

MAMMALIAN SYNTHETIC BIOLOGY: FROM PARTS TO MODULES TO THERAPEUTIC SYSTEMS

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Key Words: synthetic biology, genetic circuits, computer-aided design

Synthetic biology is revolutionizing how we conceptualize and approach the engineering of biological systems. Recent advances in the field are allowing us to expand beyond the construction and analysis of small gene networks towards the implementation of complex multicellular systems with a variety of applications. In this talk I will describe our integrated computational / experimental approach to engineering complex behavior in mammalian cells. In our research, we appropriate design principles from electrical engineering and other established fields. These principles include abstraction, standardization, modularity, and computer aided design. But we also spend considerable effort towards understanding what makes synthetic biology different from all other existing engineering disciplines and discovering new design and construction rules that are effective for this unique discipline. We will briefly describe the implementation of genetic circuits and modules with finely-tuned digital and analog behavior and the use of artificial cell-cell communication to coordinate the behavior of cell populations. The first system to be presented is a genetic circuit that can detect and destroy specific cancer cells based on the presence or absence of specific biomarkers in the cell. We will also discuss preliminary experimental results for obtaining precise spatiotemporal control over stem cell differentiation for tissue engineering applications. We will conclude by discussing the design and preliminary results for creating an artificial tissue homeostasis system where genetically engineered stem cells maintain indefinitely a desired level of pancreatic beta cells despite attacks by the autoimmune response, relevant for diabetes.

**BIOENGINEERING COAGULATION FACTOR VIII:
INSERTIONS OF UNSTRUCTURED POLYPEPTIDES (XTEN) RESULT IN ENHANCED EXPRESSION AND
EXTENDED *IN VIVO* HALF-LIFE**

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Key Words: XTEN, hFVIII, human coagulation factor

Fusion of unstructured polypeptides of defined amino acid composition, known as XTEN, is an effective means of extending the *in vivo* half-life of biotherapeutics. We are employing the XTEN technology to improve the half-life of human coagulation factor VIII (hFVIII). One or more XTENs were inserted at inter-domain sites, intra-domain sites, and/or the C terminus of hFVIII. The aim of this study was to determine the effect of XTEN insertion(s) on the expression and *in vivo* half-life of recombinant hFVIII.

Potential insertion sites were identified based on comprehensive structure analysis of FVIII and by excluding sites reported implicated in hemophilia A. Expression vector constructs encoding human B domain-deleted FVIII (BDD-FVIII) bearing XTEN modifications were transiently expressed in HEK293 cells, and the activities of the secreted FVIII measured by a chromogenic assay. The pharmacokinetic (PK) properties of hFVIII variants were evaluated in mice models by monitoring plasma FVIII activity over time.

Analysis of the effect of 42 and 144 residue XTEN insertions at 64 different sites showed that the expression level of FVIII-XTEN variants is highly dependent on the site of insertion. FVIII variants remained active with single XTEN insertions at multiple sites, predominantly in the A domains, while insertions at other sites abolished expression. We identified three insertion sites, in the B domain (N745), the a3 domain (Q1656) and at the C terminus, where a single 144 residue XTEN resulted in consistently higher expression levels and activities than those of unmodified BDD-FVIII. Increasing the number of insertions (up to six) decreased the observed activity in a progressive fashion. An XTEN insertion within the a3 domain (Q1656) partially rescued otherwise low activities resulting from multiple insertions.

XTEN insertions improved the half-life of hFVIII in mice also in an insertion site-dependent manner, with single XTEN insertions in the A1, A2 and A3 domain extending the half-life to 3.5 h, 2.5 h, and 4.5 h, respectively, as compared to 0.25 h for unmodified rBDD-FVIII. The effects of multiple XTEN insertions on PK were generally additive when insertion sites were in different domains.

The hFVIII retained activity and exhibited extended *in vivo* half-life following the insertion(s) of one or more XTENs when insertion points are appropriately selected. Single XTEN insertions at three specific sites enhanced the FVIII expression level.

DEVELOPMENT OF COMPLEX ANTIBODY-CYTOKINE FUSION PROTEINS AT HIGH QUALITY BY AN INTEGRATIVE EARLY DEVELOPMENT APPROACH

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Key Words: antibody-cytokine fusion protein, targeted immunotherapy, product-related impurities, cell line development, manufacturing process

Targeted immunocytokine therapies represent therapeutic options which can lead to durable responses in cancer patients and, at the same time, circumvent the issue of significant toxicities of highly potent non-targeted cytokines. In this study, a fast and elegant strategy for cell line selection and development of a manufacturing process for novel antibody-Interleukin 2 (IL2) fusion proteins for cancer therapy will be described.

Here, we developed a novel class of monomeric tumor-targeted IL2 fusion proteins where a single engineered IL-2 variant (IL2v) with abolished CD25 binding is fused to IgG1 antibodies with a heterodimeric Fc-part. FcγR and C1q binding is completely eliminated by a novel Fc mutation. High affinity antibodies against tumor-specific or tumor stroma-specific targets represented the targeting moieties.

The complexity of these molecules entails the formation of various product-related impurities, whereby certain IL2-containing species might have an unfavorable toxicity profile. Here, a concerted approach involving and cross-linking cell line development (CLD), upstream (USP), and downstream processing (DSP) was mandatory in order to be capable of reducing critical impurities. In CLD, it was achieved to exclude certain side products, especially IL2-bearing side products, by tailored, high throughput screening methods. Impurities still being produced by selected clones could be successfully removed by a 4-step DSP process. Moreover, tailoring of the USP process resulted in sufficiently high titers and in decreased generation of lower molecular weight antibody-IL2 fusion proteins.

In summary, an elegant approach for cell line selection and development of a manufacturing process was applied for the development of novel, complex IL2-based immunocytokines. Cross-linking CLD, USP and DSP is of outstanding importance to ensure a high quality of novel and complex therapeutic proteins.

ATTRIBUTE-BASED CELL LINE SELECTION AND PROCESS OPTIMIZATION TO IMPROVE PRODUCT QUALITY AND REDUCE TIMELINES FOR DEVELOPMENT OF NOVEL MOLECULE DRUGS

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Key Words: Novel molecule development, in-process analytics, monitoring product attributes, bioprocess optimization

Non-mAb drugs are representing an increasing percentage of the pipelines of biopharmaceutical companies. The successful development of these novel molecules represents a significant challenge given the increased complexity and wider range of post-translational modifications that are often observed. Defining the quality attributes early in the project lifecycle is essential to ensure the key product attributes for each molecule (e.g. attributes affecting activity and/or half-life) can be monitored during the development of bioprocesses. This in-process monitoring is crucial for controlling the product quality from early material supply through to toxicology batches to enable the rapid, and successful, progression of these molecules through the drug pipeline. The limiting factors for obtaining this data with conventional techniques are often a combination of throughput, amount of sample required and data generation/analysis time. However, if product attribute information is to be used to drive decisions during early development activities, the techniques used must be able to generate the data for a large number of samples in a short timeframe.

At MedImmune we have developed an analytical toolbox using state-of-the-art equipment to monitor product attributes (e.g. glycosylation, truncation, aggregation, terminal clips) throughout the bioprocess. These methods are high throughput, require low sample volumes and have a fast turnaround to enable timely decisions to be made. In most cases the techniques can be applied to crude supernatants for the analyses permitting screening during early cell line development stages as well as process development and process optimizations in bioreactors. These data, together with the growth and productivity information on each cell line, has enabled product attribute-driven cell line selection. Case studies describing the use of these tools in the development of novel molecules will be described.

RATIONAL DESIGN OF IMMUNOTHERAPEUTICS TO TREAT DISEASE CAUSED BY BORDETELLA

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Key Words: pertussis, adenylate cyclase, human IgG

In spite of near-universal vaccination, pertussis remains a significant public health problem with no specific therapy for established whooping cough disease. Because disease is not driven by bacterial infection at the time pertussis is diagnosed, antibiotic therapy is often ineffective. High-titer polyclonal antibody has been evaluated previously in mice and humans, with demonstrated ability to protect mice up to seven days after infection but with less clear outcomes in infants. We hypothesize that cocktails of anti-toxin antibodies, selected to bind key protective epitopes with high affinity will be more effective at ameliorating disease in a passive immunotherapy setting. We are pursuing two parallel lines of investigation towards this goal.

To identify novel antibodies binding protective epitopes, we are characterizing the anti-pertussis immune repertoires after immunization or infection. We have examined individual domains of the adenylate cyclase (ACT), an important virulence factor which is not currently included in any vaccine formulation, with poorly characterized protective epitopes as well as the pertussis toxin and filamentous hemagglutinin. The antibody sequence and epitope diversity and quality, in terms of binding affinity and neutralizing ability, have been characterized. To identify synergistic antibody combinations, we are evaluating the protective capabilities of cocktails comprised of antibodies with well-defined epitopes and affinities *in vitro* and *in vivo*, in mice. We have produced two anti-pertussis toxin antibodies and one anti-ACT as humanized IgG molecules for assessment in a murine model of disease. The biochemical mechanisms by which these antibodies block toxin activity and the basis for synergy have been characterized.

CELL CULTURE ENGINEERING-THRIVING AFTER ALL THESE YEARS

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Key Words: process technology, continuous bioprocessing, cell line genome

Three decades after cell bioprocessing first saw its surge, cell culture engineering is at its prime from many respects. Its products are increasing their global reach and its research and production activities are expanding relentlessly in many regions of the world. A couple important questions are worth asking while the technology is at its prime. First, what we, the process technologists, have done right since its inception to make cell culture a triumphant process technology. Second, how to maintain the success and to continue the intellectual stimuli to draw the best talents to the field. The aim of this presentation is to stimulate further discussion on a couple potentially transformative ideas that have emerged in the past few years, from continuous bioprocessing to cell line genome engineering. One cannot over-emphasize the importance of the interdisciplinary root of our profession. It may serve us well to be reminded of the critical role of engineering in the success of cell culture engineering.

CASE STUDIES ON IMPROVING CELL LINE PRODUCTIVITY, PRODUCT QUALITY, AND THE EFFICIENCY OF CELL LINE GENERATION THROUGH CELL LINE ENGINEERING

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Key Words: zinc finger nucleases, TALE nucleases, cell line engineering

Central to the needs of the biopharmaceutical industry to support the development of innovative biologics and biosimilars are more effective and efficient manufacturing processes which require highly-productive cell lines with desired quality attributes. New designs of molecules such as antibody humanization have greatly reduced immunogenicity concerns, and advances in cell culture technology including media optimization and process control have driven monoclonal antibody productivities in excess of 10 g/L with peak cell densities in bioreactors climbing to over 35 million per milliliter. However, over this same timeframe, the fundamental processes utilized for cell line generation have not change significantly. Cell line generation processes remain time consuming, labor intensive and have become the timeline limiting step for the majority of the industry.

Recent advancement in several key technologies including zinc finger nucleases (ZFNs), meganucleases, TALE nucleases, and CRISPR/Cas9 has made it practical to engineer cell lines by targeting modification of key genes related to cell culture process, metabolic pathway, as well as protein quality attributes. At Eli Lilly, efforts have been made to leverage ZFN and TALEN technologies to engineer GS-CHO host cells, in combination with expression vector engineering to improve the effectiveness and efficiency of cell line generation processes. Three case studies are presented, covering 1) more than 8-fold improvement in cell line generation efficiency with the combined usage of GS-knockout cells and weakened SV40E promoter driven GS gene; 2) both specific and volumetric productivity improvement for one hard to express mAb molecule through alternative secretion signal peptide, re-transfection and host cell engineering; 3) the elimination of a key impurity from drug substance accomplished by disruption of the CHO endogenous antigen encoding gene.

MODULATION OF MONOCLONAL ANTIBODY QUALITY ATTRIBUTES USING MICRO-L SCALE FED-BATCH CULTURES

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Key Words: high-throughput, CHO cells, micro-scale, quality attribute, glycosylation

A high throughput DoE approach was used to explore the impact of media and feed components on the main quality attributes of a monoclonal antibody. The experiment was performed using a new cultivation system based on shaking 96-deepwell plates, a system which is predictive of fed-batch performance in bioreactor cultures. Each individual well contains a micro-scale suspension culture which is operated according to the same protocol as the manufacturing fed-batch platform (i. e. same culture duration and feeding regime). The experimental plan was designed in order to test 6 CHO-S derived clonal cell lines expressing the same monoclonal antibody in 2 different cell culture media and with 6 components added on day 5 of the culture at 3 different levels. The resulting 384 culture conditions, including a number of controls, were simultaneously tested in fed-batch conditions in the 96-deepwell plate system.

Performance features such as viable cell density, culture viability and product titer were monitored in all cultures on day 3, 5, 7, 10 and 14. On day 14, the remaining culture volumes, approximately 0.5 mL, were purified using small-scale affinity columns. The product was then analyzed for charge variant distributions, N-glycan profiles, aggregates and low molecular weight forms. As a result, the early screening described here provided highly valuable insights into the factors and combination of factors that can be used to modulate the desired quality attributes of a molecule in further process development steps. Moreover, it indicated to what extent the attributes can be impacted within the selected experimental design space. The approach also revealed some of the intrinsic differences of the selected clonal cell lines. Some cell lines were very responsive in terms of changes in performance or quality attributes whereas others were less affected by the factors tested in this study. Overall, this integrated early cell culture development approach was found to be particularly fast and resource effective and the outcome correlated ideally with confirmations performed in larger cell culture volumes such as shake tubes and small-scale bioreactors.

