-specific optimization. AAV affinity resins are commonly evaluated for primary chromatography of the common serotypes; however, function, capability, and performance with novel or less common serotypes is a gap. For polishing chromatography, the primary objective for an AAV process is enrichment of full capsids, where anion exchange chromatography (AEX) is commonly used in a bind and elute mode. A common challenge with the enrichment of full capsids is achieving the necessary resolution to convert the elution to a step gradient while not sacrificing recovery or purity. Here, a comparison of a commercially available affinity resin to Sartorius CIM SO3 for capture chromatography was completed, demonstrating the Sartorius CIM SO3 as beneficial option for capture chromatography of a novel AAV serotype. The study demonstrates a >40X improvement in recovery when compared to the affinity resin as well as an improvement in processing time. A polishing step was developed utilizing the Sartorius CIM QA demonstrating a step gradient elution is achievable while maintaining high recovery and enrichment of full capsids.

You don't need affinity to purify rAAV Part 2 & beyond empty/ full separation

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Improved analytical methods allow us to better understand the capsid heterogeneity in AAV therapeutic preparations. This better understanding allows us to develop manufacturing processes that produce AAV therapeutics with fewer non-functional capsids. Four different two-column processes at 50-L scale will be compared to our 200-L ultracentrifugation (UC) process for yield, purity, and potency. All five runs were derived from the same triple-transfected, 500-L, HEK-293 suspension-culture. We consider the UC process the gold-standard for functional-capsid purification. Simplified process descriptions for the capture and empty/full (E/F) separation steps are listed below along with the analytical methods used to characterize these drug substance lots.

Process Descriptions:

1. Capture: CEX monolith (NaCl gradient), AEX filter (flow-through), E/F: UC
2. Capture: CEX monolith (NaCl gradient), E/F: AEX monolith (NaCl gradient)
3. Capture: CEX monolith (pH gradient), E/F: AEX monolith (NaCl gradient)
4. Capture: CEX monolith (pH gradient), E/F: 2-cycle AEX (NaCl Gradient)
5. Capture: Affinity resin (pH step gradient), E/F: AEX monolith (NaCl gradient)

Analytical comparability of Drug Substances:

1. Vector yields: total vg by ddPCR and total capsids by ELISA
2. Process Impurities: Host-cell protein, plasmid-DNA, and host-cell DNA

3. Product Impurities: Empty, partial, & full capsid content by SV-AUC, CDMS, and Mass Photometry, aggregation by size exclusion chromatography, and capsid protein charge heterogeneity by icIEF
4. Vector protein purity: CE-SDS (capillary electrophoresis);
5. Vector DNA purity: DNA gel electrophoresis, long-read and short-read NGS
6. Potency: *In vitro* eGFP expression

Sedimentation-velocity AUC analysis showed that the UC method and three of four two-column processes produced AAV9 Drug Substances with > 90% full capsids.

Advancing AAV empty/ full capsids separation with ExoReady™ platform: harnessing the power of monolithic columns

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Exothera, Jumet, Belgium

ExoReady™ is a viral vector technology platform that offers an off-the-shelf solution for producing adeno-associated viruses (AAVs). Within this process, one of the biggest challenges lies in achieving an efficient polishing step to enrich full capsids. During the production phase in the bioreactor, the majority of expressed AAVs lack the transgene, rendering these empty capsids devoid of therapeutic potential and classifying them as product-related impurities requiring removal. This task is often accomplished through anion exchange chromatography. However, separating empty and full capsids is challenging due to their closely matched physicochemical properties, often resulting in overlapping peaks and a complex trade-off between yield and purity.

In this study, we delve into how a CIM QA AAV2 polishing protocol was improved through upstream modifications. AAV recovery increased from 45% to 75%, while maintaining a three-fold enrichment factor. The same protocol proved successful when applied to serotype 8.

Furthermore, we conducted comprehensive process characterization and optimization using a Design of Experiments (DoE) approach, ultimately achieving 80% recovery and 80% full capsids in the final eluate. Notably, MgCl2 was identified as a critical process parameter. The protocol underwent a transition from a linear gradient to a step elution while preserving its high performance.

New monolithic modalities, such as PrimaT and PrimaS, were tested and compared to CIM QA. It is demonstrated how performance was maintained while improving separation power to facilitate the transition to step elution.

The work performed encompasses the characterization of elution peak fractions and an understanding of how the initial feed quality impacts the polishing chromatography step. Additionally, it highlights the utility of HPLC-AEX as a rapid tool to support process development.

GET+VVIRAL: a purification toolbox for the gene-editing tools and viral vectors

Stefano Menegatti
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Gene Editing Products (GEPs) are poised to become the focus of next-generation treatment of severe cardiovascular, muscular, metabolic, neurological, hematological, and ophthalmological disorders, infectious diseases, and cancer. In this context, CRISPR nucleases (e.g., Cas3, Cas9, and Cas13) and viral vectors (e.g., adenovirus, adeno-associated virus, and lentivirus) are playing a growing role owing to their superior targeting and therapeutic efficacy. The research on increasing the potency and safety of GEPs is progressing at outstanding pace, with 100s new products accessing the clinical pipeline every year. Along with medical uses, GEPs are successfully applied in numerous biotech fields, including the development of new biomaterials, plants, and livestock. As a result, the GEP market is undergoing an explosive growth, with a revenue generation estimated at $20B by 2030, while GEPs are projected to become mainstream therapeutics by 2050, impacting 60 - 80 million patients per year worldwide.
With such explosive growth, GEPs are posing critical questions to the biomanufacturing industry: how to produce affordable, high-quality nucleases in sufficient amount to supply the global biotech and biopharma demand? How to manufacture disease-/patient-specific viral vectors affordably? When is continuous manufacturing of GEPs needed and how can it be achieved? In response to these challenges, our team has introduced a portfolio of purification tools for next-generation biomanufacturing of GEPs. In this talk, I will present our current efforts: (1) LigaGuardTM adsorbents for continuous purification of protein and virus therapeutics by capturing process-related and product-related impurities via \"flow-through affinity chromatography\"; (2) SMART technology for the purification of CRISPR ribonucleoproteins, namely Cas nucleases primed with the desired guide RNA; and (3) adsorbents for the serotype/pseudotype-agnostic purification of AAVs, AdVs, and LVVs.

Two streams, a single process: optimizing AAV production for improved purification performance

Ivana Petrović Koshmak
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Maximizing the production efficiency of adeno-associated virus (AAV) vectors while maintaining the final product's purity and safety remains a critical challenge in the gene therapy field. Yield and impurity compromises made in the upstream stage of the bioprocess can have a detrimental effect on downstream success. In this study, we demonstrate how the PATfix AAV Switcher and other analytical tools may be utilized to optimize upstream production and enable in-process monitoring throughout the production process. To determine the effectiveness of upstream optimization in reducing key impurities, we compare two bioreactor-scale production runs in a downstream process that uses CIMmultus SO3 capture and CIMmultus QA polishing steps.

Limitations of various analytical techniques in characterizing recombinant adeno-associated virus empty capsids

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Adeno-associated viral vector (AAV) has demonstrated its efficacy for gene transfer in vitro and in vivo, establishing its prominence as a safe viral vector for clinical applications. Nevertheless, a recent tragic incident in the Duchenne muscular dystrophy clinical trial has highlighted concerns regarding the safety of AAV gene therapy. Adverse events in this trial could be linked to high-dose AAV, triggering an elevated immune response. Empty capsids, which lack vector genomes and therefore lack efficacy, are considered product-related impurities. Detecting these empty capsids in final AAV products has become a focal point of interest. Various methods such as HPLC-anion exchange column (AEX), transmission or cryo-electron microscope (EM), size-exclusion column-multi-angle light scattering (SEC-MALS), analytical ultracentrifugation (AUC), mass photometry, and charge-detection mass spectrometry have been explored for this purpose.

In a previous study, we compared the ratio of empty capsid to full capsid in highly purified AAV8 using HPLC-AEX, EM, AUC, and SEC-MALS. HPLC-AEX, EM, and AUC failed to detect empty capsids, whereas SEC-MALS identified 6.5% empty capsids. Notably, empty and full capsids are eluted simultaneously in SEC-MALS, indicating the challenge of differentiation. Further analysis using AXE-MALS yielded similar results, suggesting that the separation matrix did not significantly impact the variation in the empty capsid ratio.

Recognizing the known variability in AAV capsid content, we isolated high-density AAV8 (HD-AAV8) by the CsCl ultracentrifugation. Surprisingly, all methods except AEX-MALS detected no empty capsids. AEX-HPLC detected 1.6% empty capsids, emphasizing the limitations of individual methods in accurately determining empty capsids. The findings underscore the necessity of employing multiple orthogonal methods to independently confirm Critical Quality Attributes, ensuring the precise determination of empty capsids.

Clinical potential of MSC-EVs and translational challenges

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Human mesenchymal stromal cells (MSCs) are a therapeutically relevant, heterogenous cell entity with immunomodulatory and pro-regenerative potentials. Apparently, MSCs mediate a huge proportion of their therapeutic effects via extracellular vesicles (EVs). Connected to several advantages in using cell-free products for the therapeutic setting, MSC-EVs emerged as promising novel therapeutic agent for various diseases, including graft-versus-host disease (GvHD), ischemic stroke, COVID-19 and sepsis.

