



EUROPEAN ASSOCIATION OF
PHARMA BIOTECHNOLOGY

Conference book

Science to Market

Posttranslational Modifications of Proteins - Consequences and Therapeutic Use

**A Discussion and Partnering Conference
for Experts from Academia, Biotechnology
and Pharmaceutical Industry**

Vienna, Austria, February 24 - 25, 2010



**Organised by
the European Association of Pharma Biotechnology (EAPB)**

in cooperation with



Vienna Institute of BioTechnology



EUROPEAN ASSOCIATION OF
PHARMA BIOTECHNOLOGY

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Aim & Scope

As a European event, the »Science to Market« EAPB Conference aims to promote partnership between academic settings and industry. The goal is to enhance the economical output of scientific research and direct them into marketable technologies and biopharmaceutical products. Recent biotechnological developments that may be of economic interest shall be identified and transfer of the academic know-how into product development shall be facilitated.

Therefore, European academic research scientists from universities and research institutions as well as representatives of the biotech and pharmaceutical industry are invited to exchange ideas that will lead to successful co-operations.

To achieve the conference goals, the congress offers plenary lectures on state-of-the art technologies and findings in biopharmaceutical developments as well as poster presentations on current projects and networking opportunities.



Wieland W. Wolf
President of EAPB

Welcome to the Participants of the 3rd “Science to Market” Conference of the European Association of Pharmaceutical Biotechnology (EAPB)

On behalf of the EAPB Board I cordially welcome the participants at this 3rd “Science to Market” conference.

The first two „Science to Market“ conferences took place in Hannover, Germany, in 2008 and 2009, and were spanning the whole range of topics in the area of pharma biotechnology.

In order to live up to the Pan-European mission of EAPB, we decided that the locations of the future „Science to Market“ conferences will alternate, considering the home countries of our European members. Moreover, the program of the plenary lectures will focus at each conference on another major subject of biopharmaceutical importance.

We are happy that through the support of the Vienna Institute of Biotechnology this year’s meeting can be held in Vienna, Austria, at the institute’s premises and we would like to express our gratitude to the Sponsors which enabled us to organize this 3rd conference in our “Science to Market” series.

With “Post Translational Modifications” (PTM) we have selected a subject of increasing interest to the biopharmaceutical community, since PTMs have a broad, often underestimated impact on the biologic activity of proteins and in consequence on their therapeutic potential. PTMs may be crucial for wanted and unwanted effects of biopharmaceutical drugs. Therefore, the knowledge about and the control of PTMs is highly relevant.

We are confident that the mixture of a high quality scientific program, poster presentations, and open informal discussions will give plenty of opportunities for exchange and networking and thus we wish you an interesting and inspiring meeting.

A handwritten signature in blue ink, appearing to read 'W. Wolf', written in a cursive style.

Dr. Wieland W. Wolf

Organiser



EUROPEAN ASSOCIATION OF
PHARMA BIOTECHNOLOGY

The **European Association of Pharma Biotechnology** - EAPB - is a non-profit association and the representative and central network to promote and develop Pharma Biotechnology in Europe, linking academia, industries and regulatory bodies. It is dedicated to the advancement of biotechnology in pharmaceutical sciences, specifically as applied to industrial materials, processes, products and their associated problems. Its members are scientists employed in industry, government and university laboratories, biotech companies and scientific organisations.

Scientific and Planning Committee

Dr. Karoline Bechtold-Peters (Boehringer-Ingelheim Pharma GmbH & Co. KG, Biberach/Riss, GER)

Dr. Fabio Carli (Università di Trieste, ITA)

Dr. Jörg Knäblein (Bayer Schering AG, Berlin, GER)

Dr. Marion Kronabel (EAPB Managing Director, Heidelberg, GER)

Dr. Inge Mühdorfer (Rentschler Biotechnologie GmbH, Laupheim, GER)

Dr. Joachim Noeller (University of Leipzig, GER)

Dr. Gary Walsh (University of Limerick, IRE)

Dr. Axel Wenzel, Pharma Scientific Services Team, Ltd., German

Dr. Wieland W. Wolf (Laupheim, GER)

Cooperation partner



Vienna Institute of Biotechnology combines competences in microbiology, nanobiotechnology, food science, applied genetics, chemistry and watermanagement. It defines itself as a think tank for research, education and development of new technologies. Researchers at VIBT are collaborating trans- and interdisciplinary for solutions in medicine, nutrition, environmental protection and sustainable usage of natural resources. The network of disciplines in VIBT is outstanding, using technologies like genomics, proteomics metabolomics and bioinformatics.