DEVELOPMENT OF A PHASE I/II TRANSIENT GENE EXPRESSION UPSTREAM PLATFORM PROCESS FOR AN ENVELOPED VIRUS-LIKE PARTICLE VACCINE

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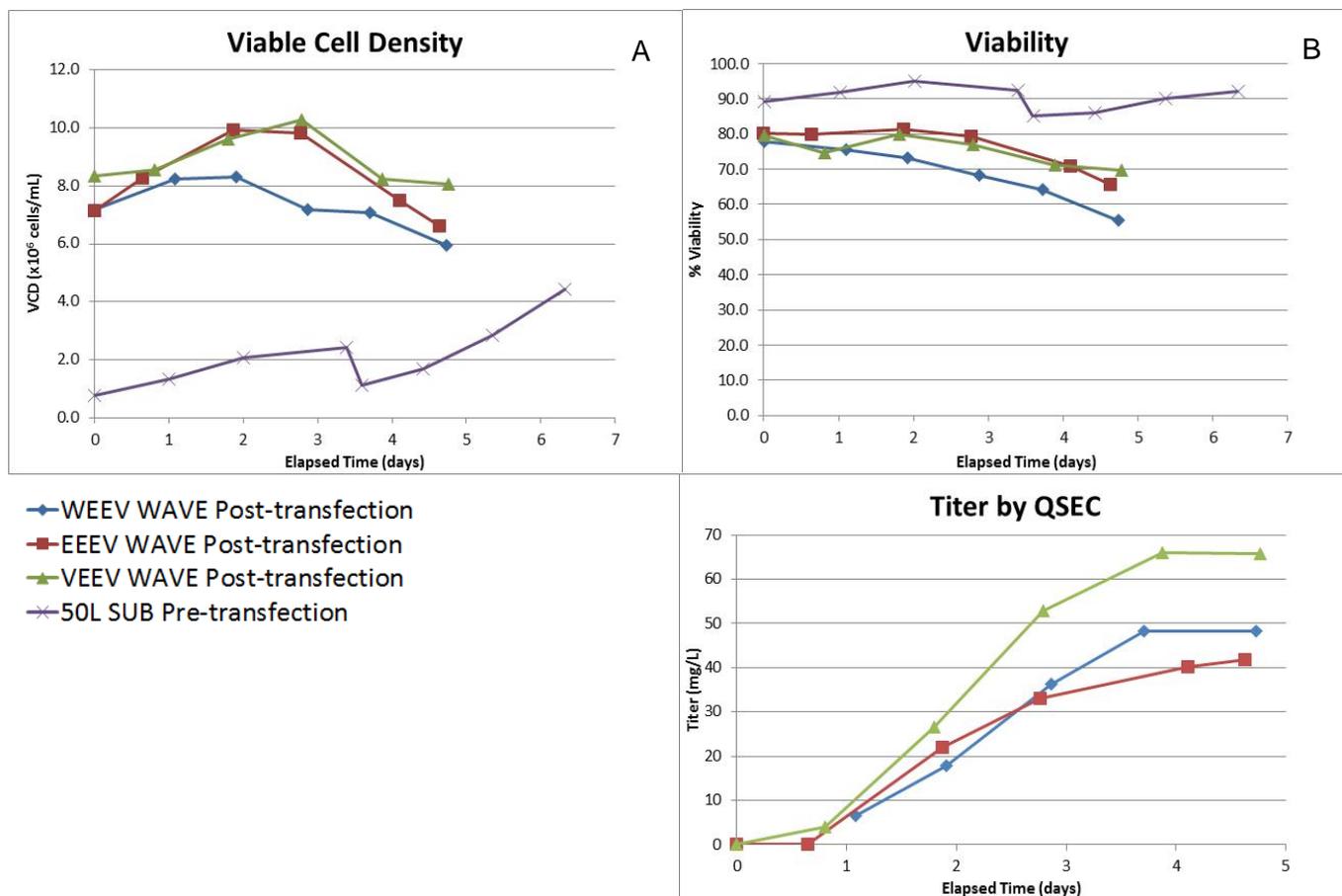
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Key Words: Vaccine, VLP, TGE

Western, Eastern and Venezuelan Equine Encephalitis Viruses (WEVEE) are alphaviruses in the Togaviridae family. These alphaviruses are highly infectious with severe, long-term neurological effects and varying levels of mortality. In addition, they constitute an ongoing and possibly increasing bioterrorist threat. The VRC is developing a trivalent virus-like particle vaccine for the WEVEE alphaviruses. The VLPs are replication incompetent enveloped VLPs that are formed in vitro by the expression of the structural proteins of each virus.

Production of alphavirus VLPs are challenging as the VLPs are intrinsically lytic to the expression cells in culture. Therefore, generation of stable cell lines for VLP expression has not been possible with techniques commonly used for cell line development. To develop a clinical product, a PEI-based transient gene expression platform process has been developed. The GMP production process for each alphavirus has been developed at the 50L-scale using completely disposable upstream equipment. The Phase I process has been transferred for GMP manufacturing. Background and optimization data for our Phase I manufacturing process of these vaccines will be presented.

Figure 1. Production of WEVEEV VLP . Viable cell density (A) and viability (B) pre- and post-transfection and titer post-transfection (C)



MULTI-GENE ENGINEERING OF MAMMALIAN CELL METABOLISM: NEW APPROACHES AND TOOLS IN THE PURSUIT OF HYPERPRODUCTIVITY

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Key Words: metabolic gene engineering, recombinant retrovirus

Metabolic gene engineering holds the potential to develop high-producing hosts for the manufacture of complex biopharmaceuticals. Hyperproductivity has been conceptualized as the orchestrated combination of superior attributes from different biochemical pathways. However, gene engineering involves labor-intensive steps, from the introduction of the target gene to the isolation and characterization of the candidate clones, turning multiple manipulations extremely difficult.

In this work, we established the basis for a pioneer project of multi-gene engineering of mammalian cell metabolism using, as study model, a human cell line producing recombinant retrovirus. To face the challenge of clone screening, a novel method for fast isolation of high-titer clones was established by merging cell cloning and product titration: the single step cloning-titration method [1]. Assisted by a previous study on the metabolic networks recruited when establishing a producer cell line [2] more than 30 candidates, including metabolic and regulatory genes, were chosen for manipulation. More than half of those candidates have already been evaluated spanning across: polyamines metabolism, glutathione metabolism, amino acid metabolism, protein processing in the endoplasmic reticulum, apoptosis and energy generation metabolism. Specific productivity improvements up to 22-fold increased were obtained. Genes found to yield high-producing phenotypes are now being combined and the resulting clones characterized. Additionally to higher productivities, relevant phenotypes – such as reduced lactate production, serum independence and increased apoptosis resistance – were engineered.

This work encompasses a challenging multi-gene engineering project, to explore the multiples routes to higher productivities. This project will contribute to deliver better producer hosts and new insights on the metabolic dynamics of manufacturing complex bioproducts in mammalian cells.

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PLURONIC F-68 CELL CULTURE RAW MATERIAL EVALUATION: SCALE DOWN MODEL DEVELOPMENT AND MECHANISM INVESTIGATION

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Key Words: pluronic F-68, lot-to-lot variation, bubble surface properties

The addition of a protective surfactant, such as Pluronic F-68, for bubble-associated cell damage in a bioreactor plays an important role in the success of suspension cell culture technology. It is probably one of the most important additives to cell culture media. Pluronic F-68 is a tri-block copolymer with a wide molecular weight distribution, which is more complicated than other chemicals in the media. Over the last few decades, lot-to-lot variation has been observed and reported. In recent years, this issue has become more of a problem for the biopharmaceutical industry as the shift to chemically defined media is stripping out naturally occurring cell protectants found in plant and animal derived raw materials.

A manufacturing campaign case study demonstrating the significance each Pluronic lot has on performance will be shared. While standard small scale satellite bioreactors showed normal performance, following intensive troubleshooting, a few scale down models were developed including an efficient and convenient shake flask method. This method also works with commercially available media and cell lines after minimal optimization. It was also found that there are different characteristics among identified suspicious lots. The mechanisms causing underperformance were investigated. A novel device was developed to test bubble surface properties between good and suspicious lots. In addition, the effect of Pluronic F-68 on other interfacial phenomenon was checked which showed a correlation with its efficiency as applied to cell culture processes.

DEVELOPMENT AND MANUFACTURABILITY ASSESSMENT OF CHEMICALLY DEFINED PROTEIN FREE MEDIUM TO SUPPORT THERAPEUTIC PROTEIN PRODUCTION IN MAMMALIAN CELLS

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Keywords: statistical design of experiment, protein-free media, mAb production

Advantages of using internally developed chemically defined, protein free media for cell culture-based therapeutic protein production over commercial media include better raw material control and medium vendor options, and most importantly, having flexibility for process development and optimization for specific products. Using statistical design of experiment (DOE), we successfully developed several CD basal media for CHO cell culture. The CD medium development was completed through several rounds of high throughput DOE screening studies. The internally prepared CD media demonstrated compatible cell culture performance to that from control media.

However, when the same formulations were submitted to two major commercial medium suppliers for the manufacturing of the final CD media, cell culture performance utilizing these media was significantly reduced compared to the in-house prepared counterpart. Through extensive troubleshooting investigation two key components were identified to be depleted in the large scale preparation of the final bulk media by the vendors. Further work necessitated the re-formulation of the original formulation into a core medium that was suitable for large-scale media manufacturing. The modified preparation of the final bulk CD media recovered their expected cell culture performance and enhanced monoclonal antibody (mAb) manufacturing robustness. This active pursuit in defining and characterizing all components for mAb production processes is our continuous effort in improving our mAb manufacturing processes, as well as the introduction of new platforms for future therapeutic protein production.

RESOLUTION OF PROCESS DEVELOPMENT AND SCALE-UP CHALLENGES WITH A SENSITIVE CHO CELL LINE

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Sensitivity of CHO (Chinese Hamster Ovary) cells in bioreactor cultures to environmental and operational factors, such as hydrodynamic shear stress, bubble damage, and metabolite/ dissolved gas levels, has been well characterized and documented. Tolerable ranges have been proposed and tested at manufacturing scales to potentially mitigate the impact in most CHO cell lines. However, when a production clone does not conform to predicted behavior at these “safe” ranges, novel approaches to process development are required to meet the complex challenges that can arise.

This presentation focuses on a production CHO cell line for an IgG4 monoclonal antibody which exhibits sensitivity to many operational and culture conditions, particularly agitation, bubble damage, and ammonia and lactate levels. For example, with a 3X increase in power/unit volume (from 20 to 60 W/m³) during scale translation, a 50-70% decrease in titer was observed. Similar effects were seen after marginal increases in superficial gas velocity. Analysis of cell morphology revealed an unusual cell shape quite different from the parental cell line or a typical CHO production cell. The cell line sensitivity manifested itself in several ways during process development and scale translation, including significant culture growth and production variability, observed medium lot to lot variability (particularly after transition from a liquid to powder medium formulation format), and potential susceptibility to antibody reduction during downstream processing.

To resolve these issues, a statistical design of experiments and scale-down model approach was employed to decouple, characterize and control the impact of numerous process parameters to ensure process robustness. This case study is a model for the rapid troubleshooting and resolution of complex development challenges, introduced with a cell line that may be impacted by slight changes in its environmental and culture conditions, without impacting timelines and product quality.

DEVELOPMENT OF A SCALEABLE AND PRODUCTIVE INSECT CELL CULTURE BASED PROCESS FOR MAKING FLUBLOK, THE FIRST FDA LICENSED RECOMBINANT INFLUENZA VACCINE

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Key Words: Flublok, recombinant baculovirus, bioreactors

Flublok was licensed by the FDA as the first recombinant influenza vaccine in January 16, 2013 and was made at the 600 liter scale using a proprietary process based on insect cell culture infected with baculovirus. The process for making Hemmagglutinin protein (HA) has now been successfully scaled-up to the 2500 liter scale in Pearl River, New York and to the 18,000 liter scale by UMN, a licensee in Japan. Details of how an existing 2500 liter Bioreactor previously used for microbial growth was retrofitted for cell culture will be described. Parallel work at the small scale demonstrated that carbon dioxide concentration was an important variable and we will illustrate how this impacted the design and operational choices at the 2500 liter scale.

A new 100 liter single use bioreactor was installed for propagating recombinant baculovirus. Operating parameters related to acceptable shear forces in the Bioreactors, acceptable oxygen levels and dissolved CO₂ levels will be provided.

An important, and unique, characteristic of this process is that it can be rapidly converted to make a new HA protein using an FDA licensed Universal Process. An example will be given with timelines for the emerging new influenza virus H7N9. Given that there is widespread cell culture capacity in many countries the implications for pandemic preparedness are significant. The same principles are also applied for introducing a strain change for the anticipated annual change in the seasonal influenza vaccine. The vaccine response to a strain change is that the DNA sequence for HA is all that is required for the Protein Sciences recombinant based approach.

The basic cell culture process (the Universal Process) is based on batch culture and is very simple. We have demonstrated that by applying fed-batch approaches the cell concentration and the product concentration can be increased several fold. This fed-batch process has been demonstrated up to the 500 liter scale and a strategy for implementation to create a second generation Universal Process will be discussed.