It is our current mission to optimize the MSC-EV production strategy in a scaled, GMP compliant manner, and to set up an appropriate quality control platform to translate MSC-EVs into the clinics. One of the challenging aspects in this context is inherited from the MSC field, i.e. contradictory reports on the efficacy of MSC therapies. Apparently, not all MSC products mediate therapeutic effects when applied into patients. Similarly, we observe functional differences among independent MSC-EV preparations; even when same MSC stocks were used as starting material. Thus, to avoid draw backs as they occurred in the MSC field by failing to show efficacy in a phase III clinical trial for GvHD treatment, it is one of our most important missions to address and appropriately handle the heterogeneity aspect. To this end, we have set up a lentiviral, hTERT-based immortalisation strategy and raised MSC lines at the clonal level. EVs released by these clonally expanded immortalized MSCs (ciMSCs) reveal immunomodulatory activities and confer therapeutic activities in vivo. According to our understanding, we thus have fulfilled an essential milestone towards scaled and standardized production of MSC-EV-based therapeutics.

The next milestone to be achieved is the definition of appropriate upstream (USP) and downstream processing (DSP) strategies. For now, for the production of our MSC-EV products, MSCs are raised in 10% human platelet lysate (hPL)-supplemented media. Since sizes of hPL batches limit the scalability of our products and also challenge DSP procedures, we are currently searching for GMP-compliant media supporting both, massive ciMSC expansion as well as the secretion of potent EVs.

Leveraging new CIMmultus QA HR for successful AAV polishing - Optimization and scale up

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Adeno-associated viruses (AAV) have emerged as leading vectors for gene therapy applications due to their low pathogenicity and ability to mediate long-term gene expression in a wide range of tissues. In each AAV downstream process, one of the key steps is enrichment of full capsids. To achieve a robust chromatographic separation of empty, partial and full capsids, one needs to establish a highly reproducible process. To address the recent needs Sartorius BIA Separations developed new CIMmultus HR line (HR stands for High Reproducibility) columns with better resolution and narrower acceptance criteria. These columns allow for batch-to-batch and scale-to-scale reproducibility within just 3% of the AAV8 empty capsid iso-conductivity elution. Such columns allow for step gradient elution using the same buffer strength at any scale. A complete enrichment process design on AAV8 case study will be presented. Starting from initial screening of conditions using 96-well plate or small monolithic columns, following with the determination of column capacity, considerations for formation of step gradient and finally a successful scale-up from 1 ml to 80 ml unit.

Monoliths to bridge the gap between purity and yield in viral vectors downstream processing

Hana Jug
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Convective Interaction Media monoliths are proven tools for the purification of large biomolecules, such as viruses, virus-like particles, and nucleic acids. Whether for vaccines, cancer immunotherapy, or gene therapy, products need to meet certain levels of purity and concentration. Downstream processes that are based on monolithic chromatography turned out to be very efficient in achieving those needs. This was confirmed with many cases and different types of viruses including Lentivirus, Influenza virus, NDV, and Adenovirus. Four different case studies of virus purification will be presented.
Lentiviral vectors can be successfully purified on anion exchange column, CIMmultus QA with a 6 µm channel size. Chromatographic purification of Lentivirus, as well as associated PATfix analytics, with recoveries up to 60 % will be presented as the first case study. The Influenza virus downstream process will be demonstrated with a combination of purification and formulation in one chromatographic step, with a yield exceeding 75%. The developed process using CIMmultus SO3 is simple and robust and can be used with minor adjustments for the purification of Newcastle Disease Virus (NDV). The last case study will present Adenovirus chromatography purification using the CIMmultus QA monolith column.

Navigating successful characterization of AAV vectors: key steps to success

Alicja Fiedorowicz
Dark Horse Consulting Europe, London, UK

This talk will provide an in-depth exploration of the critical steps involved in the successful characterization of AAV vectors. It delves into the complexities of navigating through the latest regulatory guidance and industry trends, offering insights into how researchers can stay ahead of the curve in this dynamic landscape.
Moreover, the talk will highlight various technologies available for AAV vector characterization and discusses best practices for assay setup, execution, and tech transfers. Additionally, it's important to selecting appropriate assays and technologies that are suitable for GMP environments, ensuring assays being 'fit-for-purpose' and regulatory compliance throughout the development and manufacturing processes.
By outlining these essential steps and best practices, this talk equips attendees with the knowledge and tools necessary to navigate the complexities of AAV vector characterization successfully.

Orthogonal chromatography analytics to allow for faster process development and better in-process control

Aleš Štrancar
Sartorius BIA Separations, Slovenia

In-process analytics are essential tools for process engineers who aim to visualize process-related execution data, providing a clear representation of the process flow. These tools are also crucial for monitoring and improving process execution. The production of biomolecules is a highly complex process, and the analytical data obtained can sometimes be misleading, potentially leading to incorrect conclusions. To reduce errors and enable faster and more cost-effective biomanufacturing processes, the use of orthogonal analytical methods is necessary. Chromatography stands out as the orthogonal method of choice when compared to molecular biology methods. This presentation will discuss examples of two-dimensional liquid chromatography in-process methods that are applied in the production of viruses and lipid nanoparticles (LNPs).

Gene therapy for retinal disorders - achievements and remaining challenges

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Inherited retinal disorders (IRDs) are severe eye diseases that lead to visual impairment and even blindness. IRDs are caused by defects in genes encoding key proteins of the visual process, most of which are specifically expressed in photoreceptors or retinal pigment epithelial cells (RPE). With the advent of efficient gene therapy vectors based on recombinant adeno-associated viruses (AAV), it is now possible to develop potentially curative therapeutic approaches for these previously incurable eye diseases. Since 2012 seven AAV-based gene therapies have been authorized and one of them addresses an IRD caused by mutations in the RPE expressed gene RPE65. Additional gene therapies for IRDs are expected to be approved, as more than 30 clinical trials for retinal gene therapy are currently underway. Despite these achievements, challenges remain, and technological improvements are needed to treat all IRD, especially those that are autosomal dominantly inherited or caused by mutations in large genes.
In my presentation, I will provide an introduction to AAV biology, discuss current therapeutic approaches for gene therapy of ocular diseases, and highlight key unmet needs. I will then present new technologies that are currently being developed to address the remaining challenges.

Key points of process development and characterization of AAV gene therapy products

Ella Chan
Skyline Therapeutics, Shanghai, China

rAAV has demonstrated great promise as a DNA-delivery vector to treat serious human diseases. Skyline Therapeutics has two lead AAV programs that entered clinical phase in both US and China. We established fully integrated AAV platform from research to CMC which allows us to implement a well-designed and wholistic strategy for process development, quality control, and product characterization to ensure product safety and efficacy. This talk is to share key considerations for process development and characterization of AAV vectors for investigational gene therapy product development.

Vesicles and vehicles; EVs on the road to clinics

Jens Gruber, Stefan Schneider, Cyrille Girard, July Buitrago, Jagan Mohan Kaipa, Sarah Morris, Astrid Backhaus, Anna Botvinnik
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Extracellular vesicles (EVs) derived from mesenchymal stromal cells (MSCs) have garnered significant attention in recent years due to their diverse molecular cargo and their potential therapeutic applications. These EVs, ranging from exosomes to microvesicles, serve as vital mediators of intercellular communication, facilitating the transfer of proteins, lipids, and nucleic acids between cells. Here, we explore the emerging understanding of MSC-derived EVs and their roles in modulating various physiological and pathological processes, including inflammation, tissue repair, and immunomodulation. Furthermore, we highlight the promising medical applications of MSC-EVs in regenerative medicine and discuss manufacturing steps starting with iPSC-derived MSCs in different stress conditions in the USP and enrichment of relevant EV-subpopulations in the DSP. Understanding the complex interplay between MSC-derived EVs and recipient cells holds immense potential for the development of innovative therapeutic strategies aimed at addressing unmet medical needs. In addition to the MSC-EV work we will briefly touch our recent development on additional therapeutic and cosmetic nano-tools, such as plant derived EVs for repair of aging skin or viruses and virus-like particles for directed drug delivery in a personalized medicine setting.

Separation and functional characterization of stem cell-derived exosomes from scalable manufacturing processes

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Stem cells and stem cell-derived products, such as extracellular vesicles (EVs), provide an outstanding therapeutic perspective for multiple diseases. To ensure translation into viable products, established lab-scale cell proliferation procedures have to be evolved into scalable manufacturing processes. Consistent and scalable cultivation of sensitive adherent cell lines, such as multipotent Mesenchymal Stromal Cells (MSCs), is challenging. Phoenestra has developed a stable process that uses telomerized MSC (MSC/TERT) lines and cell carriers inside an agitated packed bed bioreactor system under serum-free conditions. With this system, we are able to produce clinically meaningful EV quantities in small bioreactor volumes and in a fairly short amount of time. The downstream process results in high yields of EVs, determined and characterized by a combination of analytical methods. The PATfix™ analytical system was used to evaluate the composition of the samples and track several surface markers of EVs. Produced EVs show relevant biological functionality e.g., in cell-based anti-inflammatory or anti-fibrotic bioassays. In addition, bioassay results are put into the perspective of protein and miRNA profiles analyzed from EV preparations harvested from different MSC/TERT. In this presentation, we will show results of in-depth analytical characterization of samples processed from EV production runs, with the goal of getting closer to identifying mediators of biological activity. In our view, these insights will help product definition and accelerate clinical translation in the future.