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Services Deutschland
GmbH



Programme Overview

Day 1: Wednesday, February 24th, 2010

09:30 - 10:30 **Registration**

10:30 - 10:45 **Welcome, Introduction & Opening remarks**

Wieland W. Wolf, President of the EAPB, Laupheim (GER)

10:45 - 11:30 **Keynote lecture: Post-translational Modification of Protein Biopharmaceuticals**

Gary Walsh, University of Limerick, Limerick (IRE)

11:30 - 12:00 **Compromised posttranslational modifications of proteins as source of diseases**

Harald S. Conradt, GlycoThera GmbH, Hannover (GER), invited speaker

12:30 - 13:00 **New challenges in safety assessment of biopharmaceuticals**

Roy Forster, CIT, Evreux (FRA)

Lunch Break

14.00 - 14.30 **Complex structures in simple organisms: the N-glycosylation of invertebrates and protozoa**

Iain Wilson, University of Natural Resources and Applied Life Sciences, Vienna (AUT)

14.30 - 15.00 **Concepts of Protein Quality Control**

Markus Kaiser, Chemical Genomics Center, Max Planck Institut, Dortmund (GER)

Coffee Break

15:30 - 17:00 **Oral poster presentations - Session 1: Novel therapeutics, vaccines and diagnostics**

1a) Novel therapeutics

1b) Novel vaccines

1c) Novel diagnostics

17:00 - 18:00 **Networking at poster boards**

18:00 - 19:00 **Special key note lecture: Biopharmaceutical manufacturing trends for maximizing antibody yields**

Andreas Werner, Boehringer Ingelheim, Ingelheim (GER)

19:00

Social Event

Day 2: Thursday, February 25th, 2010

- 08:30 - 09:00 **Production of biopharmaceuticals in human amniocytes**
Gudrun Schiedner CEVEC Pharmaceuticals GmbH, Cologne (GER)
- 09:00 - 09:30 **Developing a new antibody against a combined peptide-carbohydrate target and improving it by GEX-technology**
Franzpeter Bracht, Glycotope GmbH, Berlin/Heidelberg (GER)
- 09:30 - 10:00 **Secretion of mAb in 100 l bioreactors void of plant specific fucosylation and xylosylation**
Ronald Bassuner, greenovation Biotech GmbH (GER)
- Coffee Break**
- 10:30 - 11:00 **Development of Pegylated biopharmaceuticals**
Giancarlo Tonon, Bio-Ker, Pula (Cagliari), Sardinia (ITA)
- 11:00 - 11:30 **HESylation® technology**
Helmut Knoller, Fresenius Kabi, Bad Homburg (GER)
- 11:30 - 12:45 **Oral poster presentations –Session 2: Novel technologies**
2a) Novel up- and downstream developments
2b) Novel biopharmaceuticals drug formats and pharmaceutical developments
2c) Novel analytical tools
- 12:45 - 13:00 **Closing remarks**
- 13:00 - 14:00 **Networking at poster boards**
- 14:30 - 17:15 **Visit of Boehringer Ingelheim facilities, Vienna**
(restricted to 30 participants – pre-registration required)
- Fingerfood at Boehringer Ingelheim Center (BIZ)
 - BI Austria Biopharmaceuticals and activities at the site
 - Guided tour through facilities of BI Austria Biopharmaceuticals

Schedule of oral poster presentations

Poster no.	Title, authors/ presenters*, institutions/ companies	Schedule
Workshop 1: Novel Therapeutics, Vaccines, Diagnostics		
Session 1a: Novel Therapeutics		
1a-1	Histidine residues as a tool for protein nanoparticle formation Gorazd Hribar, Tatjana Milunovic, Simon Caserman, Spela Jalen, Ana Lenassi Zupan, Mateja Novak Stagoj, Vladka Gaberc-Porekar National Institute of Chemistry, Ljubljana, Slovenia	Wednesday, Feb 24, 2010
1a-2	In Nude Rats, Tumor Growth and Osteolytic Lesions induced by MDA-MB-231 human Breast Cancer Cells were effectively treated by a monoclonal Rat Antibody against Bone Sialoprotein II Michael Zepp ¹ , Franz Paul Armbruster ² , ¹ dkfz Heidelberg, Germany ² Immundiagnostik, Bensheim, Germany	Wednesday, Feb 24, 2010
Session 1b: Novel Vaccines		
1b-1		
Session 1c: Novel Diagnostics		
1c-1		