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THE COLORFUL SIDE OF SCALING-UP TO 20,000 LITERS

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Key Words: Scale-up, troubleshooting, color, comparability, acidic species

Late stage bioprocess development includes the scaling-up of the manufacturing process to the final commercial scale in preparation for Phase 3 clinical drug substance (DS) production, process validation, and eventual filing for market authorization. Qualification of a new site and scale of manufacture requires the demonstration of analytical comparability of the DS to material previously used in Phase 1 and Phase 2 trials.

We have recently gone through one such scale-up exercise for a monoclonal antibody produced by a platform-based fed batch process. Phase 2 clinical scale was 800-liters while the chosen scale for Phase 3 scale-up was 20,000-liters (Lonza, USA). Three cGMP runs at 20kL scale were performed. Batch 1 DS met all of its release specifications and process performance both in cell culture and throughout DSP was as expected based on 800-liter data. The relative content of acidic variants (the acidic peak group, APG) was measured to be twice as high (40%) in Batch 1 DS, which posed some concern to comparability, however the APG specification was under development, hence no specific investigative action was taken. Batches 2 and 3, executed 2 months after the first batch, failed to meet their release specifications on the basis of Color (yellow-brown compared to control, spec <Y4), while the APG was measured at 50% and 60%, respectively (out of trend). All other product specifications were met in Batches 2 and 3. Revisiting of the DS color result of Batch 1 revealed that it was significantly different than 800-liter material and had marginally met its specification.

The rigorous and systematic investigation by the UCB / Lonza team working together relied on data analysis, a deep dive into differences in operating scales, the availability of in process samples retained from every step, and on established scale down models for both the cell culture and the downstream process. Within 9 weeks of initiating the investigation, we were able to identify the unexpected root cause and reproduce its effects in small scale. In a nice example of collaboration between customer and CMO, changes were introduced to the process and to the large scale plant and two batches were repeated, delivering in both cases drug substance that was fully comparable to 800-liter material with no color issues and APGs at 20%.

In this presentation we will reveal details of the investigation, the fascinating root-cause for both color and high APGs, the corrective actions taken and the overall lessons learned from the experience.

MULTIVARIATE STATISTICAL DATA ANALYSIS FOR CONTINUOUS CELL CULTURE MANUFACTURING PROCESS IMPROVEMENT: A CASE STUDY OF TEMPERATURE CONTROL IMPACT ON CELL CULTURE PERFORMANCE

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Key Words: CHO, Cell culture manufacturing, Statistical data analysis, Continuous improvement, Temperature control.

Data collected over time from complex cell culture manufacturing processes can be analyzed to assess performance and product quality consistency between batches and between campaigns to support continuous improvement, corrective actions and preventive maintenance programs in the biopharmaceutical industry. The application of statistical data analysis as data driven knowledge facilitates engineers to cut through the complex data sets to discover underlying patterns and opportunities for improvement. In this presentation, a case study will be shared to discuss how a complex issue related to temperature control, causing 25% recombinant protein product titer loss per run compared with other contemporary runs, in a commercial CHO cell culture manufacturing process was discovered, confirmed and resolved through the application of multivariate data analysis and statistical methods. Furthermore, the associated GMP changes as corrective actions, which have been being implemented for improving the accuracy, consistency, performance and reliability of the overall cell culture manufacturing processes, will be discussed. The key findings highlighted in this presentation are intended to establish manufacturing process knowledge, which is valuable for other partners in the cell culture manufacturing network.

CELL CULTURE TECHNOLOGY IN THE 21ST CENTURY: FROM EMPIRICISM TO PREDICTIVE MODELING

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Key Words: protein therapeutics

The history of producing recombinant protein therapeutics in mammalian cells spans a little more than 30 years, but over this time period the industry has experienced enormous progress. In the beginning it was challenging to make enough, high quality drug product to meet even small market demands. We can now reliably deliver enough high quality therapeutic drug to meet the most demanding markets, requiring metric tons of product. Rapidly improving process, analytical and computational tools are enabling a future we could only dream about when the modern biotechnology era began. In this future a wide variety of protein therapeutics will effectively treat disease and be accessible to much of the world population, not just those who live in the wealthiest nations. The best is truly yet to come!

INTENSIFICATION OF A BATCH/RE-FEED PROCESS FOR A LICENSED PRODUCT

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Key Words: bioreaction optimization, manufacturing constraints, glycoprotein production

A low-productivity batch re-feed process for a licensed product was intensified within the constraints of the existing manufacturing facility, purification process and rapid timelines. The production of the complex glycoprotein was increased by enriching the production medium currently used in order to support increased cell densities.

Rapid screening of medium modifications at the bench scale was followed by optimization of the component levels using a design of experiments approach, and where appropriate, multivariate analysis.

An iterative risk assessment scheme was used to identify and prioritize parameters for lab scale process characterization. The bench scale-down bioreactor model was used to characterize the bioreactor parameters in a series of multi-factorial and single-factor experiments. Bench scale harvests were partially purified and the resulting pools were characterized to ensure product quality comparability with the current process.

The results of this exercise were verified successfully in pilot and large scale development batches with appropriate analysis of product quality for development drug substance batches.

ADVANCES IN INTEGRATED CONTINUOUS BIOPROCESSING: ACHIEVING HIGH CELL DENSITY AND HIGH VOLUMETRIC PRODUCTIVITY FOR THERAPEUTIC PROTEINS

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Key Words: Therapeutic proteins, integrated continuous bioprocessing, quality by design

To improve the economics of biomanufacturing and the flexibility of plant capacity management, Genzyme is developing a new biomanufacturing platform suitable for all therapeutic proteins. Key design goals are to reduce facility complexity while simplifying technology transfer and production capacity changes. This platform utilizes the power of integrated continuous processing, spanning both upstream and downstream operations. With enhanced process understanding and a robust control strategy, we have demonstrated that a production process can be maintained at an optimum steady state for long durations. Thus, productivity may be maximized while ensuring product quality consistency, reflecting quality by design (QbD) principles. In our view, realization of these key benefits at full manufacturing scale will translate to lower manufacturing risks, lower manufacturing and facility costs, shortened plant turnaround times, and more flexible capacity utilization.

This presentation will focus on the upstream operations in an overall integrated continuous bioprocess. Process intensification via this integrated approach does bring challenges to upstream development due to increased culture cell density, e.g. nutrient deprivation, shear stress, foaming control, mass transfer and cell retention device longevity. We will discuss recent progress in overcoming these challenges to achieve long-term steady-state high cell densities and high volumetric productivity cultures. We have demonstrated the ability to achieve a volumetric productivity of 2.5 g/L-day for a monoclonal antibody product, with potential for further improvement. We also have demonstrated the successful scale up of a high cell density perfusion process to a 50 L single-use bioreactor, integrated with a continuous capture system, for a therapeutic enzyme product. The impact of the integrated continuous platform on product quality will also be presented. We believe this platform has the potential to markedly transform how therapeutic protein drugs will be manufactured in future due to the numerous strategic advantages this approach offers.

INTEGRATED CONTINUOUS PRODUCTION – A BENCH-TOP FACTORY FRAMEWORK FOR RAPID PRECLINICAL SUPPLY OF FRAGILE PROTEINS

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Key Words: Integrated continuous manufacturing, cell culture, purification.

Continuous process designs offers several advantages including streamlined process flows and high volumetric productivities. This enables a reduction of equipment size and removal of intermediate holding steps which in turn allow for compact facilities and reduced capital costs. For bioprocessing, a reduced overall processing time and elimination of intermediate storage also decreases the product's exposure to enzymatic, chemical and physical modifications. This makes continuous processing particularly attractive for the production of fragile proteins.

We have developed an integrated continuous framework for end-to-end production of complex fragile proteins based on perfusion cultivation and automated multi-step purification. Upstream, the integrated system consists of a stirred tank bioreactor with an ATF cell retention system. Automatic feedback control of viable biomass using an on-line capacitance probe ensures robust long-term operation at steady-state and hence a constant and consistent product stream for downstream processing. The clarified harvest directly enters an off-the-shelf ÄKTA chromatography system converted into a continuous purification unit. Two alternating capture columns precede a multi-step purification train with full flexibility and control of individual columns.

The integrated set-up provides a compact automated bench-top factory converting cell culture media to purified protein in an efficient manner without intermediate storage. It reduces the lead time from start of expression to purified protein compared to traditional batch-wise processing. Furthermore, the integrated approach also provides continuous monitoring of the process allowing for "just-enough" production and better use of resources. Examples from production of fragile proteins for pre-clinical supply will be presented.

INTEGRATED BIOPROCESS DEVELOPMENT – PURIFICATION OF EXTRACELLULAR PROTEINS USING MEMBRANE CHROMATOGRAPHY

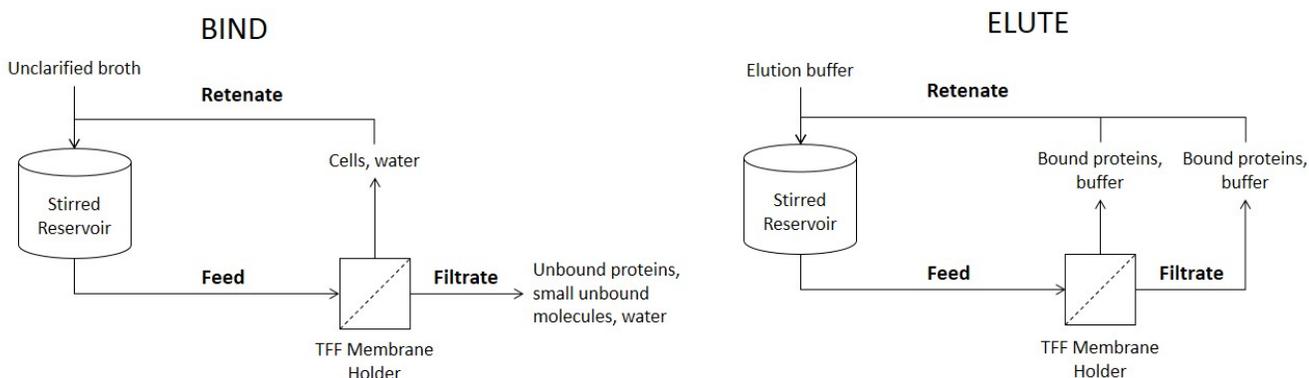
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Key Words: Membrane chromatography, recombinant protein purification, tangential flow filtration, ion-exchange chromatography, extracellular

As desirably high protein titers are now often attainable in state-of-the-art bioprocesses, advancements in downstream processing are becoming the focus of many intensification efforts. Conventional purification processes are operation intensive, technically complicated, product specific, and time consuming. To address these issues, an integrated approach to bioprocess design was developed eliminating energy intensive and tedious steps such as clarification and desalination. This was achieved by using an emerging technique, tangential flow filtration (TFF) membrane chromatography, which combines the filtration and cross flow capabilities of a membrane with the separation abilities of a chromatography resin. The process was developed using the model system of extracellularly expressed penicillin G acylase. Directly following cultivation, the unaltered broth - containing our extracellular protein of interest- was applied directly to the TFF anion exchange membrane [1, 2]. By combining the filtration, clarification, and purification steps, the operation was significantly simplified and the batch time was decreased while high purity and high recovery was attained. Advances in membrane binding capacities, increased availability of ligands, and simple scale-up are making membrane chromatography a valuable tool in increasing process productivity [3]. Due to the versatility of the developed bioprocess, it could be applied for the effective purification of recombinant proteins from a variety of hosts.

Figure 1 – Two modes of operation of tangential flow filtration membrane chromatography. In bind mode, unclarified broth is added to the reservoir and circulated parallel to the membrane. Small unbound molecules pass through the microfiltration membrane into the filtrate while cells cannot. The broth is circulated until all desired protein has been bound. Once the membranes are saturated, they are operated in elute mode to remove bound proteins from the membrane in an isocratic method.



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THE PROFILE OF INTRACELLULAR LIPID-LINKED OLIGOSACCHARIDES AND NUCLEOTIDE-SUGARS DETERMINE THE DISTRIBUTION, SITE OCCUPANCY AND N-GLYCOSYLATION PROFILE OF A CHIMERIC HUMAN-CAMELID ANTIBODY

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Key Words: glycosylation, monoclonal antibody, CHO cells, lipid-linked oligosaccharide, nucleotides and nucleotide sugars.

The demand for high yield recombinant protein production has focused on feed strategies that minimize the concentrations of media components such as glucose and glutamine. Although this may maximize protein productivity, it is important to ensure that product quality attributes such as glycosylation are maintained. The first steps in the glycosylation pathway involve the synthesis of lipid-linked oligosaccharides (LLOs) via addition of sugars through nucleotide sugar donors. Glycan macroheterogeneity is introduced by variation in site-specific glycosylation with the transfer of the oligosaccharide to the protein. Further modification of the oligosaccharide can occur through processing reactions, where some sugars are removed and additional sugars added through nucleotide sugar donors. This produces microheterogeneity of the glycan pool. Both macroheterogeneity and microheterogeneity may be affected by the availability of precursors.

We have shown that the glycosylation profile of a chimeric heavy chain antibody (EG2) produced from CHO cells was affected by the glucose concentration (0-25 mM) of cultures established at high density ($>10^6$ /ml) over 24 h. The resulting proportion of non-glycosylated Mab was directly correlated with the exposure time of cells to media depleted of glucose. Deprivation of glucose for the full 24 h resulted in a 45% non-glycosylated Mab fraction.