Mechanistic modeling for the purification of a replicant-competent enveloped virus on a monolithic ion-exchange chromatography18

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Virus particle based therapies are on their way to market due to significant advancements in safety and efficacy the last decades. Challenges in analytical methods and manufacturing are persisting and are needed to be addressed in process development. Mechanistic modeling can support the development work by increasing process understanding and digital optimization of process parameters. In this work the development of a mechanistic model of a chromatographic purification step for a replicant-competent enveloped virus is presented. The model describes the bind-and-elute behavior on a monolithic ion-exchange column using salt as displacement eluent. A scale-down approach in combination with an online light scattering detector was used to acquire required calibration runs for parameter determination. A virus particle quantification based on HPLC-SEC separation and UV detection was used for offline analysis of fractions and mass balancing. The determination of model coefficients is carried out by iterative parameter estimation using error norm and generic optimization algorithms. The feasibility and its limitations in mechanistic modeling of enveloped viruses are demonstrated.

The CCX cell line, a novel avian substrate for efficient virus production and seamless (CIM based) virus purification

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Nuvonis, Vienna, Austria

A novel avian cell line termed CCX was developed by Nuvonis. This quail-derived cell line was generated without the use of foreign genes and viral sequences an therefore CCX is a non-GMO cell line. The CCX cell line grows in animal component free commercially available medium and is available as suspension cell line termed CCX.E10 or as anchorage-dependent cell line termed CCX.2C4.
The CCX cell line was established by sub-passage of primary embryonic quail cells (Coturnix japonica) using low concentrations of pharma-grade serum for the first passages. Single cell clones were isolated and propagated, and adaption to growth in suspension was carried out leading to CCX.E10 suspension cells.
Both cell lines exhibit stable growth over multiple passages at doubling levels of 0.8-1.0 per day. To use the CCX cell line for manufacturing of vaccines and vectors, large pools of GMP master cell bank and working cell bank ampoules were generated and extensively tested according to ICH guidelines. This includes the testing of tumorigenicity at the end of production generation level.
CCX are very robust and can be grown in various standard cell culture systems (e.g. shaker, spinner, cell factories or bioreactors) to high cell densities. Quail derived cell lines can support the growth of a broad range of virus families (Poxviridae, Herpesviridae, Reoviridae, Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, Flaviviridae).
Here examples for New Castle Disease (NDV) and Vesicular stomatitis virus (VSV) will be presented. Both NDV and VSV are the basis for promising vectors against SARSCoV-2, Ebola, H5N1 influenza, West Nile, and simian immunodeficiency viruses. Oncolytic NDV and VSV vectors also hold much promise for immunotherapies against various cancers.
In CCX cells, NDV and VSV can be grown to high titers of up to 9 log TCID50/ml. The successful application of monolithic columns for the purification of CCX derived viruses with high yield and purity was demonstrated, providing a very fast and efficient downstream process.
The CCX cells are of great potential for future manufacturing of a range of viruses and vectors in scalable systems, and offer manufacturing solutions that are independent of SPF chicken-egg supply.

delNS: Harnessing interferon induction and antigen expression for enhanced immunotherapy against cancer

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The use of viral vectors for cancer immunotherapy has garnered significant attention due to their ability to induce potent immune responses against tumor cells. Here, we present delNS, a novel influenza-based viral vector designed to simultaneously induce interferon production and express foreign antigens within the tumor microenvironment. This unique combination fosters an immunologically active milieu, augmenting the immune system's ability to recognize and attack cancerous cells.

Interim results from a phase 1 clinical trial targeting cervical neoplastic lesions highlight the promising safety and efficacy profile of delNS. Notably, delNS demonstrated a favorable safety profile, with only mild and moderate transient adverse events reported, and no treatment-related serious adverse events observed. Moreover, a substantial number of treated patients exhibited elimination of neoplastic lesions alongside the causative papillomavirus.

The efficacy of delNS extends beyond cervical neoplasms, showcasing remarkable outcomes in equine sarcoid tumors, a challenging-to-treat veterinary malignancy. In a cohort of 29 horses, delNS treatment led to significant tumor regression in 20 cases, with 16 achieving complete response. Importantly, the absence of tumor recurrence over a 3-year follow-up period underscores the sustained therapeutic benefit of delNS.

Overall, delNS represents a promising therapeutic avenue for overcoming cancer immunosuppression and eliciting robust anti-tumor immune responses, offering hope for improved outcomes in cancer patients.

Efficient purification of lymphocytic choriomeningitis virus using monolithic chromatography

Binod Prasad
Abalos Therapeutics, Düsseldorf, Germany

Virus-based cancer immunotherapies have received increased attention in recent years. Abalos Therapeutics has generated lymphocytic choriomeningitis virus (LCMV) strains with optimized anti-tumoral properties that induce a strong and persisting innate and adaptive immune response against the cancer cells including distant metastases. The mode of action of LCMV immunomodulatory virotherapy therein differs from other novel oncolytic virus therapies. As a member of the Arenaviridiae family, LCMV is a 60 - 300 nm enveloped virus with a bi-segmented genome made up of two single-stranded negative sense RNAs. Abalos Therapeutics has developed a robust upstream manufacturing and downstream purification process to enable safe and high-quality production of LCMV at different scales to support preclinical and clinical studies. This presentation covers the development of monolithic cation-exchange and hydrophobic-interaction chromatography for the purification of LCMV from clarified cell culture media. The chromatographic purification process, which has been the bottleneck during LCMV purification scale-up, was further streamlined to meet the quality and purity requirements set by regulations. Reliable analytical techniques were employed to ensure precise monitoring of the processed material. Both upstream and downstream processes have been defined and consolidated at the scale and successfully transferred to the CMO for manufacturing at the clinical scale.

Process development for an oncolytic rhabdovirus

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ViraTherapeutics, Rum, Austria

Vesicular stomatitis virus (VSV) is a rhabdovirus with excellent potential to function as an oncolytic viral therapeutic due to its potential to infect many different types of cancer cells where it replicates quickly and results in their destruction. There is little preexisting immunity in humans against VSV and rare natural infections are generally asymptomatic. Wildtype VSV is neurotoxic in rodents and non-human primates. However, this limitation can be circumvented by replacing the VSV glycoprotein (GP) with that of the non-neurotropic lymphocytic choriomeningitis virus (LCMV).
At ViraTherapeutics we have developed a scalable CMC process for the production of VSV-GP from suspension cell culture using a combination of different chromatography and filtration steps. The resultant drug substance contains only low levels of impurities and high infectious titers suitable for use in clinical studies.

Young Researcher's Corner

Development of immobilized enzyme reactors on monolith backbone for rapid plasmid DNA linearization

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The manipulation of plasmid DNA (pDNA) plays a pivotal role in different applications of molecular biology and genetic engineering. For example, linearized pDNA is essential tool in in vitro transcription reaction for messenger ribonucleic acid (mRNA) production. Immobilized enzyme reactors (IMERs) stand as innovative biotechnological constructs, seamlessly merging the catalytic proficiency of enzymes with the advantages of solid support matrices. Macroporous monolithic stationary phase, characterized by highly interconnected channels, highly accessible surface area and efficient mass transfer properties provides an ideal backbone for enzyme immobilization. Opting for immobilized enzymes over enzyme solutions offers notable benefits such as improved stability, the potential to operate within a continuous system over extended durations, reusability of the enzyme, as well as reduced production costs and product purification steps. The aim of this study was to prepare a functional IMER on monolithic support for efficient pDNA linearization, that could be used in in vitro transcription reaction for mRNA production. The restriction enzyme EcoRI was selected as a model molecule. This enzyme recognizes G^AATTC sites, hence, a 6.7 kbp large pDNA with such motif was selected as substrate for assessing enzyme activity.

We immobilized EcoRI restriction enzyme on monolithic supports exploiting different coupling strategies. A chromatographic method using CIMac pDNA analytical columns was developed in parallel to assess the degree of pDNA isoforms linearization. Kinetic parameters of immobilized enzyme and enzyme in solution were compared to assess enzyme functionality upon immobilization and optimal reaction conditions were defined as well as storage conditions enabling suitable IMER half-life.

Based on collected data and within optimized conditions, a suitable linearization is achieved in about 10-15 minutes and the IMERs reusability goes up to at least one month. The EcoRI- IMER activity was also assessed by performing in vitro transcription reaction for mRNA production.