Workshop 2: Novel Technologies

Session 2a: Novel up- and downstream developments

- | | | |
|------|---|---------------------------|
| 2a-1 | Could be used downstream processes for increasing the antibiotics activity? Studies on Gentamicins separation
Dan Cascaval, Nicoleta Nicuta,
Technical University of Iasi, Romania | Thursday,
Feb 25, 2010 |
| 2a-2 | Evaluation of residual moisture in lyocakes and corresponding lyophilization stoppers of different rubber formulations
Heike Kofler,
West Pharmaceutical Services, Eschweiler, Germany | Thursday,
Feb 25, 2010 |
| 2a-3 | FACS-Assisted Directed Evolution to obtain a CHOK1 host cell line growing in glutamine free medium
Juan Antonio Hernández Bort ¹ , Beate Stern ²
¹ Institute of Applied Microbiology, Vienna, Austria
² Unitargeting Research, Bergen, Norway | Thursday,
Feb 25, 2010 |
| 2a-4 | MicroRNAs – small RNAs with big future in Chinese Hamster Ovary cell culture technology
Matthias Hackl, Nicole Borth
Institute of Applied Microbiology, Vienna, Austria | Thursday,
Feb 25, 2010 |
| 2a-5 | Efficient Removal of Aggregates from Impure Pharmaceutical Active Antibodies
Sibylle Ebert,
Rentschler Biotechnologie, Laupheim, Germany | Thursday,
Feb 25, 2010 |
| 2a-6 | Terminal radiation sterilization (25kGy) and long time storage of biopharmaceuticals enabled by stabilizing nanocoating technology
Jens Altrichter, Margraf, Breuer, Scholz
Leukocare, Munich, Germany | Thursday,
Feb 25, 2010 |

Session 2b: Novel biopharmaceutical drug formats and pharmaceutical developments

- | | | |
|------|--|---------------------------|
| 2b-1 | Novel Excipients Stabilizing Salmon Calcitonin against Aggregation
Gerrit Borchard ¹ , Claudia Müller ¹ , Martinus Carpelle ²
¹ University of Geneva, Switzerland
² Therapeomics, Geneva, Switzerland | Thursday,
Feb 25, 2010 |
| 2b-2 | Conversion of a Therapeutic Cytokine into Protein-coated Microcrystals Enabling Powder-based Dosage Forms
Kristine Berkenhoff ¹ , Verena Baum ² , Karoline Bechtold-Peters ²
LMU, Munich, Germany
Boehringer Ingelheim, Biberach, Germany | Thursday,
Feb 25, 2010 |
| 2b-3 | A new approach for preparing pegylated pharmaceutically interesting Proteins
Tatjana Milunović, Tina Maver, Simon Caserman, Vladka Gaberc Poreka
<i>National Institute of Chemistry, Ljubljana, Slovenia</i> | Thursday,
Feb 25, 2010 |

2b-4	<p>Correlation of the osmotic second virial coefficient with protein solubility and colloidal stability</p> <p>Patrick Garidel, Virginie Lebrun, Stefan Bassarab Boehringer Ingelheim, Biberach, Germany</p>	Thursday, Feb 25, 2010
2b-5	<p>Development of a pilot-scale process for protein-coated microcrystals (PCMC)</p> <p>- mixing and precipitation - part I - solvent reduction - part II - final drying - part III</p> <p>Karoline Bechtold-Peters, Corinna König, Steffan Bassarab, Verena Baum Boehringer Ingelheim, Biberach, Germany</p>	Thursday, Feb 25, 2010

Session 2c: Novel analytical tools

2c-1	<p>CHO Host Cell Lines Optimised by Cell Sorter Assisted Directed Evolution</p> <p>Nicole Borth University of Natural Resources and Applied Life Sciences, Vienna Austria</p>	Thursday, Feb 25, 2010
2c-2	<p>Analysis of product comparability: using a novel risk based approach</p> <p>Martin Blüggel, Katja Aschermann, Andreas Wattenberg Protagen, Dortmund, Germany</p>	Thursday, Feb 25, 2010
2c-3	<p>Improved Primary Structure Characterization of Therapeutic Proteins by Mass Spectrometry combined with massive permutations of potential modifications</p> <p>Daniel Chamrad, Gerhard Koerting, Katja Aschermann, Andreas Wattenberg, Martin Blüggel Protagen, Dortmund, Germany</p>	Thursday, Feb 25, 2010
2c-4	<p>MS based Primary Structure Mapping vs. Complete MS Data Interpretation for Characterization of Therapeutic Proteins</p> <p>Martin Blüggel, Gerhard Koerting, Andreas Wattenberg, Katja Aschermann, Daniel Chamrad Protagen, Dortmund, Germany</p>	Thursday, Feb 25, 2010

CVs and Abstracts of plenary speakers

Post translational modification of protein biopharmaceuticals

Gary Walsh, University of Limerick, IRE



Gary Walsh was awarded his Ph.D. degree from the National University of Ireland at Galway in 1989. Prior to joining the University of Limerick in 1994 he worked within the pharmaceutical and biotech industries and was a visiting Fulbright professor at the University of Georgia, in the United States in 1998. His research interests span various aspects of pharmaceutical and enzyme biotechnology, and he has or continues to supervise 20 post graduate and post doctoral researchers. His 191 publications include 7 authored books, 2 edited books, 11 book chapters and 55 journal articles. Three of his books are international students textbooks in the area of pharmaceutical/protein biotechnology (Pharmaceutical Biotechnology, concepts and applications; Biopharmaceuticals: biochemistry and biotechnology and Proteins, biochemistry and biotechnology, all published by John Wiley & Sons, UK).