Analysis of steady state levels of intracellular LLOs showed that under glucose limitation there was a reduction in the amount of full length LLO ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$), with a concomitant increase in the smaller mannosyl-glycans ($\text{Man}_{2-5}\text{GlcNAc}_2$). Glucose limitation also reduced the cytoplasmic concentrations of nucleotides triphosphates (i.e. ATP, CTP, GTP, UTP) as well as nucleotide-bound hexosamines (i.e. UDP-GlcNAc, UDP-GalNAc) which could affect the metabolic state of the cell and ability for glycosylation. A lower adenylate energy charge (AEC) (0.3) was also detected under low glucose concentrations compared to a maximal value of 0.9.

Glycan microheterogeneity was quantified by galactosylation and sialylation indices (GI and SI) which showed a direct correlation to the exposure time of cells to media depleted of glucose. The GI increased to 0.83 following media supplementation with a cocktail of uridine, manganese and galactose. This is significantly higher than for a fully humanized antibody (DP12) produced under the similar conditions or for similar antibodies reported in the literature. The high GI of the chimeric antibody (EG2) may be due to its low molecular weight and unusual structure. These findings are important in relation to the low substrate that may occur in fed-batch cultures for Mab production.

THE EFFECTS OF ALTERNATIVE CARBON SOURCES ON CHO CELL METABOLISM AND PRODUCT QUALITY

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Key Words: Recombinant protein production, glycosylation, GCMS analysis

Recently it has been observed that Chinese hamster ovary (CHO) cells expressing recombinant protein grown using alternate sugar sources have resulted in the production of proteins with dramatically different glycosylation phenotypes. To better understand the cause for changes in glycosylation, CHO cells were grown in media containing different sugars, uptake rates were measured as well as product quality, growth and titer. CHO cells were then grown in media containing a combination of ¹³C-labeled sugars. Metabolites were extracted from cells during exponential growth and analyzed by GCMS to measure isotopic ratios. Using a metabolic network that included alternate sugar metabolism, the ¹³C-metabolic flux for each sugar was modeled. Distinct metabolic outcomes were identified for each sugar, resulting in novel mechanisms for the formation of observed glycosylation patterns as well as a more complete understanding of glucose metabolism during recombinant protein production.

MODEL PREDICTIVE CONTROL OF PRODUCT QUALITY IN CHO CELL PROCESS

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Key Words: Product quality, control, PAT, glycosylation

There are many important motivators for the development of active control of product quality in cell culture processes. Control of critical product quality attributes would enable more robust manufacturing processes, better product matching between early clinical and commercial processes, as well as between innovator and biosimilar products. In this work we report the ability to achieve feedback control of the fraction of a specific glycoform in a CHO cell culture process. After the initial demonstration of the ability to modulate the glycoform distribution via media composition, the goal was to develop an automatable control scheme that would work with the existing constraints of assay frequency and analysis time. A kinetic model describing cell growth, product formation, product composition, and critical metabolite concentration, was developed and used as the basis of a Model Predictive Control (MPC) scheme. Offline measurements were used to periodically correct the assumed state of the predictive model. The model was then used to make daily adjustments to the feed composition projected to achieve the desired product quality. The benefits of MPC when dealing with relatively infrequent measurements with significant delays will also be discussed.

OPTIMIZATION OF A BIOPROCESS FOR PRODUCTION OF A NON-PROTEIN BIOPHARMACEUTICAL: PRODUCTION OF A BIOENGINEERED HEPARIN

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Heparin, a carbohydrate drug with a \$7 billion annual market, is the most widely used pharmaceutical to control blood coagulation in modern medicine. Currently, heparin is derived from animal tissues, primarily porcine intestines and bovine lung, reminiscent of insulin production before the advent of recombinant DNA processes. A contamination crisis in 2008, resulting in ~100 deaths in the U.S. alone, has sparked interest in the development of a “bioengineered heparin” that could be produced in cell culture under cGMP conditions. We have recently metabolically engineered CHO cells to produce a heparin-like glycosaminoglycan (GAG) by introducing two enzymes, human N-deacetylase/N-sulfotransferase (NDST2) and mouse heparan sulfate 3-O-sulfotransferase 1 (Hs3st1)¹. The engineered cell lines exhibit substantially increased GAG production and a more highly sulfated GAG, with increased anticoagulant activity when compared with the parental CHO-S cells.

The engineered cell lines exhibited very different growth and metabolic characteristics from the parental cell line, including dramatically increased glucose and oxygen consumption and a dramatically decreased viable cell density. In addition, we observed that the product quality (as determined by the amount of sulfation on the GAG) varied throughout the culture, suggesting a limitation in sulfur precursors. We successively optimized fed-batch shaker and bioreactor cultures and evaluated the effects of process conditions on the expression of both endogenous and exogenous enzymes in the heparin biosynthesis pathway. We were able to achieve a 3-fold increase in product titer (from ~20 µg/mL to ~60 µg/mL) by feeding the cultures in shaker flasks and a 5-fold increase in titer using bioreactor cultures as shown below. More recently, we have explored cysteine feeding in an effort to improve the sulfation pattern while maintaining high product yields. Results from these studies may provide guidance for controlling glycosylation of protein therapeutics as well as glycosaminoglycans.

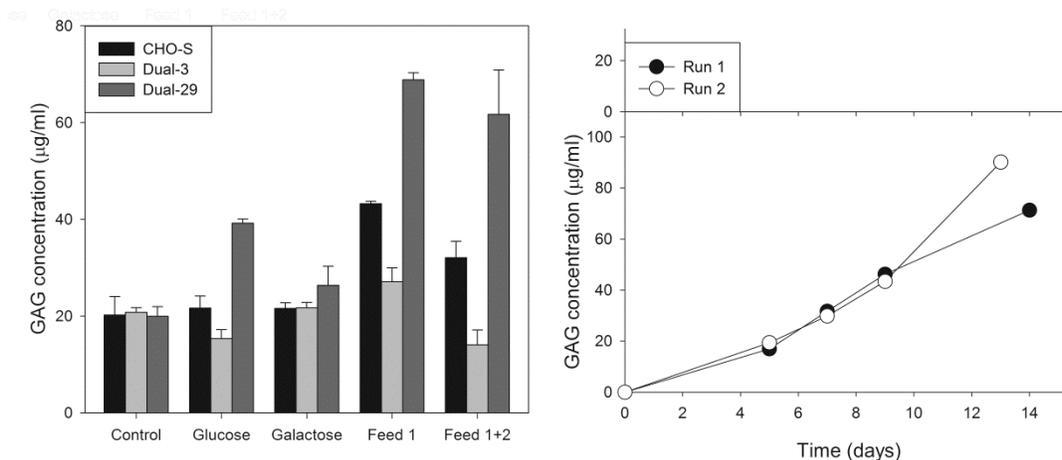


Figure 1. GAG concentrations in fed-batch shaker flasks for parental (CHO-S) and engineered cell lines (left panel). GAG concentrations in two bioreactor cultures of clone D29 (right panel).

1. J.-Y. Baik, L. Gasimli, B. Yang, P. Datta, F. Zhang, C.A. Glass, J.D. Esko, R.J. Linhardt, and S.T. Sharfstein, *Metabolic engineering of Chinese hamster ovary cells: Towards a bioengineered heparin*, *Metabolic Engineering*, 14: 81–90. (2012). doi.org/10.1016/j.ymben.2012.01.008

ESTABLISHING A LINK BETWEEN CELL CULTURE MEDIA AND FEED FORMULATIONS AND DRUG SUBSTANCE AGGREGATE GROWTH FOR MONOCLONAL ANTIBODIES

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Key Words: Therapeutic protein manufacture, aggregate formation, protein chromatography

In preparation for commercialization of therapeutic protein manufacturing, cell culture activities focus on developing a robust process that consistently delivers large quantities of high quality protein. Components in cell culture media are known to have a significant impact on both process yield and the quality of the purified protein. Recent work has demonstrated that components in cell culture media and feeds also have an impact on soluble aggregates formed in the drug substance during accelerated stability studies. This aggregate is not detected during analysis of material from cell culture or the drug substance prior to being placed on accelerated stability. To identify those components that impact aggregate formation on stability, an in-house method was used to screen cell culture samples purified using protein chromatography, and determine a relative rank for a component's propensity to form aggregate. This method was used to quickly assess media and feed formulations, and identify two key components that impact aggregate growth on stability. Following the screening studies, 5L bioreactors were used to generate protein that was purified, concentrated to 50 mg/mL, and placed on accelerated stability. Aggregate formed in the drug substance was then measured using size exclusion chromatography over a period of 12 weeks. In addition, the in-house method to measure the molecule's propensity to form aggregate was measured for each condition, to confirm the correlation between the in-house method and the total aggregate at the end of the 12 week stability study. These studies concluded that the concentrations of copper sulfate in the media and cysteine in the feeds can impact the total aggregate formed in the drug substance when placed on accelerated stability.

BIOPROCESS DEVELOPMENT FOR DIABETES CELLULAR THERAPY

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Key Words: diabetes, cellular therapy, stem cells, encapsulation.

Cell therapy products have the potential to revolutionize the treatment of degenerative diseases such as diabetes, with more and more promising cell types being effectively produced in bioreactors. For solid or glandular organs such as the pancreas, scaffolds mimicking the native microenvironment may be needed to ensure adequate cell function, with the ensuing complication of providing sufficient graft oxygenation. For type 1 diabetes and allogeneic grafts in general, an additional challenge resides in the immune rejection of the transplanted cells. We have developed an alginate bead emulsification process that allows rapid and scalable pancreatic cell encapsulation (Figure 1). Compared to conventional drop-by-drop encapsulation using nozzle-based devices, the emulsification process enables higher throughput as well as higher alginate concentrations. Improved graft survival in allogeneic mice was observed for mouse insulinoma cells encapsulated in 5% alginate emulsion-generated beads compared to 1.5% alginate beads generated by a conventional nozzle-based process. Neonatal porcine islets also were encapsulated and transplanted into immunocompromised mice. As expected, the progenitor cells within these islets differentiated into beta cells that secreted insulin after meals. However, central necrosis was observed in encapsulated cell clusters, likely due to hypoxia. To overcome this limitation, the encapsulated islets could be transplanted into a pre-vascularized site created by anastomosing small-diameter vascular grafts to the circulation. To avoid vascular graft occlusion due to thrombosis and intimal hyperplasia, we are developing biomimetic vascular biomaterials engineered to recruit circulating endothelial progenitor cells. These materials could facilitate the design of a perfused transplantation site ensuring long-term islet survival. These results illustrate how simple changes in process parameters can greatly affect the therapeutic potential of cellular therapy products. Overall, this work is addressing key bottlenecks hampering the clinical application of cellular therapy, such as the need for systematic process optimization and novel devices for cell delivery.

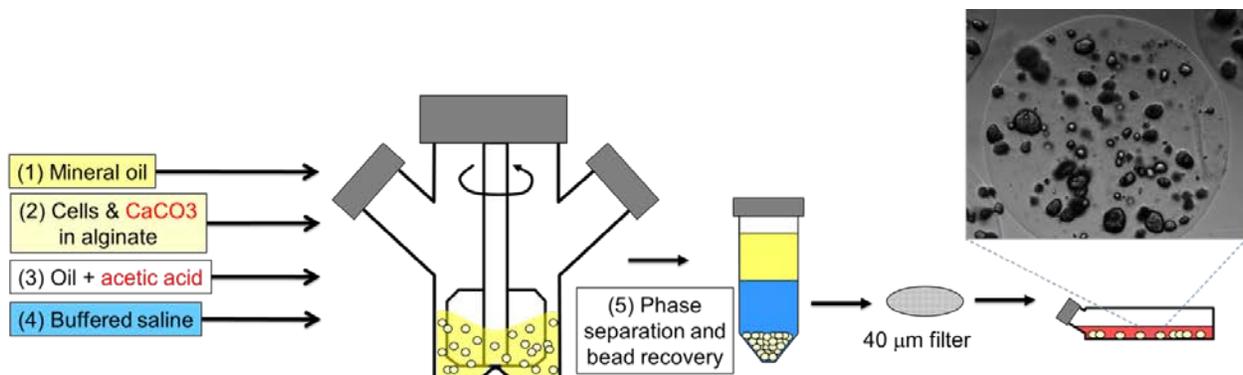


Figure 1 –Cell encapsulation by the emulsion and internal gelation process showing MIN6 beta cell clusters obtained in the beads after 2 weeks of immobilized culture.