Quality by design for mRNA platform purification based on continuous oligo-dT chromatography

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In this study, a fully continuous multi-column oligo-dT chromatography system was developed for the downstream purification of mRNA-based vaccines and therapeutics, which significantly improves the productivity and cost-efficiency. The optimisation of loading parameters including salt type, salt concentration, load flow rate and RNA load concentration both in batch and continuous mode was conducted to achieve >90% yield, >95% RNA integrity and >99.9% purity. The productivity was estimated 5.75-fold higher, and the operating cost was estimated 15% lower when comparing continuous to batch chromatography. Moreover, a QbD framework was established and the relationship of critical quality attributes (CQAs) & key performance indicators (KPIs) as a function of critical process parameters (CPPs) & critical material attributes (CMAs) was assessed.

Monolithic columns enable streamlined downstream processing and analytics of lipid nanoparticles

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Lipid nanoparticles (LNPs) have emerged as the foremost non-viral carrying vehicles for therapeutics, capable of encapsulating various payloads.
Compared to traditional viral delivery systems, LNPs provide significant advantages such as modularity, rapid production, and superior scalability. However, their modularity introduces complexities in purification and characterization due to diverse properties of different LNP formulations.
Post formulation, LNPs require downstream processing to ensure the formulation is applicable for in vivo applications and adheres to critical quality attributes (CQAs). Analytical methods for process monitoring and quality control (QC) of LNP production are mostly offline and require extensive sample pre-treatment.
In response, we have developed innovative chromatographic techniques that are widespread, easy to use, automatable and customizable.
More specifically, we introduce a method for purification of LNPs on monolithic columns, that is fast and maintains LNP integrity, while also concentrating particles. This method is also capable of achieving separation of different biomolecules allowing separation of LNPs from its free cargo. It enables purification and concentration of LNPs, while at the same time avoiding shear forces and membrane interactions. Column modifications and buffers can be tuned to achieve the desired separation.
On an analytical scale, similar chromatographic methods can be used to monitor LNP production and purification. Important characteristics can be determined, such as encapsulation efficiency, nucleic acid quantification, size distribution and lipid composition.

Optimization of in vitro transcription reaction with design of experiments

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In vitro transcription (IVT) reaction is an RNA polymerase-catalyzed production of mRNA from DNA template, and the unit operation with highest cost of goods in the mRNA drug substance production process. In order to decrease the cost of mRNA production, reagents should be optimally utilized. Due to the catalytic, multi-component nature of the IVT reaction, optimization is a multi-factorial problem, ideally suited to design of experiment (DOE) approach for optimization and identification of design space. We derived a data-driven model of the IVT reaction and explored factors that drive process yield, including impact of NTP concentration and Mg:NTP ratio on reaction yield and how to optimize the main cost drivers RNA polymerase and DNA template, while minimizing dsRNA formation, a critical quality attribute (CQA) in mRNA products.

Poly(A) tail length determination with UV detection using ion pair reversed-phase liquid chromatography

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The most distinct feature of the messenger RNA (mRNA), used as a therapeutic agent, is its polyadenylated (poly(A)) tail, which enables translation and provides stability to the molecule. The presence and length of the poly(A) tail are the key parameters of the drug substance mRNA as proposed by the US Pharmacopeia guidelines.
In this work, an analytical method to determine the length of poly(A) tails was developed. Poly(A) tail is cleaved from mRNA using ribonuclease (RNase T1), purified with affinity on oligo dT monolith, and analysed using the PATfix® analytical system with the CIMac SDVB reverse phase analytical column. The present approach enables same-day sample treatment, purification, and analysis.
The newly developed IP-RP analytical method provides poly(A) tail analysis using only UV detection. The 30-minute analytic is based on hydrophobicity-independent elution of mRNA-like species; therefore, the length is easily determined through the retention of commercially available RNA ladders. The method is sensitive to the heterogeneity of poly(A) tail length, in contrast to enzymatically added poly(A) tails post IVT reaction, more homogeneous tails are observed in samples with poly(A) tails encoded in their DNA template.

Selective hydrophobic interaction chromatography for high purity of supercoiled DNA plasmids

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High purity of plasmid DNA (pDNA), particularly in supercoiled isoform (SC), is used for various biopharmaceutical applications, such as a transfecting agent for production of gene therapy viral vectors, for pDNA vaccines, or as a precursor for linearized form that serves as a template for mRNA synthesis. In clinical manufacturing, pDNA is commonly extracted from Escherichia coli cells with alkaline lysis followed by anion exchange chromatography or tangential flow filtration as a capture step for pDNA. Both methods remove a high degree of host cell contaminants but are unable to generically discriminate between SC and open-circular (OC) pDNA isoforms, as well as other DNA impurities, such as genomic DNA (gDNA). Hydrophobic interaction chromatography (HIC) is commonly used as polishing purification for pDNA. We developed HIC-based polishing purification methodology that is highly selective for enrichment of SC pDNA. It is generic with respect to plasmid size, scalable, and GMP compatible. The technique uses ammonium sulfate, a kosmotropic salt, at a concentration selective for SC pDNA binding to a butyl monolith column, while OC pDNA and gDNA are removed in flow-through. The approach is validated on multiple adeno-associated virus- and mRNA-encoding plasmids ranging from 3 to 12 kbp. We show good scalability to at least 300 mg of >95% SC pDNA, thus paving the way to increase the quality of genomic medicines that utilize pDNA as a key raw material.

HPLC for at-line reaction monitoring and purification improves yield and purity of tRNA

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Engineered transfer RNA is an emerging therapeutic modality, particularly suited to treatment of diseases caused by genetic disorders based on premature termination codons, frameshifts, or missense mutations. It also finds use in reprogramming of *in vitro* translation systems to generate non-canonical amino acid-containing proteins and peptides, such as in mRNA display. Due to its length, chemical synthesis of tRNA is challenging and production of engineered tRNA at scale is currently limited to *in vitro* transcription from a DNA template. Previously, the highest reported *in vitro* transcription yield was 2.5 g/L, significantly below the industry standard for mRNA production of 7–10 g/L. To improve this process, we implemented monitoring of nucleoside triphosphate consumption and tRNA production during *in vitro* transcription, using at-line high-performance liquid chromatography, with a monolithic solid phase. This allowed for optimization of nucleoside triphosphate concentration, reduction of the *in vitro* transcription time to <4 h, and improvement of yields up to 4.5 g/L. This analytical approach was also used to develop a step-elution purification on a DEAE chromatographic monolith with >90% step yield. These improvements in the production and purification of tRNA by *in vitro* translation represent an important step in facilitating production of tRNA for research purposes, while paving the way for purification of therapeutic tRNAs that is scalable and compatible with GMP requirements for clinical production.

High-throughput sequencing approaches for in-depth analysis of AAV-based gene therapies

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In last decade, recombinant adeno-associated viruses (rAAVs) have been successfully used as vectors for treating monogenetic diseases. The main goal of rAAV production is to generate a large number of effective viral particles that are capable of curing diseases. However, existing manufacturing and purification protocols do not fully achieve this goal, since the final product consists of a mixture of full, partially filled and empty viral particles. To predict potential side effects of these ineffective viral particles, comprehensive characterization is essential. High-throughput sequencing techniques provide a powerful tool to explore the nucleic acid composition of rAAVs. In our study, we compared two sequencing methodologies: short-read sequencing using the Illumina platform and long-read sequencing using Oxford Nanopore Technologies platform. Our study focused on a set of rAAV samples produced via triple transfection in HEK293T cells and purified in two consecutive ultracentrifugation runs in a CsCl gradient. We compared the sequencing results with data previously obtained using various analytical techniques. In addition, we investigated the relative abundance of specific sequences and attempted to determine the origin of unmapped reads, which led to interesting findings about chimeric sequences. Our results showed that the majority of reads, as determined by both sequencing approaches, originated from the viral vector sequence. A smaller fraction of reads mapped to plasmid sequences and host cell DNA. Through various pretreatments prior to libraries preparations, we were able to get an inside of whether nucleic acid contaminates were packed inside of viral capsids or were free floating in the solution. Unlike short-read sequencing, long-read sequencing unveiled distinct vector genome length profiles between studied rAAV samples.

Production and purification of Staphylococcus epidermidis phage Cop-80B for preclinical evaluation of its safety and efficacy for the treatment of orthopaedic-related chronic infections

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Antibiotic-resistant, biofilm-forming bacteria pose a serious public health threat. Chronic infections, including periprosthetic joint infections (PJI), may cause lifelong disability in the case of postoperative complications. An alternative treatment strategy is phage therapy, used alone or as adjunctive therapy for difficult-to-treat infections. Despite its enormous potential, phage therapy has not yet been approved by regulatory authorities, due to the lack of successful clinical trials. Properly purified and characterized phages are important for ensuring the safety of phage therapy.

We developed a C57BL/6_OlaHsd mouse PJI model caused by Staphylococcus epidermidis, a common biofilm-forming bacterium, and used it to demonstrate the safety and efficacy of phage therapy. We used a phage from COBIK's phage bank, which contains several environmental Staphylococcus-specific phage isolates. The phage was extensively characterized to confirm its suitability for further tests, including antibacterial and antibiofilm activity, and absence of unwanted genetic elements. The phage was produced using a characterized propagation strain in liquid cultures and purified by centrifugation, filtration and chromatography. We used the CIMmultus OH capture step to ensure concentration of bacteriophage and removal of host DNA and protein. Polishing step on the CIMmultus QA

column was performed to provide additional removal of key process impurities. The preparation was applied to mice in which chronic PJI was established. We compared the treatment efficacy of the purified phage with that of vancomycin, the standard-of-care antibiotic for methicillin-resistant coagulase-negative staphylococci. The most successful outcome was obtained using a combination of antibiotic and phage therapy. No adverse events were observed in the treated mice regardless of the treatment.