He has presented invited/keynote papers at 32 international conferences. He has also served as editor, biotechnology section, of the European Journal of Pharmaceutics and Biopharmaceutics and as a member of the editorial boards of Biopharm. International, New Drugs and the Encyclopaedia of Industrial Biotechnology.

He is a former scientific secretary and member of the board of governors of the European Association of Pharmaceutical Biotechnology. Gary Walsh teaches various aspects of pharmaceutical biotechnology at both undergraduate and taught post graduate level and is course director of the B.Sc., Industrial Biochemistry Program.

He has/continues to serve as an external examiner for various undergraduate biochemistry/biotechnology programs at Dublin City University, as well as at 4 Institutes of Technology. He served as acting Dean, Teaching & Learning, at the university of Limerick in 2004. He is also a former recipient of both his University's excellence in teaching award and special achievement in research award.

The majority of protein-based biopharmaceuticals bear some form of post-translational modification, and such modifications can profoundly affect the biological activity and therapeutic properties of the target protein. While glycosylation represents the most common modification additional PTMs including carboxylation, hydroxylation, sulfation and amidation are characteristic of some products. This presentation considers the range and potential effects of such modifications. Emphasis will be placed upon PTMs associated with more recently approved biopharmaceuticals, as well as those in development. The relationship between PTM structure and function, the engineering of PTMs in order to optimize therapeutic potential, as well as PTM-related considerations in the context of protein expression systems and biosimilars will all also be overviewed.

Compromised posttranslational modifications of proteins as source of diseases

Harald S. Conradt, GlycoThera GmbH, Hannover (GER)

- Ph.D. (Dr. rer. nat.) Department of Biological Chemistry, University of Heidelberg, 1977
- Researcher ,1979-1982 University of Heidelberg, Medical Faculty, Department of Biochemistry
- 1982-1987 Senior Scientist at the GBF- German Governmental Center of Biotechnology in Braunschweig. Research on human T-cell activation and expression of lymphokines
Staff Member at the Department of Genetics at the **GBF**.
Head of a "Protein Design" research project: " Heterologous Expression of Proteins in mammalian cell lines and characterisation"
Since 1989:
Head Department of Protein Glycosylation (PGL), German National Center for Biotechnology Project Coordinator of several Research Projects funded by the European Union
Project Coordinator "Posttranslational Protein modification"; Project Coordinator
"Metabolic Engineering of mammalian cells"; Molecular Biology and Animal Cell Culture Technology; Initiator and Project Coordinator "Molecular Analysis and engineering of Cells for human therapy and diagnostics"
Secretory pathway of cells, dendritic cells, viral vectors, proteomics, medical diagnostics by micro methods(MS and MS/MS-MS techniques)"

1999 - : Founder of:
GlycoThera GmbH
BioTechnology of Human GlycoProtein Therapeutics :
Development of Products & Contract Analytics
Current position: CEO & CFO

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Almost all secreted and cell surface proteins from higher eukaryotic cells are glycosylated. The functions of the oligosaccharide chains of proteins are highly variable as are their complex and in most cases highly heterogeneous structures. Glycan functions include cell-cell communication, intracellular signalling, protein folding, targeting of proteins within cells and control of hormone levels.

Several inherited disorders affecting the biosynthetic pathways of N-glycans have been discovered during the past years, e.g. Leukocyte Adhesion Deficiency type II (LAD II) and a number of are a large family of genetic diseases resulting from defects in the synthesis of oligosaccharides known as *Congenital disorders of glycosylation (CDG)*.

Many malignant are accompanied by expression of aberrantly glycosylated secretory and membrane glycoproteins and specific structural motifs may be used for therapeutic approaches and in diagnosis in such diseases.

Intravenous enzyme replacement therapy (using recombinant enzymes) for the treatment of e.g. Fabry disease (human α -galactosidase) and other lysosomal diseases are available.

The glycosylation of therapeutic (recombinant) proteins also has an important impact on their in-vivo efficacy, half-life, and targeting to cells/tissues in patient. The attention of regulatory authorities regarding glycosylation of human therapeutics has significantly increased during the past few years. Decoration with structural motifs of outer antennae of N-glycans with Gal α 1-3Gal motif, presence of N-glycolylneuraminic acid (NeuGc), peripheral lewis-type α 1-3/4 or α 1-2 fucosylation, phosphorylated oligomannosidic or hybrid-type structures, sulphated structures as well as O-acetylation of sialic acids and LacDiNAc motifs are discussed as potential risks when present in recombinant therapeutics from mammalian hosts are used in humans. Examples will be presented regarding the methodology of detection and quantification of this type of modifications in therapeutic market products as well as in the development of innovative drugs and “*biosimilar*” medicines. The problem of appropriate host cells for recombinant biopharmaceutical glycoprotein production will be addressed