MINING CHO CELL 'OMICS' DATA: BEYOND DIFFERENTIAL EXPRESSION ANALYSIS

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Key Words: CHO cells, Transcriptomics, MicroRNA, Proteomics, Bioinformatics, Multivariate statistics

Experimental platforms such as gene expression microarrays and proteomic mass spectrometry have been used for over a decade to study Chinese hamster ovary (CHO) cells at the molecular level. In this talk, we will demonstrate the utility of advanced statistical analyses (e.g. multivariate statistics and machine learning) to reveal biological mechanisms associated with beneficial industrial phenotypes (e.g. cellular growth rate) and identify targets for cell line engineering. We will begin by describing the construction of a partial least squares (PLS) model to predict cell specific productivity (Qp) from CHO cell

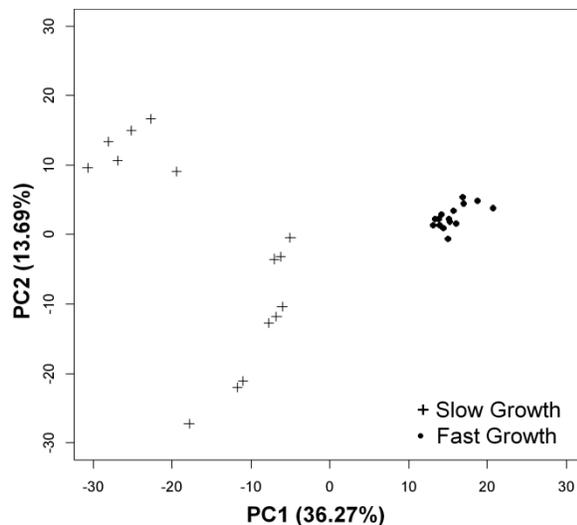


Figure 1: Principal component analysis (PCA) of microarray data from CHO clones generated during a single cell line development project. The PCA plot shows a distinct separation of the “fast” ($\geq 0.025 \text{ h}^{-1}$) and “slow” ($\leq 0.023 \text{ h}^{-1}$) growing CHO cells based on their gene expression profiles.

gene expression and discuss the potential applications of this technique in cell line development. We will also present a coexpression network analysis of a large-scale CHO transcriptomic dataset (295 microarrays spanning a diverse range of bioprocess conditions) and outline the subsequent development of an accompanying web based interface (www.cgcdb.org) to make the findings of this research available to the community. Finally, we will present a study designed to investigate the molecular basis of CHO cell growth rate through the analysis of data from multiple levels of the biological system. The sample set under investigation consisted of a panel of clones selected from a single cell line development project that displayed similar Qp yet varied significantly in terms of growth rate. These clones were profiled in parallel using qPCR, microarray and quantitative LC-MS/MS for global analysis of the CHO cell microRNA (miRNA) and mRNA transcriptomes as well as the proteome. We will outline the methods used for data integration and discuss the advantages of combining multiple omics datasets e.g. the identification of high priority miRNA targets undergoing translational repression. In the years to come advanced mining of expression data in combination with the CHO-K1 and parental hamster genomes has the potential to dramatically broaden our

understanding CHO cell biology and ultimately improve biopharmaceutical production in these cells.

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THE GENOME AS ONE STEP TOWARDS ENABLING PRODUCT ATTRIBUTE CONTROL

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Key Words: Genomics

A major milestone within the biomanufacturing community has been the establishment of the CHO K1, Chinese hamster, and related genomes. This basic knowledge provides a means to better understand and control cells and process conditions with the ultimate goal of enabling product attribute control of cell culture processes. For example, one of the important elements for product attribute control is to understand the connection between processing steps and critical product attributes and some of this is encoded by the genome of the host cell. While our community is not yet at the stage of defining clear relationships between the relevant basic science and product attribute control, there may be some relevant lessons learned from other disciplines. We will explore analogies to other disciplines as well as the impact of a community-wide effort to establish a reference genome for the CHO community.

INVESTIGATING GROWTH CESSATION IN LATE STAGES OF FED-BATCH CULTURES: GOING BEYOND THE CONVENTIONAL INHIBITORS, LACTATE AND AMMONIA

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Key Words: Metabolism, Growth Cessation, Inhibitors, Metabolomics, CHO Cells, Fed-batch Cultures.

In recent years, using robust CHO cell expression systems and efficient glucose control strategies, our group has demonstrated exquisite control of the traditional mammalian cell culture inhibitory metabolites lactate and ammonia, whilst still achieving very high cell densities, high productivities, and suitable product quality for human protein therapeutics. The techniques previously described¹ have proven practical and amenable for manufacturing-scale CHO fed-batch cultures. Even in such cultures, although conventional inhibitors accumulate below effective concentrations, cell division eventually halts.

Using this optimal system as a starting point, we will describe our systematic and thorough investigation to determine the factors that limit the peak cell density in fed-batch cultures. Our experiments eliminated the exhaustion of vital nutrients, the accumulation of proteins or peptides, and other non-optimal culture conditions (dissolved oxygen/carbon dioxide, etc.) as potential causes for cell growth slowdown. Metabolomic information generated for various processes spanning different cell lines was parsed and cross-referenced to find and quantitate the accumulating metabolic intermediates or end-products that were overlapping across the queried data sets. Using purified chemicals, the degree of growth inhibition exerted by each accumulating metabolite was quantified in well-controlled experiments and a list of the most problematic compounds was compiled.

Employing functional analysis, the carbon sources and the biosynthetic metabolic pathways for these inhibitors were postulated. Ensuing efforts to develop effective strategies for control of inhibitor production may enable fed-batch cultures to reach new levels of productivity and performance.

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SYSTEMS BIOTECHNOLOGY DRIVEN DEVELOPMENT OF CHO EXPRESSION HOST CELL LINES AND BIOPHARMACEUTICAL PRODUCTION PROCESSES

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Key Words: Bioinformatics, CHO host cell engineering, metabolomics, miRNAs, transcriptomics

The continuously growing amount of bioinformatic and omics data on CHO cells provides an immense – though not always easily accessible – knowledge base to improve and rationalize biopharmaceutical expression platforms and processes. At Boehringer Ingelheim (BI), we have in the past +5 years taken a multi-faceted approach to the bioinformatics field, and worked to combine experimental wet lab cell line and process development work with genomics and metabolomics approaches, to better understand how the macroscopic bioprocess data correlates with intracellular processes and signaling pathways. This is for example done by looking into the transcriptomics and metabolomics make-up of sub-clones derived from our BI-HEX host cell (a CHO-DG44 derivative) as well as of various engineered cell lines under different cultivation conditions. Alternatively, the cells can be actively manipulated to change their phenotype, eg by overexpressing a specific protein or a miRNA, again followed by transcriptomics and/or metabolomics analyses.

The talk will include data and learnings from several projects: In the first, the goal was to identify miRNAs capable of enhancing CHO production cell line performance with respect to productivity. A genome wide functional miRNA library screening was performed leading to the identification of several miRNAs capable of changing the CHO cell phenotype in this direction under transient and stable expression conditions. The effect was observed both in an IgG expressing cell line and in cells expressing recombinant human serum albumin, showing that the miRNAs work in a product-independent manner. Furthermore, combinations of individual miRNAs were identified that enhanced production levels further (Strotbek et al, 2013).

Next, we will discuss transcriptomics/NGS results obtained from BI-HEX1 and BI-HEX2 subclones, which were observed to have a consistent difference in their N-linked glycosylation pattern, giving rise to a higher GOF content in one line versus the other. We used NGS technologies to better understand the genetic background for this difference and could identify differential expression of glycosylation related genes between the two cell lines (Koenitzer et al, 2013). Using the same approach we recently obtained some very interesting data on a previously reported CHO cell line, conCERT, engineered to overexpress a secretion enhancing molecule, the ceramide transfer protein which is involved in protein transport from the Golgi to the plasma membrane (Florin, 2009). It was shown that certain difficult-to-express molecules expressed better in these cells. Transcriptomics analysis and comparison to our standard BI-HEX CHO-DG44 host cells revealed enhanced expression not only of the recombinant CERT, but also of other proteins likely to be involved in the ceramide-driven secretory pathway in the engineered cells. Such data could give hints to additional targets for engineering.

Finally, we will present the systematic and rational approach we took to develop a new generation of the BI media and feed platform. Here, we made use of metabolic flux analyses and metabolic profiling of our cells under different growth conditions to gain in-depth insight into changes in energy and waste metabolism. The improved understanding of cellular metabolism helped to identify media additives that efficiently prevent late stage lactic acid production and to design a balanced medium optimized for our cell lines. Surprisingly, the media and feed has turned out to work very well not only on the BI-HEX cells for which it was developed, but also on a variety of other CHO derivatives of CHO-K1, CHO-S or CHO DG44 origin.

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OMICS GUIDED CELL LINE ENGINEERING: REDUCING HIGH MANNANOSE BY OVER EXPRESSING N-GLYCOSYLATION PATHWAY REGULATORS

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Key Words: Cell Line Development, Critical Quality Attributes, CHO cells, High mannose.

High Mannose (HM) is a critical quality attribute (CQA) for recombinant therapeutic monoclonal antibodies (mAbs) that can impact biological activity by influencing Fc mediated effector functions, product stability, clearance rate and safety. In this study, we show that increased levels of N-acetylglucosaminyltransferases (Mgat1 and Mgat2) and the UDP-Galactose transporter (Slc35a2) correlate with lower levels of high mannose glycans in mAb producing Chinese Hamster Ovary (CHO) cell lines. We sought to further characterize the role of these genes by altering their expression levels and examining the effect on glycan processing. Using RNAi mediated knockdown in a low HM cell line we show that reduced levels of Mgat1 can increase the levels of HM by ~70%. Furthermore, we have overexpressed Mgat1, Mgat2 and Slc35a2 genes in a CHO cell line which produces mAbs with high HM levels. Expression of both Mgat1 and Mgat2 genes was sufficient to reduce high mannose levels by ~70 %. However, overexpression of Slc35a2 did not significantly impact HM levels. Our results show that Mgat1 and Mgat2 may represent ideal targets for the engineering of new host cell lines that can produce products with increased levels of fully processed glycan structures and hence low levels of HM.

THE METABOLIC PROGRAMMING OF INDUSTRIAL IGG EXPRESSING CHO

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CHO cells are now accumulating titers that exceed their viable cell mass. This has largely been the product of dedicated efforts in media optimization and high-throughput screening. Still, surprisingly little is known about the underlying metabolic reprogramming associated with the high-titer phenotype. Currently the biotech industry relies predominantly on two CHO expression systems, glutamine synthetase (GS) and dihydrofolate reductase (DHFR).

First, we considered a fed-batch DHFR system. During the peak antibody production phase, total nutrient consumption was reduced by 40% in comparison to exponential growth phase, indicating that substantial increases in carbon and energetic efficiency were associated with peak antibody production. Through ¹³C metabolic flux analysis (MFA) and examination of intracellular redox state, we found that elevated oxidative mitochondrial metabolism corresponded with peak antibody production. In contrast, peak cell growth was characterized by elevated glycolytic metabolism.

Second, we will discuss ongoing experiments to examine the metabolic rewiring of a fed-batch GS system using ¹³C MFA and metabolomics. In addition to comparing metabolic phenotypes at various culture phases, we will directly compare ten unique IgG-secreting GS clones to two empty-vector (i.e., non-producing) GS clones. This will enable us to systematically assess the impact of IgG production on the central metabolism of CHO host lines, in contrast to the impact of GS overexpression alone. We will discuss the central metabolic trends observed among both GS and DHFR expression systems as a means to provide potential metabolic targets to further enhance IgG productivity and titer.

CHO CELL ENGINEERING TO ALTER LACTATE METABOLISM AND IMPROVE CELL CULTURE PROCESSES

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Keywords: CHO cells; pyruvate carboxylase; metabolic engineering; monoclonal antibody

In fed-batch processes, lactate and ammonia accumulation in the culture medium over time can have detrimental impacts on cell growth and product quality. Continuous cell lines typically exhibit an inefficient metabolism whereby most of the pyruvate derived from glucose is diverted to lactate and only a small percentage is incorporated into the TCA cycle. The mitochondrial pyruvate carboxylase, which catalyzes the conversion of pyruvate into oxaloacetate, is one of the key enzymes at the junction between glycolysis and the TCA cycle. Previous metabolic flux analyses of CHO cells have revealed that only a minor fraction of the pyruvate pool is metabolized via this pathway.

In this work, a recombinant CHO cell line producing an antibody was further genetically modified with the insertion of a cytoplasmic yeast pyruvate carboxylase (PYC2) gene. Following selection, individual clones were isolated by limiting dilution and screened for their cytoplasmic PYC2 expression levels. In contrast with the parental cell line, all the clones exhibiting strong PYC2 expression consumed lactate during the stationary phase until near depletion of this metabolite from the culture environment. This metabolic shift was found to occur consistently in all commercial culture media tested. The decreased lactate production was also shown to significantly delay the acidification of the medium in uncontrolled flask cultures. In a bioreactor, it is expected to greatly reduce osmolarity changes related to pH control. Interestingly, the expression of the PYC2 gene was also associated with at least a 30% reduction in final ammonia concentrations, another highly desirable trait given the impact of this metabolite on both cellular growth and product quality attributes. Moreover, the cell specific growth rate remained unaffected, which is a significant advantage over nutrient substitutions sometimes employed to reduce waste formation.

Thus, the unique metabolism displayed by PYC2-expressing CHO cells make them highly suitable for the development of a high-performance fed-batch culture process.

MULTIPLE STEADY STATES IN CONTINUOUS CULTURE OF MAMMALIAN CELLS: MECHANISM AND CONTROL

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Cultured mammalian cells exhibit multiple steady states in their energy metabolism. Under seemingly identical conditions the glycolysis flux can be in a high flux state or a low flux state. In the high flux state, the cells consume glucose at a high rate and produce a large quantity of lactate; while in the low flux state, metabolic shift occurs such that glucose flux decreases and lactate is produced at a low rate or even consumed. In continuous cultures, such steady state multiplicity confers different cell and product concentrations. Continuous cultures of hybridoma cells demonstrated that high cell and product concentrations could be accomplished by directing cells to a metabolically shifted state compared to the cultures without a metabolic shift. Understanding the controllability of those steady states and the transient behavior that lead to those steady states are critical to achieve the desired cell density and productivity.