These results demonstrate that the developed mouse model can be used to test the efficacy and safety of phage therapy and provide experimental evidence for the need for further development of characterized and purified phages in the fight against challenging bacterial infections.

Poster Presentations

P01 | pPLUS® AAV-Helper, novel engineered pHelper plasmid to improve yield and quality of several AAV serotypes in suspension cell culture systems

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Harnessing rAAVs as viral vectors for therapeutic transgene delivery still requires improvements in yields and specificity to lower vector doses, and therefore manufacturing cost, as well as to improve patient safety. To this end, our research is focused on developing novel technologies to ensure manufacturing of high yielding rAAV particles using transient transfection, as well as enhancing features of rAAV vectors that act on the overall size of packaged material and specificity of delivery. Here we present our state-of-the art approach to design new helper plasmids (phelpers) with the aim of improving both the infectiosity (TU/mL) and the quality (full/empty ratio) of the viral particle obtained from suspension cultures. We took the opportunity to exploit our proprietary DNA assembly method technology to explore the synergies of multiple genetic features modularly assembled in synthetic plasmids. Comparison of the biological activity of several versions of rationally designed pHelpers led us to identify the optimal configuration able to outperform existing helper plasmids in every tested bioproduction conditions. Our expertise in DNA plasmid design and assembly together with our scalable transfection solutions for rAAV manufacturing gives us the potential to improve both productivity and specificity of gene therapy products.

P02 | Development of a rAAV empty to full capsid ratio quantification method by AEX-HPLC

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Adeno-associated viruses, (AAV), have emerged as promising vectors for gene therapy, offering potential treatments for a wide range of genetic acquired diseases. To ensure the safety and efficacy of AAV-based gene therapies, robust analytical methods are crucial for characterising vector properties, in assessing quality purity and ensuring batch to batch consistency within manufacture.
One key metric used to assess the quality of AAV product is the quantification of full capsids.
AEX-HPLC has been used as an assay in the pharmaceutical industry to determine the charged variants of biological molecules. In the rAAV case, due to the surface charge difference between the full and the empty capsid, they can also be visualised as charge variants to be analysed quantitatively with the AEX-HPLC method. Therefore, AEX-HPLC has been reported for the empty to full capsid analysis as a relative quantification method and combinations of buffer, column and gradient settings are investigated for various rAAV capsids.
In this study, a novel rAAV candidate has been analysed by a tetramethylammonium chloride (TMAC) based gradient elution method, which is developed in house at PureSpring therapeutics. During the method development, we evaluated a range of assay parameters to achieve this E:F ratio analysis goal including: resin column-based salt elution, monolith column-based pH-gradient elution, monolith column-based salt gradient elution and screening of the gradient length to determine the impact of each variable across the system. Finally, a magnesium chloride and TMAC dual salt gradient condition is selected, combined with the monolith column (CIMac) with quaternary amine ligand. This method is used to analyse the efficiency of full capsid enrichment process and relative tittering of capsid concentration.

P03 | Optimization of mRNA production and purification: characterization and controlling of post-transcriptional polyadenylation

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While mRNA therapeutics developed in recent years show tremendous potential, there are still technical obstacles to overcome, two of the most significant being mRNA's intrinsic instability and its immunogenicity. In order to address these challenges, several different approaches can be used, including optimization of 3'poly(A) tail length.
Even though DNA encoded poly(A) tail is commonly used, long (>120 nt) stretches of adenines in DNA can lead to cloning difficulties and plasmid instability during culture, making enzymatic post-transcriptional addition an interesting alternative. Very limited information is published on post-transcriptional polyadenylation (PA), therefore main focus of this study was on improving the understanding of in vitro PA reaction. With the use of CIMac PrimaS analytical column, which allows monitoring of ATP consumption in sub-1.2 min, we were able to control the length of poly(A) tail near real time. For additional characterization of poly(A) tails mRNAs were digested with Rnase T1. Oligo dT purified poly(A) tails were subsequently analyzed with various analytical tools, including CIMac SDVB, confirming the lengths previously determined by CIMac PrimaS.

P04 | From Variability to Consistency: A Novel Manufacturing System for Standardized Extracellular Vesicles from immortalized human Mesenchymal Stromal Cells

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Cell-based therapies are inherently complex and their broader translation into clinical efficacy has been slow over the past years. Some reasons behind are the complexity of cells, their variability and difficulties in scalable manufacturing. This holds also true for extracellular vesicles (EV) which offer great promises for many applications in Regenerative Medicine and beyond. The composition and biological function of EV are largely dependent on the cellular background, the cultivation conditions including media and the downstream processing applied. We have recently established a scalable and tunable manufacturing setup based on stable, telomerized Mesenchymal Stromal Cells (MSC/TERT), a proprietary 3D cell culture and perfusion bioreactor configuration, and standardized isolation steps which ensure the molecular integrity of the cell secretome in general and the EV fraction specifically. With this novel setup which allows for continuous harvesting of conditioned media (tested for up to 36 days and beyond), we have been successful in reproducible manufacturing of EV preparations with relevant biological functionalities and in significant quantities. In a consortium with partner companies, we have established the analytical basis for better understanding the relationship between molecular composition and these biological functionalities. The miRNA content and specific miRNA and protein patterns have been analyzed and compared with data from anti-inflammatory and anti-fibrosis bioassays. Different cell lines, harvest time points and downstream processing conditions have been assessed so far. The collected data are forming the basis for selection of EV preparations for testing in organoid and in vivo disease models. We will share an overview of performance data from this novel manufacturing approach with different MSC/TERT lines, including preparative separations, and extended characterization data from resulting EV preparations and further provide an outlook how the results will support pre-clinical and clinical translation.

P05 | Improved purification of enveloped virus-like particles (VLPs) by advanced differentiation of extracellular vesicles (EVs) and VLPs

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Virus-derived particles are utilized in a wide range of vaccine and gene therapy applications. Both modalities have strict regulatory requirements for high particle purity. However, due to the novelty of these therapeutic strategies and the variability of the products, there is currently no consensus on the downstream process unit operations that are required to meet these quality criteria. Thus, methods and model system allowing rapid screening for successful purification approaches are urgently needed.

Although being applicable as vaccines, virus-like particles (VLPs) can be considered the easier to handle virus surrogates as compared to viral vectors. The purification of their membrane-enveloped transduction-competent counterparts, such as lentiviral vectors, is even more challenging owing to their more complex structure and fragility. During budding, enveloped viruses and VLPs hijack cellular pathways required by the cell to release extracellular vesicles (EVs). As a result, EVs have similar surface characteristics to viral particles originating from the same host cell, impeding the discrimination of the two particle populations. Despite the increasing recognition of EVs as potential contaminants in virus and VLP feed streams, currently no standard methods are available to quantify EVs. Accordingly, knowledge of the EV population in virus and VLP preparations is very limited.

We used fluorescently labeled human immunodeficiency virus (HIV)-derived VLPs as a model for enveloped viruses. The lack of infectivity and ease of production make fluorescent VLPs an important tool for downstream platform optimization as they can be directly and rapidly quantified using numerous fluorescence-based techniques. As a first step, methods were used to distinguish between EVs and VLPs. Next, different membrane chromatography and filtration methods will be implemented to optimize the separation of contaminants, focusing on host cell DNA and EVs. The results will foster the establishment of a scalable downstream processing platform for enveloped virus-derived vector particles and VLPs.

P06 | Efficient rAAV8 Capsid Quantification in Upstream Process Development with two-dimensional liquid chromatography

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In the development of recombinant adeno-associated viruses (rAAV) in upstream processes, achieving a high viral titer with an abundant full capsid percentage is crucial for successful gene therapy applications.
While PCR methods can measure the titer of full capsids in crude samples, other techniques such as AUC and TEM require sample purification and concentration to accurately determine the empty/full rAAV ratio. The combination of biochemical methods, e.g. ddPCR and ELISA is a common approach to determine the empty/full ratio in crude samples, however it is time consuming, and not applicable for a real-time in-process control. To overcome these challenges, the PATfix AAV Switcher, an automated two-column analytical system was developed.
This poster introduces the two-column analytical method for determination of empty and full capsids for crude rAAV8 samples without extensive sample preparation. An analytical example presented will show the impact of different conditions on the empty/full capsids ratio simultaneous during rAAV8 production process.
In addition, the effect of impurities, e.g. proteins in the upstream lysed samples on the detection limit and the minimal concentration of full capsids that can be reliably determined will be presented.