New challenges in safety assessment of biopharmaceuticals

Roy Foster, CIT, Evreux, FRA

Dr. Roy Forster is Scientific Director of CIT, a preclinical Contract Research Organisation, located near to Paris. CIT performs preclinical safety, efficacy and pharmacokinetics studies on behalf of client companies in the pharmaceutical and biotechnology industries. In his role as Scientific Director, Dr. Forster advises many Sponsor companies on the preclinical toxicology development of their candidate drugs. Dr Forster graduated from Cambridge University in 1972, and performed postdoctoral work at the University of Sussex. He subsequently worked in research institutes in the UK (Water Research Centre, Henley, UK), Rome (LSR-RTC, Pomezia, Italy), and Milan (Italfarmaco Research Centre, Cinisello Balsamo, Italy) before joining CIT in 1995. His professional activities include: coordinator of the FP6-funded RETHINK project on the application of minipigs in toxicology; European Registered Toxicologist; Fellow of the Royal Society of Medicine, London; Founding Editor of the journal "Transgenic Research". Dr Forster is author of more than 50 scientific articles.

In the development of new biopharmaceuticals, the safety of the intended therapeutic use of the product and the clinical development plan are supported by the preclinical safety dossier, composed of regulatory toxicity studies performed in animals. For the performance of these studies, the overall characteristics of biopharmaceuticals such as recombinant proteins, monoclonal antibodies and peptides differ from those of synthetic "small molecule" drugs. As a consequence, protein therapeutics may present a number of well-known problems for safety testing. These include (i) the selection of a relevant animal species for testing (ii) immunogenicity, which can modify the pharmacokinetics and toxicity of human proteins in animals and (iii) the route of administration: practical problems can arise from the need for parenteral routes of administration. These issues can, in turn, cause further problems in the application of traditional approaches to the evaluation of some important potential hazards such as reproductive toxicity or carcinogenicity. The use of surrogate products and / or homologous models have in some cases provided a way around these problems. After a long period in which ICH guidance provided a framework for safety testing of protein therapeutics, there is currently new debate surrounding preclinical approaches to biopharmaceuticals. Current regulatory discussions are bringing some new approaches to these problems, which may lead to more pragmatic but nevertheless demanding approaches to the preclinical safety of protein therapeutics. In this talk I will focus on current regulatory expectations and issues in the preclinical safety evaluation of biopharmaceuticals.

Complex structures in simple organisms: the N-glycosylation of invertebrates and protozoa

Iain Wilson, University of Natural Resources and Applied Life Sciences, AUT



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iain.wilson@boku.ac.at ; www.chemie.boku.ac.at/7975.html
Present position: Assistant professor, Universität für Bodenkultur, since 2002

EDUCATION:

2001: Habilitation in Biochemistry, Universität für Bodenkultur Wien
1988 - 1991: University of Oxford, DPhil, Biochemistry
1991: Royal Society of Chemistry, Chartered Chemist (CChem, MRSC)
1984 - 1988: University of Edinburgh, BSc (Hons, first class), Biochemistry

CURRENT GRANTS

Fonds zur Förderung der wissenschaftlichen Forschung (Austrian Science Fund, FWF)

Re-engineering glycosylation in insect cells (2007-2010)
Glycogenomics of *Dictyostelium discoideum* (2007-2010)
Glycosylation of *Acanthamoeba castellanii* (2008-2011)

European Commission

EuroGlycoArrays Initial Training Network (one of 16 partners; 2008-2012)

Lower organisms such as unicellular protists, nematodes and insects are frequently-used tools in biological research, based on the premise that these organisms are simpler as systems and also cheaper to maintain than mammals. Furthermore, insect cells are frequently used in the heterologous expression of recombinant proteins; even amoebae can also be used for this purpose. As the optimal biological function of many proteins is determined by glycosylation, as in the case of immunoglobulins, knowledge as to the glycan structures and the pathways of glycan biosynthesis are required; this facilitates examination of the function *in vivo* of these post-translational modifications as well enabling the development of approaches aimed at optimising these pathways. Often the glycans of non-vertebrate species have certain properties such as being immunogenic, allergenic or immunomodulatory. This is because these glycans are 'foreign' to mammals, but these properties may also confer an advantage to various protists and worms which are parasites of mammalian hosts. Using both analytical and molecular biological methodologies, my group has examined the N-glycosylation capacities of amoebae (*Dictyostelium* and *Acanthamoeba*) and nematodes (*Caenorhabditis* and *Ascaris*) as well as of insects (*Drosophila* and various cell lines). Common themes include the occurrence of either core alpha1,3-fucose or beta1,2-xylose residues as found on a number of plant allergens; thereby, these residues account for the immunological cross-reactivity of plant and non-vertebrate glycoproteins. The impact of mutations or suppression of glycosylation pathways will also be discussed.