A multi-scale kinetic model taking into account the intracellular metabolism of glycolysis, PPP and TCA cycle as well as the macroscopic cell growth in the reactor was established. The glycolysis enzyme activities are regulated by allosteric regulations and growth signaling pathway. Conversely, the growth rate of the cells in the reactor depends on the metabolic state.

As a result of such multi-layered interactions, multiple steady states arise in certain region of the flux space. In this region, with a given feed glucose and dilution rate, cells can reside in either a high or a low metabolic state. The corresponding cell concentration in the reactor also exhibits multiple steady states. The actual steady state accomplished is dependent on the trajectory employed including ramping up or down of dilution rate and increasing or decreasing feed glucose concentration. The implications of those steady state behaviors on the industrial application of continuous culture will be discussed.

USING GLOBAL, UNTARGETED METABOLOMIC PROFILING AT A SYSTEMS LEVEL TO UNDERSTAND THE IMPACT OF PROCESS CONDITIONS ON A COMMERCIAL CHO CELL LINE

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Key Words: Metabolomics, CHO, systems biology, Ingenuity pathway analysis, biomarkers

Mammalian cells, and in particular Chinese hamster ovary (CHO) cells, are the primary hosts used for the production of biopharmaceutical proteins including antibodies, fusion proteins, hormones and cytokines. Since the first clinically approved CHO recombinant protein in 1987, over 140 recombinant products have been brought to market, and volumetric productivities have risen from a modest 0.05 g/L to up to 10 g/L of protein. These improvements can be largely attributed to cloning and genetic engineering techniques, clone selection, and bioprocess development optimization, such as media formulation. Despite the success of these techniques, they are labor intensive, time consuming and cannot consistently be applied in a rational manner. While approaches like multifactorial design have streamlined some aspects of process optimization, any empirical approach must be applied on an individual basis for every cell line, ultimately requiring significant time and resources. Furthermore, while improvements can be obtained empirically, there is little fundamental understanding of how or why specific process conditions result in improvements, which often leads to unpredictable behavior during process scaling. Future progress and improved efficiency may require a transition away from a purely empirical optimization approach, and efforts towards rational process modification through knowledge of mammalian cell biology and intracellular biochemical and molecular processes. Metabolomics is one such approach to capture intracellular characteristics, changes in metabolism, and understand cellular physiology at a systems level. As such, applying metabolomics to CHO bioprocess optimization has the ability to enhance and transform industrial process development.

This work sought to better understand the metabolic changes in a commercial, IgG producing cell line associated with variation in process conditions including raw material lots, vessel size, and media formulation. We utilized Global untargeted metabolomic profiling to identify and quantify metabolites present in fresh basal and feed media, supernatant, and cell pellets collected at several time intervals throughout production. Sample analysis was carried out using both LC/MS and GC/MS, and compounds were identified using a library of pure standards. This rich data set enables an understanding of how metabolite levels are changing across process conditions and over time. Using Partial Least Squares Regression, correlations between changes in metabolites and culture performance were identified. Finally, Ingenuity Pathway Analysis was used to identify pathways associated with significant metabolites.

This work highlights the utility and quality of information that can be gained by applying Global untargeted metabolomics to immunoglobulin producing CHO cell lines. Over 200 unique compounds were detected. Correlations between observed phenotypes and changes in metabolite levels were methodically identified. Furthermore, a systems biology approach was used to link these metabolites to critical pathways that may influence cellular phenotypes. Finally, the links identified between metabolites and relevant phenotypes serve as a starting point for the identification of critical biomarkers. This work contributes to recent research in the applications of 'Omics in biotechnology, and represents a contribution towards the transition from the empirical to a rational, intracellular approach to bioprocess optimization.

METABOLOMIC QUANTIFICATION OF COMMERCIAL PROCESS VARIABILITY, FACILITY TRANSFER, AND VARIABLE IMPACT

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Key Words: Metabolomics, Tech Transfer, Small Scale Model

As part of the transfer of a commercial antibody from one 15K facility to another, a metabolomics approach was taken to quantify known variability in the process and proactively measure performance at another site against it. A panel of approximately 50 metabolites was established by assessing six production runs known to show different metabolic patterns and product quality attributes as executed in the original manufacturing facility. A subsequent submission from the initial engineering and validation runs at the new facility was then measured against the variability of the original process. Multivariate analysis of the data showed metabolism and the associated variability was essentially maintained at the new facility. With a panel of metabolites established to characterize the acceptable metabolic patterns of the process, a number of variables including small scale models, the impact of raw material changes, and working cell bank changes were assessed. The entire body of data from these metabolomics studies performed with Metabolon establishes further confidence in a successful process transfer, allows for a more detailed qualification of proposed process changes or models, and creates a baseline against which any future process shifts can be measured.

INTRACELLULAR TRAFFICKING OF POLYETHYLENIMINE/SYNDECAN-1 COMPLEXES IN HEK293 CELLS

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Polyethylenimines (PEIs) are polycationic vectors widely used to transfect mammalian cells both *in vitro* and *in vivo*. Although cellular uptake is a crucial step for gene delivery, the membrane receptor(s) for PEI-DNA complexes (polyplexes) remain to be clearly identified. Furthermore, little is known about the intracellular addressing of polyplexes following their uptake. We have already shown that syndecan-1 (SDC1) may act as a membrane receptor for polyplexes. Here, we explored the intracellular fate of polyplexes/SDC1 complexes over time. Shortly after transfection, SDC1 forms clusters colocalizing with polyplexes that are rapidly endocytosed via lipid raft. Only a weak proportion of the resulting endosomes are addressed to the lysosomes and most of the polyplex/SDC1 complexes colocalize with the trans-Golgi network (TGN) within 3h post-transfection. While in the TGN, polyplexes dissociate from SDC1 between 3 to 6h post-transfection. Finally, SDC1 is degraded and polyplexes form large aggregates into the cytoplasm. Taken together, these results give new insights into the intracellular trafficking of SDC1 and polyplexes during PEI-mediated transfection.

EXTENDED GENE EXPRESSION FOR THE OPTIMIZATION OF PROTEIN PRODUCTION BY TRANSIENT TRANSFECTION

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Transient gene expression (TGE) is based on episomal plasmid DNA expression. Since episomal DNA is lost during cell division, the production period is limited, typically to 96 hours, and therefore the productivity of TGE is relatively low.

A novel strategy, extended gene expression (EGE), is proposed, having as an objective prolonging the production period by repeatedly transfecting cell cultures. The benefits of this method are discussed for the production of three model products including two recombinant proteins (GFP and Cherry) and a complex VLP (Gag-based VLPs), using HEK 293 cells.

In the proposed methodology, suspension HEK 293 cells grown in Freestyle medium containing non-animal derived additives are transfected with 25-kDa linear PEI. The initial transfection round is typically done at 2×10^6 cells/mL using 1 μ g of plasmid DNA/mL of cell culture as performed using standard TGE protocol. Transfections are repeated using different plasmid DNA concentrations, time intervals and culture feeding conditions in order to optimize protein expression.

Best production performance is achieved by re-transfecting cell cultures every 48 hours using a DNA concentration of 0.5 μ g/mL and medium exchange. Using this strategy, production time was prolonged to 10 days, maintaining cell viability and the percentage of transduced cells. Both product concentration and accumulated yields are significantly improved using this novel EGE strategy up to 6-fold, when compared to conventional TGE.

RAPID DEVELOPMENT OF A HIGH-PRODUCING CHO TRANSIENT PROTEIN PRODUCTION METHOD

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Key Words: CHO, Transient Transfection, Polyethylenimine PEI

CHO cells are the dominant hosts for commercial biopharmaceutical production processes, therefore they offer obvious advantages as hosts for producing drug candidates using transient methods during early phase research and development. However, the use of CHO cells in transient transfection is not ideal due to their poor titers. Additionally, the sheer number of variables built into any transfection protocol can make optimization an unwieldy and complicated process. In this study, we demonstrated in five experiments the development of a CHO transient transfection process that supports desirable cell growth, transfection efficiencies, and titer. In order to do this, we addressed the crucial parameters necessary to improve our initial process, which typically produced approximately 150mg/L. Beginning with a survey of growth media, we identified a medium that nearly doubled growth of the host CHO cell line. In this new growth medium, inhibitory components for transfection were identified and lowered in concentration or altogether removed. This resulted in a medium that supported a transfection efficiency of 90% or greater without sacrificing the previously discovered growth improvement. Consequently, it was necessary to re-evaluate the transfection protocol in order to exploit this improved growth profile. Transfection time and cell density were optimized along with DNA and PEI concentrations, which resulted in a significant increase in both the post-transfection cell mass and titer. Further augmentation of culture longevity and titer was achieved by screening various hydrolysate supplement blends. By collectively optimizing the growth medium, transfection protocol, and hydrolysate supplement, this study led to a process that consistently achieves protein titers of 500-700 mg/L.

USE OF AN ANTI-APOPTOTIC CHO CELL LINE FOR TRANSIENT GENE EXPRESSION FOR MULTI-SCALE APPLICATIONS: AUTOMATED TUBESPINS TO STIRRED TANK BIOREACTORS

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Transient gene expression in mammalian cells allows for rapid production of recombinant proteins for research and preclinical studies. Here, we describe the development of a polyethylenimine (PEI) transient transfection system using an anti-apoptotic CHO host cell line. The host cell line, referred to as the Double Knockout (DKO), was generated by deleting two pro-apoptotic factors, Bax and Bak, in a CHO-K1 cell line using zinc finger nuclease mediated gene disruption. As evidence of their anti-apoptotic properties, post-transfection, DKO cells maintained higher viability and had reduced levels of active caspase-3 compared to CHO-K1 cells. Nuclear plasmid DNA copy numbers and message levels were significantly elevated in DKO cells. Although DNA uptake levels, as early as 40 min post-transfection were higher in DKO cells, this was not due to differences in cell surface heparan sulfate (HS) or initial endocytosis mechanism as both cell types utilized caveolae- and clathrin-mediated endocytosis to internalize DNA:PEI complexes. These results suggest that the increased transfection efficiency from DKO cells is attributed to their resistance to transfection-induced apoptosis and not differences in endocytosis mechanism. Optimized DNA and PEI volumes for DKO transfections were 50% and 30% lower than CHO-K1, respectively. During transfection DKO cells produced relatively high levels of lactate, but this was mitigated by a temperature shift. DKO cells expressed ~3-4 fold higher antibody titers than CHO-K1 cells. This optimized high throughput process can be used for scales ranging from automated 30 mL tube spins to 25L wavebags and 35L stirred tank bioreactors.

A HIGHLY SUCCESSFUL COLLABORATION TOWARDS THE COMMERCIALIZATION OF A HUMANIZED MONOCLONAL ANTIBODY

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Key Words: PPQ, characterization, clinical campaign, IPC

A development partnership for a phase III humanized antibody was established between BMS and AbbVie. The production process using an NS0-derived cell line, fed-batch production and three-column purification was transferred to a new production site, and extensive process characterization was performed by the partner companies. This presentation will discuss the overall strategy for advancing the molecule to a commercial production process, and will include summaries of knowledge gained from characterization studies and lessons learned during three clinical supply campaigns and a Process Performance Qualification (PPQ) campaign.

The process characterization studies found a limit for viable cell density during the inoculum expansion stage. In the shake flasks, growth beyond that limit resulted in slow growth in the subsequent passage followed by rapid growth in the passage after that, oscillating expansively. In the wave bioreactor growth stalled at that limit and viability decreased rapidly. At the $n-2$ seed bioreactor, growth beyond that limit resulted in slower growth in the $n-1$ seed bioreactor. Differences in growth between two Manufacturer Working Cell Banks (MWCB's) were observed during clinical manufacturing and these differences were linked to the limit found during characterization studies. Cell growth was also impacted by a change in consumables introduced during the first clinical manufacturing campaign.

The knowledge gained from both characterization studies and clinical supply campaigns at large scale was used to develop an in-process control (IPC) strategy. PPQ runs performed mostly within the ranges established within the IPC, with the exception of brief pH excursions in the production bioreactor. This collaborative project culminated in a successful PPQ campaign.

**THE DEVIL IS IN THE DETAILS: STRATEGIES, CASE STUDIES, AND RECOMMENDATIONS
FOR SUCCESSFUL TECH TRANSFER AND PRODUCT COMMERCIALIZATION**

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The author will share his scale-up and tech transfer experience for clinical and commercial manufacturing in different organizations (Bayer, GSK, J&J). Even though the approaches taken and the strategies used changed from one CMC team to another, the recipe for success had the same ingredients; a defined and robust process designed for manufacturability, a dedicated team with clear roles and responsibilities, a detailed project plan with contingency, and a trained and dedicated workforce. Attention to details was the most critical element determining the success or the failure. All these points will be covered in detail and illustrated by the case studies.

**SIX MONTH TECH TRANSFERS:
PROCESS FIT AND SCALE UP FOR MANUFACTURING OF BIOLOGICALS**

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Accelerated tech transfers to manufacturing, with the associated parallel process fit and scale-up activities, are needed for rapid production of Phase III clinical and initial commercial supplies. This becomes even more critical when supporting fast-track timelines. Parallel process scale up and process fit require good collaboration between sending and receiving partners, so that efforts and expertise from both sides are leveraged effectively.