P07 | Depletion of production-related impurities improves the performance of self-amplifying RNA

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RNA-based vectors are revolutionizing the fields of vaccines and therapeutics. Self-amplifying RNA (saRNA) is obtained from the alphavirus genome by replacing the structural genes with a gene of interest for example a desired antigen. It allows extended durability of antigen expression and lower doses for equivalent protective immune responses compared to mRNA.
During RNA production by in vitro transcription process-related impurities are introduced that can reduce the safety and effectiveness of saRNA. Cells sense these structures and activate cell-autonomous innate immune mechanisms. This immune response might prevent the expression of the encoded gene and would lead to adverse effects in patients. Therefore, process-related impurities need to be minimized. In this project, the impact of additional purification by ion-pair reversed-phase chromatography using CIM SDVB columns was studied in context of Chikungunya virus derived saRNA expression and interplay with cell-autonomous innate immunity.
We show that the additional purification step has reduced double stranded RNA byproducts of the in vitro transcription process without reduction of the saRNA integrity. After transfection into the permissive cell line BHK-21 saRNA purified with one or two-steps resulted in similar transgene expression levels. This indicates that the purification process does not influence functionality of saRNA in absence of innate immunity. In cells with functional cell-autonomous innate immunity the additional purification step results in saRNA with lower cytotoxicity, lower level of interferon and pro-inflammatory cytokine secretion and greater transgene expression. Our time-course studies suggest that additional purification delays antiviral response of the cell thereby buying time for saRNA to establish replication.
In this study, we tested saRNA with reduced level of production related impurities. Lower immunogenicity of the resulting saRNA might result in better tolerability in patients. Furthermore, it is conceivable that such saRNA will allow lower dosing and make RNA vaccines production more cost-efficient.

P08 | Lentiviral downstream process optimisation and Design of experiments on CIM QA 0.05 mL Monolithic 96-well Plates

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Lentiviruses are being increasingly used as tools for gene delivery. Sufficient purity and infectivity, however, remain a bottleneck for their widespread use. In order to address these issues, monolithic columns have been tested for downstream purification, with good initial results on CIMmultus QA. To further improve the downstream process, we decided to determine the factors and conditions, which contribute the most to downstream recovery.
In this work we present the results from optimisation and Design of Experiments (DoE) studies, which have been performed to aid with lentiviral downstream process improvement. In order to increase throughput of the DoE studies, we used CIM QA 0.05 mL Monolithic 96-well Plates, which significantly sped up the testing process, as well as decreased variables which occur from day-to-day work. From the factors tested in our experiments, results indicate that starting salt concentration in lentiviral load has the most profound effect on lentiviral recovery.
Improved understanding of conditions important for lentiviral downstream will ease future process development. With additional process improvements, monoliths have great potential in enabling scalable and cost-effective downstream process solutions for lentiviruses.

P09 | Synthetic Enzymatically produced DNA for Gene Therapy & Vaccine Applications

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The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA for use in gene therapy and vaccines. In addition to worldwide lack of capacity and long lead times, complex sequences such as long homopolymeric sequences including long polyA tails are difficult to propagate in bacteria.
4basebio has developed a proprietary, scalable, fully enzymatic synthesis process for the production of linear DNA constructs via our Trueprime™ amplification technology. The process yields DNA at 1g/L, several orders of magnitude higher than plasmid fermentation yields, allowing a small footprint using benchtop equipment.
The process is size and sequence independent, allowing for large scale production of linear DNA with high yield and purity in less than a week. Unlike plasmid DNA, 4basebio DNA eliminates contamination from endotoxins or host proteins, and excludes bacterial sequences such as antibiotic resistance genes. Complex sequences including ITRs and homopolymeric sequences are easily produced without risk of deletion or recombination.
Currently, we make 4 types of DNA, each with unique application-specific benefits. For mRNA production, opDNA™ can be used directly in IVT processes, achieving significantly higher yields as compared to linearised plasmid, with equivalent capping efficiency and dsRNA impurities. Proinflammatory cytokine/chemokine levels in isolated primary, human PBMCs are comparable to mRNA produced from linearised plasmid.
hpDNA™ can be used in the production of AAV, replacing conventional plasmid triple-transfection. Using hpDNA™, we were able to achieve equivalency in viral genome titres, Full:Empty ratios and infectivity as compared to a plasmid controls, across a range of serotypes.
Finally, proprietary non-viral delivery system, Hermes™, is a nanoparticle vector that can encapsulate a range of payloads, and can be customised to target cells or tissues of interest for a range of applications. The particles additionally offer long term stability at 4 degrees C, overcoming cold chain requirements.

P10 | Advancements in adeno-associated virus bioprocess development: enhancing chromatographic strategies for efficient gene therapy production

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Gene therapy utilizing AAV vectors has emerged as a promising therapeutic modality for treating genetic and acquired diseases. To meet the demands of scalable and efficient AAV production, this study focused on the development of chromatographic steps in the bioprocess. Activities included conducting a DoE to optimize the capture chromatography step, evaluating different chemistries for the polishing chromatography step, conducting a scale-up confirmation study, and incorporating analytical techniques for process monitoring. In the initial phase of the study, a DoE was performed to optimize a capture chromatography step for AAV purification. Key parameters such as pH were systematically varied to identify the optimal conditions that resulted in maximum AAV recovery and purity. The DoE approach facilitated efficient process development and enhanced the understanding of critical process parameters. Furthermore, three different chromatographic chemistries were evaluated for the polishing step, aiming to establish a robust and selective purification strategy. Comparative data on the performance of these chemistries is presented, including overall AAV yield and purity (ratio of full to empty particles). The findings provided insights into the most suitable chromatographic medium for AAV polishing, ensuring the removal of product impurities and the attainment of high product quality. To validate the scalability and reproducibility of the optimized process, a scale-up confirmation study was conducted. This study provided important insights into the performance of the developed chromatographic steps at larger scales, paving the way for future process scale-up. In summary, this research emphasizes the crucial role of chromatographic strategies in the development of efficient AAV bioprocesses for gene therapy production. The outcomes of this study contribute to the advancement of AAV production methodologies, facilitating the translation of gene therapy into effective clinical treatments.

P11 | Reverse-phase chromatography removes double-stranded RNA, fragments and residual template to decrease immunogenicity and increase cell potency of mRNA and saRNA

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We present a comprehensive study of ion-pair reverse-phase (IP-RP) chromatography for mRNA polishing, demonstrating its ability to remove double-stranded RNA (dsRNA), RNA fragments and residual DNA template. We developed a step elution IP-RP strategy to simplify the purification process, with consistent recovery rates and purity across different column scales (1 – 80 mL bed volume). Column sanitization with 75% acetonitrile was replaced with NaOH cleaning methodology, thus removing the need for concentrated acetonitrile. We show how IP-RP utilizing up to 20% acetonitrile (ACN) removes double-stranded RNA hybrids. When mRNA is not treated with DNase, IP-RP separates two forms of DNA from full length mRNA, reaching residual DNA levels comparable to DNase treated mRNA. Reverse-phase polishing was validated with different RNA constructs with nominal length from 900 – 9000 nucleotides, highlighting the necessity of temperature control for effective dsRNA removal, especially for longer RNA sequences, such as self-amplifying RNA (saRNA). Our findings highlight the importance of IP-RP while simplifying the technological requirements for its adoption in clinical manufacturing processes.

P12 | Viral vector genome integrity evaluation to support process development and comparability

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Gene therapy offers new opportunities for the treatment of a range of diseases, from cancer to various genetic disorders. Therapeutic viruses, such as adeno-associated viruses (AAV), are used to deliver genes to target cells. Effective gene therapy requires functional viral vectors with intact genomes; however, current harvesting and purification methods do not fully separate the mixture of full, empty, and partially filled viral particles. Currently, even the final drug product contains at least some unwanted particles, either with contaminants or with fragmented genomes.
We addressed the problem of assessing the genome integrity of AAV vectors by developing an advanced dPCR multiplex approach. The newly developed 4-plex assay was qualified at the DNA level and tested on several different AAV vectors to demonstrate its applicability in process development. We have shown that different conditions in upstream processes can lead to up to fourfold differences in the amount of full-length vector genome. The assay represents an improvement over the simplex vector genome titer assay by providing accurate quantitative genome integrity results. The use of such an assay can provide better insight into process development and lead to the presence of as many full-length vector genomes as possible

P13 | Monolith based chromatography is a suitable tool to prepare and analyze *E. coli* outer membrane vesicles

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Intranasal administration of Escherichia coli O83 - outer membrane vesicles (OMVs) was found to reduce allergic airway inflammation induced by ovalbumin in mice. Production of sufficient amounts of OMVs to supply further clinical trials implies the need for scalable methods and precise analytics. Different orthogonal methods are required to provide a complete picture of the process. We present an exemplary OMV preparation, demonstrating robustness of our chromatographic platform. As shown before for various types of extracellular vesicles from animal and human cell origin, the platform was proved suitable also to isolate OMV´s produced by E. coli. The purification process was monitored using three common analytical techniques – nanoparticle tracking analysis, flow cytometry and PATFix chromatography. The results demonstrate that chromatographic isolation methods can be implemented to produce OMVs and that orthogonal methods are mandatory to paint an accurate picture through the whole preparative process.