Concepts of Protein Quality Control

Markus Kaiser, Chemical Genomics Center, Max Planck Institut, Dortmund, GER



Name:	Markus Kaiser
Nationality:	German
Date and place of birth:	18. 04. 1975 at Offenbach am Main
Family status:	married with Barbara Saccà (Italian), 2 children.
School education:	A-levels school degree at the Einhardschule, Seligenstadt, Germany.

University career

Chemistry at the Johann-Wolfgang Goethe-Universität, Frankfurt,
Chemistry at the Eberhard-Karls-Universität, Tübingen and
Parallel studies of romanic languages
Development of synthetic methods for the lipidation of peptides and
proteins in the research group of Prof. Luis Moroder, Max-Planck-Institute
of Biochemistry, Martinsried, Germany.
Synthesis of small molecules modulating DNA and RNA functions in the
research group of Prof. Jean-Marie Lehn at the Collège de France, Paris
Since 09/2005 Independent Research Group Leader at the Chemical
Genomics Centre, Dortmund, Germany.
01/2010 Call for a full professorship (W3) from the Univ. Duisburg- Essen

Awards and Fellowships

2002: Bert L. Schram Award (ESCOM Science Foundation)
2003: Friedrich-Weygand Preis (Max-Bergmann-Kreis)
2003: DAAD-Postdoc-Stipendium
2004-2005: Marie-Curie Postdoc-Stipendium
‘Young Leaders in Science’ (Schering Stiftung)

Small molecule approaches to Protein Quality Control

Protein quality control (PQC) is a cellular key process that ensures that all proteins are properly folded and localized. Although PQC is mostly associated with stress conditions such as heat shock, it plays a fundamental role under any environmental situation as misfolding and mislocalization errors even occur under homeostatic conditions. As a consequence, failure of PQC often results in severe pathologies, most prominently neurodegenerative disorders such as Parkinson’s and Alzheimer’ disease or cancer. On the other hand, PQC is also critical for bacterial virulence as pathogenic bacteria without sufficient PQC cannot survive in their hosts. Consequently, either selective restoration of hindered PQC or inhibition of bacterial PQC represent promising strategies for small molecule intervention. To put this approach into practice, suitable molecular targets and small molecules are required.

In the first part of my talk, I will report on a new class of proteasome inhibitors, known as syrbactins. Proteasome inhibitors are promising anticancer agents that most presumably act by inhibition of endoplasmatic PQC, leading to apoptosis in cancer cells. Syrbactins are a family of recently discovered natural products that play a pivotal role in plant pathogen interactions. Besides their interesting biological role, they also serve as a potential lead structure for drug development. The current status on syrbactins as anticancer agents will be summarized.

In the second part of my talk, I will present chemical biology studies with HtrA proteases which might represent suitable drug targets for small molecule intervention. HtrA proteases are a family of widely conserved serine proteases. They consist of a serine protease domain adopting a chymotrypsin fold and one or two PDZ domains. Contrary to common serine proteases, their proteolytic activity is controlled by cellular stress signals, allowing a reversible switch between proteolytic on- and off-states. Here, I will report our findings on their biochemical regulation, biological function and potential medicinal relevance.

Special key note lecture:

Biopharmaceutical manufacturing trends for maximizing antibody yields

Andreas Werner, Boehringer Ingelheim, Ingelheim, GER



Dr. Andreas Werner studied biology and biotechnology at the University Bielefeld focusing his research on production platforms for monoclonal antibodies & derivatives, bioartificial liver systems, inflammatory interactions and stem cell technology. After finishing his PhD at the Technical Institute of Cell Culture Technology he gave lectures in medical biotechnology as an assistant professor at the University of Bielefeld. In 2003 he started his career in the pharmaceutical industry at Boehringer Ingelheim followed by various positions heading production coordination, strategic capacity management, investment planning and the establishment of a strategic production alliance network. From 2007 to 2009 he led the planning & coordination department in Vienna and he is now responsible for the business governance of the division biopharmaceuticals at the Boehringer Ingelheim Corporate Center.

In 2009 worldwide 367 biopharmaceuticals derived from mammalian cell cultures and 249 biopharmaceuticals derived from microbial fermentation had been in development in various clinical phases. This increasing number of new biopharmaceuticals also results in a consistent number of approved products per year in the range of 6 New Biological Entities (NBE).

Most of the biopharmaceuticals produced by mammalian cell cultures are monoclonal antibodies, whereas biopharmaceuticals developed from microbial fermentation processes are antibody mimetics or a variety of protein scaffolds. This implicates the availability of a broad applicable technology platforms for mammalian cell cultures derived products and heterogeneous technology platforms for products from microbial processes.