This case study involves a monoclonal antibody cell culture process, developed and demonstrated at intermediate scales at Merck Research Labs (MRL), and then transferred in under 6 months for commercial scale up and implementation at AstraZeneca Biologics (AZB) at the 15,000L scale. The presentation will review the commercial process scale-up, process fit, and production strategies that spanned across the Merck and AZB organizations. Lessons learned from each step of the process will be shared.

**CASE STUDIES FOR UTILIZATION OF CONVENTIONAL AND CFD APPROACHES FOR
SUCCESSFUL SCALE UP AND SCALE DOWN OF BIOREACTOR PROCESSES FOR MONOCLONAL
ANTIBODIES**

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Conventional approaches to scale-up of bioreactor process parameters to new facilities have historically been successful, but not all monoclonal antibody processes are created equal. For some programs, these methods can result in suboptimal process performance and require optimization to be performed at manufacturing scale. More recently, use of both conventional and computational fluid dynamic approaches to develop scale-down, pilot-scale models of production bioreactors have resulted in improved process understanding and more successful transfer for late stage processes. The new scale-down models are more predictive of manufacturing and are used to map out impact of scale-up parameters to process performance. Case studies of both approaches will be presented.

HIGH PERFORMANCE GLYCOANALYSIS TO IMPROVE BIOLOGICS DEVELOPMENT

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Key Words: glycoanalysis, high-throughput, development, biologics.

Glycomics is a rapidly emerging field that can be viewed as a complement to other „omics“ approaches including proteomics and genomics. Hence, there is a dramatic dynamic increase in the demand for sophisticated databases and analytical tools in glycobiology respectively, glycobotechnology. In order to enhance and improve the comparatively small existing glycoanalytical toolbox, fully automated highly sensitive, reliable, high-throughput and high-resolution analysis methods including automated data evaluation are required - i.e. high-performance methods. Especially one glycoanalysis approach, based on multiplexed capillary gelelectrophoresis with laser induced fluorescence detection (xCGE-LIF), shows high potential for high-performance analysis of glycoconjugates.

The development of such an innovative xCGE-LIF based glycoanalysis system (method, software and database) and its application to different fields with respect to sample preparation, separation and data analysis is presented. First, an optimized modular sample preparation method and workflow are presented with respect to performance and feasibility regarding high-throughput [1,2,3]. Second, with up to 96 capillaries in parallel, the fully automated separation with an impressive sensitivity is shown to result in massive reduction of the effective separation time per sample [3]. Third, automated data analysis with a newly developed modular software-tool for data-processing and -analysis, interfacing a corresponding oligosaccharide-database [4,5] is demonstrated. Using this high-performance glycoanalysis system, the generated “normalized” electropherograms of glycomoieties (“fingerprints”) can be evaluated on three stages: (1) “simple” qualitative and quantitative pattern comparison (“fingerprint“-analysis), (2) identification of compounds in complex mixtures via database matching (“glycoprofiling”) and (3) extended structural analysis using exoglycosidase sequencing in combination with xCGE-LIF based glycoprofiling. The smart applicability of the system is demonstrated for different types of glycosamples such as the “glycome” of single (recombinant) glycoproteins with respect to biopharmaceuticals and vaccines [1,2], of human milk [6], respectively of whole human blood serum [3]. This novel modular high-performance glycoanalysis system allows fully automated, highly sensitive, instrument-, lab- and operator-independent “real” high-throughput glycoanalysis. This is in contrast to the currently prevailing methods, where multiplexing with respect to high-throughput is highly cost and lab-space intensive and ties up a lot of manpower.

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TUNING THE GLYCAN COMPOSITION IN CHO CELLS BY GENETIC AND METABOLIC APPROACHES

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Glycosylation is one of the most complex and critical quality attributes of biotherapeutics produced by mammalian cell culture. Glycans modulate binding characteristics and often participate in the molecular interaction with other proteins, mask immunogenic epitopes or hydrophobic surfaces and contribute to structural stability. They have a dominant impact on half life and activity and can be responsible for mounting an immune response to the product.

Being not as rigidly defined as the primary sequence the glycan composition depends on the starter cells line, individual features of specific producer line, the media composition and the fermentation process. Individual structures can be enriched during purification. While enhancing desired structures may be advantageous for novel biologics reproduction of the original pattern is of uttermost importance in development of Biosimilars. With a different starting cell line, new chemically defined media and the aim for increased titers the latter is particularly challenging.

We have engineered CHO K1 and DG44 cell lines to constitutively express the Pseudomonas enzyme RMD that shuts down the fucose de novo synthesis pathway and efficiently depletes the cytosolic sugar nucleotide pool of GDP-L-Fucose. Afucosylated antibodies with enhanced antibody dependent cell-mediated cytotoxicity (ADCC) and related anti-tumor activity are generated. Because fucose can still be taken up by the cells from the culture medium and utilized via the salvage pathway a predefined fucosylation level can be set via fucose feeding. With this technology fully fucosylated or afucosylated antibodies can be generated from the same cell line.

Similarly, the degree of galactosylation and sialylation is tunable: While overexpression of the respective glycosyltransferases induces more drastic changes that can be predetermined by the expression level of the modulating enzyme, fine-tuning is achieved with media variations. In contrast, content of incompletely processed glycans with terminal mannose is strictly dependent on process duration and less susceptible to pronounced changes. Case studies for each of those modifications will be discussed.

THE SYNTHESIS AND ENRICHMENT EFFICIENCY OF CARBOXYMETHYL CHITOSAN FOR 'PULL DOWN' GLYCOPROTEOMICS

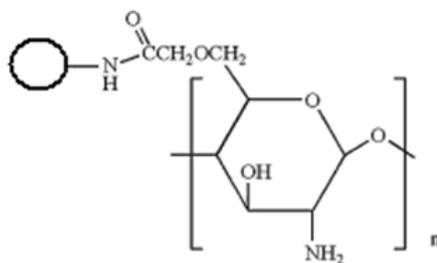
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Keywords: glycopeptide, enrichment, SPE, weak anion exchange, WAX, HILIC, hydrophilic, sialylation, sialic acid, mass spectrometry, glycoproteomics, tandem mass tag

At present, glycans have been demonstrated to have various roles throughout the body including implicated roles in diseases such as rheumatoid arthritis and various metastatic carcinomas. Moreover, glycans have been demonstrated to be key in immunological pathways, resulting in interest as targeted molecules of study for potential drug candidates such as Herceptin. In particular to many immunological mediated processes, is the nine carbon vertebrate sugar known as sialic acid (neuraminic acid) which bears a free carboxylic acid and is often found at the distal end of molecules perhaps highlighting its importance in these events. At present, glycoproteomics is dominated by mass spectrometry (MS) as the benchmark tool by which to study and investigate these molecules. However, due to the complexity inherent amongst glycoconjugates, including an array of branching, and vast microheterogeneity, as well as limitations present in current glycoproteomic workflows, the necessity for enrichment of glycoforms prior to MS analysis is crucial.

Here, the synthesis of carboxymethyl chitosan onto amine derivatized silica is described and utilized for the enrichment of glycopeptides. The stationary phase is designed to work through both hydrophilic interaction liquid chromatography (HILIC) as well as weak anion exchange (WAX) principles in an attempt to target sialylated glycoconjugates. To assess the efficiency of this novel approach, a commercially available glycopeptide kit is used in parallel, along with the use of tandem mass tags (TMT) for relative quantification between the two approaches.



Silica-CMCH

A FRAMEWORK FOR REAL-TIME GLYCOSYLATION MONITORING (RT-GM) IN MAMMALIAN CELL CULTURE

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Keywords: Glycosylation, real-time, monitoring, glycan

Glycosylation is a critical characteristic of biotherapeutics because of its central role in *in vivo* efficacy. Multiple factors including medium composition, and process conditions impact protein glycosylation and characterizing cellular response to these changes is essential to understand the underlying relationships. Current practice typically involves glycosylation characterization at the end of a fed-batch culture, which in addition to being an aggregate of the process, reflects a bias towards the end of the culture where a majority of the product is made. This approach masks the dynamics of glycosylation over the course of a fed-batch culture where transients are expected. In an attempt to rigorously characterize the entire time course of a fed-batch culture, a real-time glycosylation monitoring (RT-GM) framework was developed. It involves using the micro sequential injection (μ SI) system as a sample preparation platform coupled with an ultra performance liquid chromatography (UPLC) system for real-time monitoring of the antibody glycan profile. The μ SI system is capable of performing automatic sampling and sample preparations in micro fluidic channels with assay-specific configurations and assay-specific software. Prepared samples are delivered to the UPLC for analytics and the analytical data are available for real-time decision making. This RT-GM framework was applied to multiple fed-batch bioreactor runs under varying process conditions that influenced glycosylation and changes in protein glycosylation were monitored in real time. During process development, the rapid availability of glycosylation data can guide process and medium changes which can result in the desired product quality attributes. In a more mature process, the RT-GM framework described in this study can serve as a monitoring and diagnostic tool. Overall, the ability to obtain time-course data on glycosylation improved process understanding and can help optimize process development activities.

MODULATING THE EFFECTOR FUNCTION OF RMABS BY HOST CELL GLYCOENGINEERING

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Glycosylation has attracted major interest from biopharmaceutical industries as a means to control the safety and modulate the efficacy of biopharmaceuticals. The N-glycan attached to the conserved glycosylation site at Asn 297 in the Fc region of recombinant monoclonal IgG's (rMAbs) for example, plays a multifunctional role in the physiochemical and biological properties of the IgG molecule (1). These N-glycans sequestered within the interstitial space enclosed by the CH2 domains maintain the thermodynamic stability and quaternary structure of the two heavy chains for the correct binding of the IgG molecule to the Fcγ receptors as part of the antibody cell-mediated cytotoxicity (ADCC) and C1q of complement. The absence or addition of specific sugar moieties to the N-glycan has been shown to modulate the immunoeffector function of the molecule. The absence of core fucose on the IgG N-glycan can greatly increase antibody dependent cellular cytotoxicity through an enhanced IgG-FcγIIIa receptor interaction while the addition of terminal galactose and α2,6 linked sialic acid enhances the anti-inflammatory activity of the molecule (2,3).

The selection of the mammalian Chinese Hamster Ovarian (CHO) cell line as the primary producer of rMAbs is largely influenced by the host cells ability to generate therapeutically acceptable glycoprofiles. Building upon the recent availability of sequence data from the CHO genome, we implemented a number of CHO cell based glycoengineering strategies to alter the composition of the IgG1 N-glycan structure and improve various critical characteristics of our selected rMAb. To reduce the α1,6-linked content of IgG N-glycan we transfected the host cell line with a bacterial enzyme which strongly reduced a key metabolic intermediate in the synthesis of GDP-Fucose. UPLC analysis of the IgG1 N-glycans from a selected clone revealed a 98% to 20% shift in the relative percentage of α1,6 linked fucose content which resulted in an 11-fold increase in ADCC activity from an in vitro plate based assay. In an effort to increase terminal galactose content we overexpressed a CHO β1,4 galactosyltransferase I enzyme in the host cell line followed by a combined high-throughput screening strategy which resulted in terminal galactosylation of over 80% of the IgG1 N-glycans from selected clones. Finally, the relative percentage of IgG1 N-glycan with terminal sialic acid content also increased from >2% to 65% with coexpression of β1,4 galactosyltransferase and α2,6 sialyltransferase enzymes. Our studies highlight the various glycoengineering strategies which may be applied to improve various critical characteristics of rMAbs.

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BREAKING THROUGH THE PARADIGM SHIFT OF CELL THERAPIES- THE DEVELOPMENT OF A FULLY DEFINED ENZYME AND INHIBITOR FREE METHOD OF SUB-CULTURING HUMAN STEM CELLS

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Key Words: stem cells; sub culturing; scale-up; thermo responsive polymers.

The success of mammalian cell derived biologics owes much to the development over the last thirty years of robust processes capable of reproducibly producing large complex proteins under fully defined conditions. The concept of developing a tested master cell bank from a single cell, of always using cells from this bank to start the process, and then running a carefully controlled suspension culture where it has been shown that the producing cell line is stable for 50 or 60 generations forms the basis for the GMP production of biologics. Mixed suspension culture allows the accurate control of the environmental conditions seen by the cells during the process, and provides the opportunity for tight environmental control for consistent product quality.

Human stem cells represent a considerably greater bioprocessing challenge. For such cells, the genomic integrity is essential but can readily change in a stem cell depending on the environmental conditions, unlike for protein producing cell lines where only the DNA for the product needs to be genetically stable, and minor changes in the genome in what are comparatively stable cell lines will usually not impact on product expression. Any disruption from the optimal conditions will see the stem cells starting to differentiate to other lineages. The optimal conditions for pluripotent growth of the cells is dependent on the composition of the medium, environmental conditions, the substrate on which the cells are attached, and contact between cells. When the conditions are changed to promote differentiation to a specific lineage, all of the above variables may need to be changed/optimized, to reach the challenging goal of having most of the population differentiate to the desired cell type. Considerable challenges exist to get such bioprocesses operating in a consistent, reproducible fashion required of a GMP process.

In this paper we describe a novel thermo responsive polymer system which solves one of the key bottlenecks for the scale-up of GMP stem cell bioprocesses by allowing stem cells (hESC) to be sub-cultured under non-invasive conditions. Currently sub culturing stem cells requires either manual dissection, when the cells are grown on two dimensional surfaces (2D), or if the cells are grown as aggregates in 3D suspension cultures, the addition of enzymes to release the cells as single cells or small clumps, often with the need to add small molecule inhibitors such as a ROCK inhibitor to reduce cell apoptosis and improve self-renewal after each passage. These added enzymes and inhibitors concomitantly modify the cell surface and signaling pathways, thereby impacting on future cell behavior and fate.