P14 | Development of a full capsid enrichment polishing step in AAV9 and AAV8 purification process with CIMmultus® PrimaT monolithic column

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Gene therapies treatments are promising for rare diseases especially, at Genethon, for muscular dystrophies and metabolic disorders. However, while producing AAV vectors for these gene therapies, empty capsids are also produced in a certain amount. Clinical effect of empty capsids is unclear. Empty capsids are not the intended therapeutic product and could include a toxicological effect, process teams need to reduce their presence. This objective could be achieved by designing new capsids and transgenes, by changing cell culture conditions or by implementing a polishing step on current purification process. The latter approach was evaluated using the CIMmultus® PrimaT monolithic column developed by Sartorius. Parameters like viral loading, residence time and conductivity of loading and elution were tested. Based on the generated data, mainly the full capsids enrichment rate and the viral genome recovery, conditions were defined. Results obtained confirm the performance, repeatability, and scalability of CIMmultus® Prima T monolithic column to enrich the product in full capsids and to remove some product-related impurities.

P15 | Purification of vesicular stomatitis virus (VSV) by immunoaffinity monolith chromatography with native elution

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Recently we invented a novel strategy termed "native elution" for effective elution of fully functional viruses bound to virus-specific antibodies in immunoaffinity monolith chromatography. This advancement moved immunoaffinity chromatography closer to the real purification option in downstream virus processing. A highly concentrated solutions of selected amino acids at neutral pH effectively eluted captured mumps virus (MuV) from the monolith-based stationary phase with covalently bound MuV-specific antibodies as ligands. MuV infectivity and functionality were preserved through the process.

The aim of our work was to demonstrate that this innovative concept could be adapted and used to purify other viruses. Vesicular stomatitis virus (VSV) was selected due to its oncolytic properties and attractiveness in oncolytic virotherapy. It is a non-pathogenic, negative-strand RNA rhabdovirus with a short replication cycle.

VSV-specific immunoaffinity column was prepared by oriented immobilization of human polyclonal anti-VSV IgGs isolated from the serum of an oncolytic VSV-treated patient via the antibody\'s carbohydrate moiety on a CIMmic™ hydrazide activated monolithic column. The dynamic binding capacity of the prepared column was determined and the native elution efficiency of the bound VSV was examined using several high-molarity amino acid solutions at physiological pH. The purification efficiency of immunoaffinity chromatography was evaluated by degree of preservation of both infectious virus (CCID50 assay) and total particles (Nanoparticle tracking analysis), but also by degree of the removal of host cell proteins (ELISA) and host cell genomic DNA (RT-PCR).

In the preliminary experiments the native elution of captured VSV with Arg, Gly and Ser in different combinations was examined and the presence of Arg in the amino acid solution was found to be crucial for the effective elution of VSV. Immunoaffinity monolith chromatography demonstrated effectiveness in reducing host cell protein content (up to 70%), and achieving recoveries of infective virus above 50%.

P16 | Cell Type-Specific Genetic Therapy of Cancer, Virus Infection and Mitochondrial Disease

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Cell type-specific gene delivery which restricts gene transfer to therapy-relevant cells, hence reducing unwanted side effects caused by the ectopic expression of transgenes, remains one of the biggest challenges. To mitigate potential off-target effects, we developed a programmable cell type-specific gene therapy using an RNA trans-splicing-based approach for gene replacement therapy or suicide gene therapy that can selectively express therapeutic proteins or suicide signals based on the cell's endogenous pre-mRNA expression profile. Using the liver as a medical target organ, we developed a hepatocellular carcinoma-specific trans-splicing based Herpes simplex virus thymidine kinase/ganciclovir suicide gene therapy approach. We rationally designed suicide RNAs that can target multiple HCC-related pre-mRNA biomarkers thereby increasing the overall concentration of targetable pre-mRNA. For delivery, non-viral redosable dumbbell-shaped DNA vectors were designed, manufactured, and tested in tissue culture cells, in 3D patient-derived xenograft spheroids and in mouse model. For cell-type specific delivery, the conjugation of dumbbell vectors with GalNAc3 residues enabled targeted delivery into hepatoblastoma-derived human tissue culture cells. To deliver nucleic acids into the mitochondria of human cells, we studied the long non-coding β2.7 RNA of the human cytomegalovirus which was previously reported to colocalise with mitochondrial complex I. Using thermodynamic profiling, we identified four thermodynamically conserved structural subdomains within the β2.7 RNA that are responsible for its mitochondrial targeting activity. A vector comprising eight mitochondrial targeting domains exhibited a three times higher mitochondrial targeting activity as compared with the parental β2.7 RNA. The amalgamation of mitochondrial targeting RNA and trans-splicing allows cell type-specific transcription of mRNA which can be delivered into mitochondria for mitochondrial gene therapy. Trans-splicing based gene therapy represent a technology that is highly translational offering promising perspectives towards suicide gene therapy of any cancer types or incurable infections with integrating viruses and may enable cell type-specific mitochondrial gene therapy.

P17 | Comparability Study Between Ion-Exchange Monolith and Affinity Resin for Purification of AAV8

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The main objective of every downstream process (DSP) for AAV is to achieve high recovery while delivering the purest, most potent product possible. The capture step in AAV gene therapy is either affinity or cation exchange chromatography, which both concentrates the product and removes impurities. Following the capture, the eluate is generally further processed to enrich for full capsids and further purification. For this enrichment and polishing of full AAV capsids, CIMmultus QA, a monolith-based anion exchange chromatography, is widely used.
To achieve this goal robust and orthogonal analytical methods are needed and will be presented in this paper.

P18 | Integrated cGMP Manufacturing of mRNA: From DNA Template to Drug Product – the CDMO's Point of View

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The increasing demand of clinical-grade mRNA has led to significant changes in biopharmaceutical cGMP production. Manufacturers, as biotech CDMOs, developed innovative processes and unit operations, sourced and qualified novel raw materials, and optimized new assays for analytical mRNA characterization.
In this case study we developed and characterized a comprehensive mRNA GMP manufacturing platform, covering the whole process chain. This required the consideration and integration of the DNA template for in-vitro-transcription (IVT) into the mRNA manufacturing concept. It was the goal to evaluate the manufacturing processes of both – template-DNA and mRNA – by parameters of economy, compliance and quality.
The target was an mRNA coding for a CRISPR/Cas9-nuclease. The transcript was 5´-capped with a cap analogue, 3´-tailed with a 45 base-long poly(A) unit and base-modified with N1-methyl-pseudouridine. GMP manufacturing was performed at full scale in clean rooms. Manufacturing of the DNA-template for the IVT consisted of circular plasmid cloning, *E. coli*-cell banking, fed-batch cultivation, cell lysis, enzymatic linearization and several chromatography steps. With the GMP-produced DNA-template, manufacturing of the mRNA molecule was performed by enzymatic IVT (T7-RNA-polymerase), with co-transcriptional capping. After IVT, mRNA purification was finished by oligo-dT chromatography.
The process resulted in an excellent mRNA yield per applied DNA (factor 65:1) and an overall yield of ~60% related to IVT bulk. All quality parameters (content, identity, purity, safety) were within pre-defined specifications. Bulk mRNA was highly homogenous (90 % by CE). 5´-capping efficiency was at nearly 95%, and 3´-poly(A) tail length was confirmed (LC/MS). Double-stranded RNA was below 0.1%. Stability of the mRNA batch was confirmed by multi-conditional stability studies. Biological functionality was demonstrated by

in-vitro translation with a rabbit-reticulocyte-lysate extract based system and analysis by western blotting. As a conclusion, with the established mRNA platform, the parameters and requirements of yield, throughput time, GMP compliance and quality were successfully achieved.

P19 | Utilizing CIMmultus® OH for efficient purification of MSC EVs

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Mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) have promising therapeutic potential in diverse applications, such as tissue regeneration and immunomodulation. On the pathway to the clinic, there is a substantial need for developing robust and scalable methods for EV production and purification. In this work, we prepared MSC EVs using two different production systems. 2D production in flasks was compared to a 3D microcarrier-based bioreactor process. Conditioned media EVs harvested from both production strategies were captured using preferential exclusion chromatography on the CIMmultus OH column. This way we concentrated the vesicles and removed the majority of impurities in a single step. Finally, samples from different stages of EV bioprocess were evaluated based on particle yield and the presence of common exosome markers using NTA and PATfix analytics. CIMmultus OH chromatography offers a valuable tool for the EV capture step and demonstrates robust performance on different upstream materials.

P20 | Chromatographic characterization of cation-exchange carboxymethyl/nanofibrilated cellulose-based membranes using lysozyme as a model protein

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Bio-based materials are becoming important low-cost and readily available chromatographic supports for the purification of biomacromolecules. In this research, we fabricated microporous cellulose-based membranes by using carboxymethyl cellulose (CMC) as weak cation-exchanging polymer and cellulose nanofibrils (CNFs) as stabilizing and structural filler, in which the macropores were generated by variation of their mass weigh ratio and using a freeze-casting process, while their stability was obtained by crosslinking through esterification of available hydroxyl groups using citric acid (CA) mediated reaction performed in situ during the fabrication. The membrane's charge (pKa ~4.7-5) enables ionic interaction with positively charged Lys (pKa ~11), while its transport mechanism depends on the membrane's morphology and porosity, therefore the membranes were evaluated by potentiometric titration, SEM imaging and mercury porosimetry. The membranes were then analyzed for pressure drop, static (SBC) as well as dynamic (DBC) binding capacities of Lys at different flow rates and Lys concentrations by being packed in a chromatographic housing and compared to the existing solutions.