For a time-to-clinic development the existence of such validated technology platforms is of utmost importance. For the development of monoclonal antibodies high titer Chinese Hamster Ovarian (CHO) are mostly applied in conjunction with animal-free chemically defined media and feeding processes. Presently titers are reached in the range of 10 g/L after a fourteen day's fermentation period.

Preferentially, separation of cells during the harvest process are conducted by centrifugation, to avoid cost for filter consumable material.

The technology platform for the downstream processing is established with affinity chromatography on protein A, followed by cation- and anion exchange chromatography. To avoid high cost for chromatography material alternatively to protein A, a contaminant precipitation is performed followed by cation chromatography and Q-membrane filtration.

For galenic formulation a technology platform has to be available for high concentration of up to 200 mg/ml of the therapeutic antibody to allow small volume prefilled liquid syringe application of the high dose therapeutic antibody in oncology and immunology.

In order to improve efficiency of the antibody, protein engineering or glyco engineering of the Fc part of the antibody is required to enhance the Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), extension of serum half life or decreased antigenicity. For treatment of solid tumors cytotoxic drug conjugates are required.

For the development of antibody mimetics or protein scaffolds serum half life extension technologies such as pegylation, recombinant poly-amino acid aducts, and human serum albumin- or Fc fusion protein technologies have to be in place. Antibody mimetics or protein scaffolds preferentially are produced in E. coli or yeasts with titer of up to 15 g/L.

Due to the high titer and chemically defined media, crystallisation platforms can be applied for direct crystallisation of the therapeutic protein from the fermentation supernatant or as an intermediate process step in the downstream processing. In downstream processing yields up to 90 percent are achievable.

Based on the availability of these technology platforms an integrated approach for process development and clinical development can be performed allowing shortest timelines from genetic engineering to clinic of 21 months and lowest resources investment considering attrition rates of the products. Such attrition rates are 80% in phase I, 70% in phase II and 17% in phase III. This financial risk has to be considered during process and product development for a just enough approach.

The competitive environment and the therapeutic needs require shortest timeline for the development of biopharmaceuticals for early return on investment and patient needs.

To achieve such short timelines in advance, development of competitive technology platforms have to be performed on an ongoing basis years before product development of biopharmaceuticals starts. Innovation from in-house and external process sciences collaborations with universities and research institutes are highly supportive in such processes.

Thursday, February 25, 2010

Production of biopharmaceuticals in human amniocytes

Gudrun Schiedner, CEVEC Pharmaceuticals, Cologne, GER



Dr. Gudrun Schiedner has received her academic training as Biologist and Molecular Geneticist from the University of Konstanz and obtained a PhD in Molecular Genetics and Virology from the University of Cologne. For several years as Senior Scientist at the Baylor College of Medicine in Houston/USA, and then by joining the Gene Therapy Division of the Center of Molecular Medicine in Cologne, she gained several years of experience in gene therapy and cell line development. Gudrun Schiedner has published numerous scientific papers in high-ranking journals and is co-inventor of several patent applications. Together with a group of scientists and clinicians from the University of Cologne she started CEVEC Pharmaceuticals in 2004 and is in the position as Chief Scientific Officer since 2005.

Human CAP (CEVEC's Amniocyte Production) cells allow for stable production of recombinant proteins with excellent biologic activity and therapeutic efficacy as a result of authentic posttranslational modification. Among others, high expression levels, human-like glycosylation, high cell densities, fast and easy cell line development and complete cell documentation are key benefits of the CAP technology. Based on human CAP cells an additional transient expression system has been developed, that is scalable and serum-free and that enables extremely high production yields of recombinant proteins within a few days. The novel CAP-T transient human cell expression system is a rapid and versatile tool for fast and serum-free protein expression for screening, assay development, drug discovery and early stage pre-clinical drug development. With fast process times, high yields and authentic glycosylation patterns CAP-T cells outperform currently used transient production systems. Thus, CAP and CAP-T technologies offer the use of only one unique platform for customer's need from very early preclinical development up to clinical supply of recombinant biotherapeutics.

Developing a new antibody against a combined peptide-carbohydrate target and improving it by GEX-technology

Franzpeter Bracht, Glycotope GmbH, Berlin/Heidelberg, GER



early 2007: Joining Glycotope Chief Financial & Business Officer
2001:AplaGen GmbH: CEO/CFO/Co-Founder
1997:Kienbaum Management Consultants: Principal, Head of Pharma & Life Sciences
1996: Ernst &Young: Consultant
1996: PhD in Pharmacology
Co-Founder and investor in other small biotech

In recent years, glycosylation, the attachment of carbohydrates to a protein's peptide backbone as means of post-translational modification, has received a great deal of attention. The molecular composition of these glycan-structures has been found to have a profound influence on a biotherapeutic's clinical characteristics such as half-life time, activity or immunogenicity.