The system we have developed utilises hESC natural ability to come together as aggregates, satisfying their inherent requirement for cell-cell and cell- extracellular matrix contact. The system uses thermo responsive polymer worms decorated with recombinant vitronectin fragments (an extracellular matrix protein) that binds to and bridges between adjacent cells. The polymer worms aid in aggregate formation, and also facilitates their breakdown to smaller aggregates when the aggregates grow to the optimal size where nutrient diffusion becomes limiting. By a simple temperature shift from 37° C to 25° C, the thermo responsive polymer chains repel each other, allowing the shear in the reactor to break the aggregates to smaller sizes. Dilution of the culture with fresh medium and polymer components, and raising the temperature to 37° C allows the polymer chains to condense and hold together the smaller diameter aggregates as growth continues. Cycling between the two temperatures allows cell expansion until the desired number of cells have been obtained, providing a simple, non invasive and scalable method for sub culturing.

We have shown that it is possible to grow cells for 18 days (after 3 passages) in such a system and maintain a pluripotent hESC phenotype. The stem cell aggregates produced at the end of the process have been shown to be in the ideal form for subsequent differentiation to the required lineage.

INTEGRATING BIOPROCESS OPTIMIZATION AND OMICS TOOLS TOWARDS THE DESIGN OF NOVEL CARDIAC STEM CELL THERAPIES

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Key Words: Human Stem Cells, Up- and Down- stream Processing , Metabolomics, Proteomics, Cell Therapy

Stem cell (SC) transplantation has emerged as an exciting treatment option for patients with heart failure and acute myocardial infarction. Today, strong business opportunities exist for companies looking to pursue SC-based therapies; within the next years upcoming phase 3 studies will provide definitive answers of whether such therapies truly show potency in the clinic [1]. The challenges of developing cell therapies are manifold including the lack of expertise in product development and characterization as well as specialized cell manufacturing which are imperative to bring SC-based products closer to the clinic [2]. Within this context, our work has been focused on production and characterization of challenging SC-based products for application in both autologous and allogeneic cell therapies: i) cardiomyocytes (CM) derived from induced pluripotent SC (iPSC), which are capable to regenerate myocardium in infarcted hearts [3], and adult SCs, namely human mesenchymal and cardiac SC (hMSC and hCSC, respectively), which trigger paracrine mechanisms that activate endogenous SC to promote regeneration [4]. For this purpose, a systematic approach was developed using robust methodologies for both up- and down-stream bioprocessing and high-throughput proteomic and metabolomic tools for product characterization and process optimization.

Our strategy for iPSC-derived CM production consisted in designing an integrated bioprocess by combining expansion, differentiation and cell lineage purification steps in environmentally controlled bioreactors (stirred tank and Wave bioreactors) operating in perfusion. By optimizing different bioprocessing parameters we were able to improve by 1000-fold the cardiomyocyte differentiation yields (up to 60 CMs/input iPSC) and obtain a highly pure population of CM. Cell expansion, differentiation and purification processes were monitored along culture time using fluorescence microscopy, flow cytometry and qRT-PCR analysis. Metabolome and fluxome analysis is being applied along the differentiation process to disclose which metabolic pathways are differentially activated and/or repressed in low versus high yielding bioprocess conditions. Cell characterization and functional analysis confirmed that iPSC-derived CM presented a typical cardiac morphology, high deposition of extracellular matrix such as collagen type I, reproducible electrophysiological profiles and drug responsiveness.

Regarding hMSC and hCSC biomanufacturing, we were able to implement an efficient protocol for cell cultivation using microcarrier-based stirred culture systems. For downstream bioprocessing, our strategy was focused in combining membrane technology and novel chromatographic tools in a robust and scalable manner while compliant with Good Manufacturing Practices. Cell characterization assays showed that hMSC and hCSC retained their identity, differentiation and proliferation capacity as well as potency throughout the entire bioprocess. State-of-the-art mass spectrometry tools (nanoLC-MS) have been applied to obtain a comprehensive characterization of hMSC and hCSC secretome and receptome. hCSC Receptome analysis rendered the identification of numerous plasma membrane proteins and several cell surface markers (e.g. myoferlin), including more than 100 plasma membrane receptors (e.g. epidermal growth factor receptor, frizzled family receptor 6, etc), overall more than 2000 proteins were identified, including different proteins allied to cardiac function. Secretome data are still under evaluation. Up to now about 500 proteins were identified, and although we are working with highly complex samples, with a broad dynamic range of protein concentrations, we were able to confidently identify the low abundance cytokine IL-8.

The cell manufacturing platforms developed herein, along with the robust proteomic and metabolomic tools implemented for product characterization, provide important insights to streamline the design of novel cell-based therapies for cardiac repair.

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PRODUCING AND HARVESTING CULTURE-DERIVED PLATELETS WITH FUNCTIONAL ACTIVITY FROM BLOOD STEM CELLS

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Production of platelets in culture by megakaryocytic cells (Mks) derived from hematopoietic stem and progenitor cells (HSPCs) would supplement volunteer donations for transfusions, while also overcoming problems associated with storage and patient alloimmunization. We showed that a 3-phase process with increasing pH and pO_2 yields 36 Mks, including 4 high-ploidy ($> 4N$) Mks, per HSPC from mobilized peripheral blood (mPB). Nicotinamide decreased Mk production by 1/3, but doubled the number of high-ploidy Mks. Culture-derived platelets displayed a wider size distribution and a larger mean size than donor platelets, but exhibited functional activity similar to that of fresh donor platelets, as evidenced by basal tubulin distribution and the expression of CD62P and CD63 surface markers and spreading in response to platelet agonists [1]. However, further advances are needed for this technology to become clinically relevant. One challenge is to efficiently purify platelets from the precursor Mks, as well as other cell types that arise during heterogeneous differentiation of HSPCs in culture. Recent studies on platelet production from 2D culture have used successive centrifugation to pellet large cells, then platelets, but this multi-step method is subject to error-prone manual manipulation and is unlikely to generate a pure platelet population. As an alternative, we are developing a rapid, one-step approach to separate platelets from larger cells using spinning-membrane filtration. Furthermore, because Mk maturation and subsequent platelet release have been shown to occur asynchronously in culture, we have explored how this separation technique could be used to separate platelets and cells at several time points during culture. In this way, one could harvest and prevent pre-activation of platelets generated early in culture, while reseeding immature Mks to generate proplatelets and platelets later in culture. To first characterize the method, 3 replicate experiments were performed wherein phorbol 12-myristate 13-acetate (PMA)-stimulated (up to 32N) CHRF megakaryoblastic cells were suspended in PAS-V platelet additive solution and mixed with apheresis platelets collected using the Amicus Separator. The mixture of CHRF cells and platelets was processed on a cell-washing device consisting of a polycarbonate spinning membrane with 4- μ m cylindrical pores. Cells that passed through the membrane ("platelet fraction") or were retained by the membrane ("cell fraction") were collected in separate bags. The separation process efficiently recovered platelets in the platelet fraction, while CHRF cells were excluded. Platelets recovered in the platelet fraction showed minimal pre-activation, and CHRF cells recovered in the cell fraction had a similar distribution of viable and apoptotic cells compared to the input population. The ploidy distribution of recovered CHRF cells was also very similar to the input population, suggesting that both smaller and larger cells were eluted from the membrane. Finally, when the recovered CHRF cells were transferred to an adhesive culture surface, they formed proplatelet-like structures similarly to unprocessed cells. To apply the method to primary cells, mPB CD34⁺ HSPCs were differentiated to Mks, which formed proplatelets and released platelets in culture. The mixture of in-vitro-derived Mks and platelets was then processed on the cell-washing device. Similar to experiments with the CHRF Mk line, primary Mks were excluded from the platelet fraction and the viability and ploidy distribution of the recovered Mks were similar to those of the input Mks. Further, recovered in-vitro-derived platelets spread in the presence of thrombin similarly to unprocessed in-vitro-derived platelets. However, recovery of CD41⁺CD42b⁺ in-vitro-derived platelets in the platelet fraction was lower than that observed with apheresis platelets (70% vs. $91 \pm 4\%$ at 3000 rpm). This is likely due to the larger size of culture-derived platelets and preplatelets ($5.6 \pm 2.3 \mu\text{m}$ vs. $3.3 \pm 0.8 \mu\text{m}$) in relation to the 4- μ m pores in the membrane. Based on later experiments separating larger Day 8 red blood cells ($7.3 \pm 1.2 \mu\text{m}$) from CHRF cells (80% RBC recovery in the platelet fraction at 2000 rpm and 90% at 1500 rpm), we are confident that we will be able to greatly increase culture-derived platelet recovery without substantial Mk contamination. One other concern from the primary cell experiment is that the recovered Mks formed fewer proplatelets when reseeded into culture and exhibited increased spreading on the culture surface compared to proplatelet-forming Mks prior to processing. Proplatelet formation is negatively regulated by Mk contraction caused by engagement of activated integrins. Since decreasing the membrane rotation rate will also decrease the maximum and average shear to which Mks are exposed during processing, we believe that decreasing the membrane rotation rate will also decrease integrin activation and improve proplatelet formation after reseeding.

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THE CANADIAN APPROACH TO THE REGULATION OF SUBSEQUENT ENTRY BIOLOGICS (BIOSIMILARS)

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Health Canada has maintained an approach that regulatory decisions regarding the quality, safety and efficacy of medicinal products should be based on scientific evidence and accepts that copies of biologics originally licensed by innovator companies will have a role in health care. Such products should not be considered as generics; however, information in the public domain regarding safety and efficacy of an innovator product over many years of use can be considered relevant if suitable data is provided demonstrating comparability/similarity to that specific reference product. Health Canada released a detailed regulatory guideline on Subsequent-Entry Biologics in March, 2010. The presentation will cover the origins, important elements, and challenges of demonstrating comparability to a reference product and will discuss important issues such as the appropriate choice of reference product (comparator), post-market requirements and interchangeability.

**DEVELOPMENT OF A SAFE AND EFFECTIVE BIOSIMILAR MONOCLONAL ANTIBODY
FOLLOWING INTERNATIONAL GUIDELINES**

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Several companies have decided to develop Biosimilar Monoclonal Antibodies and will soon deliver the promise of offering alternative options to physicians and patients worldwide. Each development group is confronted with studying current regulatory environments, available production systems, state of the art analytical methods and scientific information on the reference product and with this information sketch a development plan. We will present the rationale for the development of a biosimilar rituximab showing how similarity is the driver from clone selection to purification and scale up and how the developed product, as a consequence, shows similarity when compared to the reference in terms of purity, identity, folding, glycosylation, affinity, biological activity, in vivo testing in monkeys and clinical testing.

OVERCOMING BIOSIMILARITY CHALLENGES THROUGH OPTIMIZATION OF PARAMETERS FOR A CHO CELL CULTURE PROCESS

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Biosimilar development holds the promise of increasing access of low cost medicines to patients in emerging markets and the world at large. From a process standpoint, the complex structure of biologic medicines calls for rigorous parameter optimization to ensure that the analytical, clinical and physio-chemical/ non-clinical data generated for the biosimilar is similar to the innovator drug with respect to the structural properties, safety and efficacy. In this presentation, taking an in-house CHO cell based process as a case-study, a target-directed strategy for development of a biosimilar product will be discussed. Key aspects of process scale up and impact of upstream process parameters on final product quantity and quality will be outlined. An understanding of the role of these process parameters led to implementation of process changes for commercial runs and had a net positive impact on the COGs for the biosimilar product.

QUALITY CONSIDERATION IN BIOSIMILAR DEVELOPMENT

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Key Words: mAb biosimilars, CHO cells, charge heterogeneity

It is well-known that safety and efficacy are key factors in term of mAb pharmaceuticals development. This means that all kinds of quality attributes are assessed by how much impact on safety and efficacy. Since biosimilar are emerging as important issues in pharmaceutical industry, however, similarity has been added as a key factor. This is because that good similarity in various physicochemical analyses could reduce scientific/experimental effort to prove its qualification and guarantee easier regulation tract in biosimilar development. In general, the quality of mAb biosimilar is assessed by over 50 kinds of analytic methodologies to identify how much different between reference drug and in-house biosimilar in term of safety, efficacy, and similarity. Also, diversified approaches are employed to control those qualities during production process, which are type of host cell, clonal selection, components of culture medium, environmental condition of cell culture process, and purification process.

In our experiences developing mAb biosimilar using CHO cells, unknown charge heterogeneity had been observed by cation exchange HPLC. This was properly a major crisis to promote further work in term of securing similarity even though in vitro biological activity and other physicochemical quality had no marked difference. Our results of in depth analyses were that partial processing of signal sequence during expression caused the charge heterogeneity of monoclonal antibody. Also, we observed that altering type of signal sequence eliminated the problematic variants during production. The design of expression cassette have been usually investigated in term of productive efficiency, which is single sequence engineering, enhance/promoter engineering, intron design, codon optimization, poly A tail engineering, and etc. However, we have identified that the expression cassette design, particularly usage of signal sequence, could affect to the quality of monoclonal antibody that is human antibody expressed from CHO cell.