It was ascertained that mono-esterified/bound CA contributes the most to the total carboxylic groups (2.2- 3.5 mmol/g), while CMC induced CNF orientation, resulting in a highly porous (96% porosity, ~94 μm pore size) microstructure with longitudinal channels in a cross-section for the sample with higher total solid content, and a SBC for Lys of between 300-600 mg/g membrane. A Lys DBC evaluated in a chromatographic regime for the selected membrane was in the range of 28-66 μg/mg at a throughput of 0.5 mL/min and with 95% recovery. Although the Lys adsorption and transfer were reduced by the increasing flow rate and membrane density (due to higher CMC content and compression pressure, resulting in smaller and irregularly distributed pores), the DBC was comparable with the commercial CIM® monoliths.

P21 | PATfix analytical method for Orf virus sample characterization

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In recent years, the Orf virus (ORFV) has become a promising technology for the generation of recombinant vaccines and oncolytic therapy. However, suitable analytical methods for characterisation of ORFV materials at various stages of production, including up- and downstream are limited.
In the present study, we demonstrate a high-performance liquid chromatography (HPLC) PATfix analytical method, suitable for rapid fingerprinting of ORFV materials used for assessment of sample composition at various stages of ORFV production and determination of ORFV particles. The principle of the PATfix HPLC method is anion-exchange (AEX) chromatography, performed on CIMac QA-0.1 (6 µm) monolithic analytical column. Multiple-detector PATfix technology enables simultaneous detection of absorbance, two different fluorescence and light scattering emitted/scattered from the sample.
We show that fast, reproducible, and robust PATfix method that detects ORFV and records impurity profile at the same time is suitable for ORFV samples of various complexity and could therefore represent a valuable tool in ORFV production/purification process monitoring.

P22 | Processing CD and DSC data on protein folding with Zimm-Bragg model in water

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Circular Dichroism (CD) and Differential Scanning Calorimetry (DSC) data are processed with a novel model incorporating water effects and inter-/intra-molecular hydrogen bonding energies to better fit experimental data on protein folding as compared to the two-state approach.

P23 | 2D-LC evaluation of complex samples containing extracellular vesicles

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Analytics still represents a bottleneck for the breakthrough of extracellular vesicle (EVs) exploitation in the fields of diagnostics and therapeutics. New techniques are constantly emerging, but fast, reproducible, and robust methods that allow high-throughput and reliable analysis are still needed.
In the present study, we demonstrate a two-dimensional liquid chromatography (2D-LC) method. The method is especially suitable for the analysis of particles, including EVs, in complex samples that contain heterogeneous populations of nanoparticles together with variable amounts of biological macromolecules and smaller components. Experiments were performed using a valve-switch upgraded PATfix system, with incorporated detectors for monitoring light absorbance, fluorescence, and light scattering. The valve-switch module enables the resolution of sample components based on two completely different chromatographic principles in a single experiment. In the present study, we coupled the isolation of nanoparticles employing size exclusion chromatography (SEC), with their subsequent separation using ion-exchange chromatography (IEC). The result of the analysis is firstly size separation of sample components and secondly charge separation of excluded particle fraction.
The proposed approach suits for evaluation of small sample constituents (free proteins, nucleic acids, and their small complexes), while at the same time revealing heterogeneity in the population of particles. The main advantage of SEC-IEC separation is the identification of different sub-populations of EV and non-EV nanoparticles that naturally overlap in size. Implementation of SEC before IEC minimizes the interferences of buffer composition for IEC evaluation, allowing for the evaluation of samples with a very wide range of matrices. In the case of well-defined EV subpopulations of interest, the method also suggests potential purification strategies.
We show that the method is applicable to different types of EV-containing samples (e.g. conditioned cell culture media from various cell lines, and various EV-enriched preparations) and therefore represents a valuable tool in EV production and purification process development.

P24 | Grafting chromatographic monoliths with charged linear polymers for highly productive and selective plasmid DNA purification

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Ion exchangers (IEX) with charged groups on surface extenders (surface modified polymer chains formed by grafting reaction) have attracted attention due to their greatly enhanced productivity for biomacromolecules purification compared with traditional IEX. The goal of our investigation was to increase the dynamic binding capacity (DBC) for plasmid DNA (pDNA) on a monolith stationary phase by grafting the surface with linear polymethacrylate brushes, while retaining high pDNA recovery and chromatographic selectivity between pDNA and RNA impurities.
The inner walls of the CIM Convective Interaction Media® monolith channels were successfully coated with polyglycidyl methacrylate linear chains by a controlled radical polymerization reaction. Glycidyl groups of formed polymers of varying densities and lengths were converted to anion-exchanging (AEX) moieties. DBC for 7.3 kilobase pair pDNA increased with the thickness and density of grafted weak AEX layer from 2.3 mg/mL (for non-grafted) up to 17.6 mg/mL, at the expense of pDNA elution recovery, which decreased due to entanglement of pDNA molecules inside the dense grafted layer. A satisfactory compromise between high pDNA DBC and elution recovery was found with relatively long and low-density polymer chains. The optimal grafted AEX column with 1 mL bed volume was evaluated for pDNA capture from neutralized *E. coli* lysate. With a capacity of 13.5 mg pDNA per mL support, ≥95% elution recovery, and complete RNA clearance, the pDNA was successfully purified at loading flow rate of at least 15 column volumes per min. According to our knowledge and literature search, these process characteristics would enable one of the highest pDNA isolation productivities to be achieved with currently tested IEX.

P25 | High reproducibility, homogeneity, and scalability of CIM QA HR chromatographic monoliths demonstrated by separation of Empty and Full AAV capsids

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One of the key challenges in adeno-associated virus (AAV) viral vector manufacturing is effective and consistent separation of full AAV particles (containing the full recombinant DNA of the gene of interest) from non-functional (empty, partially filled etc.) capsids. Small deviations in chromatographic process performance arise from variability in upstream sample, variability in chromatographic conditions and variability in the chromatography media and can change the AAV capsid separation into a nightmare. One part of the solution is providing highly reproducible (HR) chromatographic columns intended for empty and full AAV capsid separation. For this purpose we have first set up reproducible and highly controlled chromatographic conditions for separation of AAV8 capsids in an ascending KCl gradient using quaternary amine (QA) modified monolithic columns. Separation of empty and full AAV8 capsids on 1, 8, 80 and 800 mL CIMmultus QA columns demonstrated reproducible elution conditions as empty capsids eluted at KCl concentration in the range of 89.4–91.4 mM. Extracting 200 µL units from the 800 mL monolith and testing them for AAV8 separation with the same method confirmed the same elution pattern as obtained with the parental column. The principle of extracting small units (specimen) and testing them for AAV separation was further developed to prove excellent scalability and homogeneity of CIM QA chromatographic monoliths. Specimen (200 µL or 1 mL bed volume) enabled implementation of AAV8 separation as one of column release test methods in the production of the commercial CIMmultus QA HR monolithic line (1–8000 mL). All columns in this new column line fit a strict release criteria of empty AAV8 capsid elution at KCl concentration between 89.5 and 95.1 mM and demonstrate good batch-to-batch and scale-to-scale material consistency.

P26 | Rapid desalting of *E. coli* cell lysate for CGE analysis using monolithic spin columns

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Neutralised *E. coli* cell lysate is a complex intermediate sample in the production process of plasmid DNA (pDNA) for use in vaccines and gene therapy. It contains various amounts of pDNA isoforms together with bacterial RNA and remains of bacterial genomic DNA, together with different ions (Na^+, Ca^{2+}, K^+, NH^{4+}, acetate, SO_4^{2-} etc.) in millimolar to molar concentration range. These salts interfere with different analytical techniques, used for lysate characterization, thus decreasing method accuracy, sensitivity and/or robustness. One example of extremely matrix-dependant analytical technique is capillary gel electrophoresis (CGE) with LIF detection, which is often used for the analysis of pDNA isoform composition. Low sample conductivity is particularly essential for robust injection, which is not easily achieved for complex samples such as *E. coli* cell lysate. Currently, centrifugal filters with membranes of different materials and pore size cut-offs are used for sample desalting via ultrafiltration, where the degree of desalting is linked to the number of centrifugation cycles. Here we present the use of an innovative method for desalting of *E. coli* cell lysate using monolithic spin columns and compare its performance with traditional centrifugal filters. In both cases, CGE was used for sample characterization after desalting. Utilizing the bind-elute mechanism, monolithic spin columns can efficiently remove unwanted salts as the column binds only nucleic acids, while salts pass through it. Under optimal conditions, samples prepared with monolithic spin columns retain the nucleic acid profile of the lysate, enabling precise analysis of nucleic acid/pDNA isoform composition in the *E. coli* lysate. Furthermore, one device and one general procedure can be used for rapid desalting of various nucleic acids (oligonucleotides, pDNA, RNA), regardless of their size, which is not attainable with centrifugal filters. Finally, the desalting procedure can also be transferred to CIMmultus column line and applied for preparative desalting of nucleic acids.

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