TA- MUC-1, a cancer marker, when expressed in tumors, carries an altered glycosylation as compared to healthy tissues. Glycotope has used these differences to develop a monoclonal antibody targeting both the MUC-1 peptide backbone and the attached cancer-specific carbohydrates. As a result, GT-MAB 2.5-GEX combines a very high affinity, mediated by the backbone, with an exceptionally high tumor-specificity, as the particular carbohydrate is present on tumor-cells only.

With GlycoExpress, Glycotope's proprietary platform of human cell-lines for the improvement of glycosylated proteins, the antibodies glycosylation has been optimized to confer a strong ADCC-activity already at low doses, combined with an extended half-life time. Same was done with Cetuximab, a marketed EGFR-Receptor antibody (GT MAB 5.2-GEX, CetuGEX).

After very encouraging pre-clinical data-sets, GT-MAB 2.5-GEX is currently undergoing a clinical dose-finding study. The IMPD for CetuGex will be filed in March 2010.

Secretion of mAb in 100 l bioreactors void of plant specific fucosylation and xylosylation

Ronald Bassuner, greenovation Biotech, GER



Since 2009: Chief Technology Officer, Greenovation Biotech, Freiburg, Germany
2005 – 2009 Business Development Manager, Sigma-Aldrich, St. Louis, MO, USA
2002 – 2005 Director Pilot & GMP Production, SemBioSys Corp., Calgary, AB, Canada
1998 – 2002 Scientific Project Manager, Monsanto Corp., Madison, WI, USA
1994 – 1998 Visiting Assistant Professor, Purdue University, West Lafayette, IN, USA
Before 1994 Group Leader, Institute of Crop Plant Research, Gatersleben, Germany
Habilitation in Genetics, Martin-Luther-University, Halle-Wittenberg, Germany
PhD. in Plant Biochemistry, Academy of Sciences, Berlin, Germany

Moss (*Physcomitrella patens*) is a novel expression platform for pharmaceutical proteins, providing a number of advantages, as well as challenges when compared to other platforms. In its regular, vegetative appearance *P. patens* harbors a haploid genome which facilitates greatly the stable introduction of foreign genes. During normal vegetative growth of the biomass these genes remain stably integrated into the genome. As the complete genome has been sequenced, we were able to specifically knock out two genetic loci that encoded for plant-specific glycosylation (β -1,2-XylT and α -1,6-FucT), eliminating the potential risk of immune responses to these glycan structures of proteins when administered to humans. Due to the precise and nearly homogeneous structure of the glycan moiety we demonstrated a significantly enhanced ADCC activity of an antibody. To further enhance the utility of our platform, we established a biphasic production protocol whereby biomass expansion is followed by the phase of efficient secretion into the simple, salt-containing media. This approach delivered mAbs already with a high level of initial purity to the downstream processing train. We transferred the production process from pilot scale successfully into two different 100 L photo-bioreactors. While both reactor types are suitable for cGMP production, one reactor uses disposable technology that is familiar to most contract manufacturing organizations. I shall discuss our current challenges to develop scalable photo-bioreaktor technology to a level where it is transferable to the cGMP production environment of a partnering CMO.

Development of pegylated biopharmaceuticals

Giancarlo Tonon, Bio-Ker, Pula (Cagliari), Sardinia, ITA

- Graduated in Medicine at the University of Milan
- Postdoctoral fellow in the Department of Pharmacology, at University of Milan and at University of Texas (Houston).
 - Research experience in neuroscience and biochemical Pharmacology
- R&D Director at Dompè Farmaceutici (Milan) .
 - Managing of drug development projects at the pre-clinical and clinical stages (pulmonary, gastroenterology and inflammatory diseases).
- Vice President R&D at Pierrel (Milan)
 - Engaged in infectious, cardiovascular and endocrinological diseases and recombinant proteins projects
- Vice President at Pharmacia (Milan)
 - Projects management and drugs development in CNS area
- C.E.O. and General Manager at Bio-Ker (Pula, Cagliari)
 - R & D in Biopharmaceuticals, recombinant therapeutic protein and protein delivery and conjugation
- Extensive experience in biotechnological product development
- Inventor in several international patents related to developed and registered bio- pharmaceutical products
- Author of many scientific publications
- Contract professor at San Raffaele University in Milan, State University of Milan and State University of Pisa

Biotechnology-derived drugs represent now a significant market and are estimated to be approximately one third of all drugs. The most important biodrugs include insulin, interferons, several growth factors, erythropoietin and monoclonal antibodies. Currently, the biodrugs are administered to over 300,000,000 patients and about half of all drugs currently under development are biotechnology-derived. Moreover, therapeutic treatments became less intense, both in the frequency of administration and in the smaller quantity of active drug substance per dose. Consequently, many controlled-release techniques were developed, such as slow or sustained-release, to improve therapeutic treatment using biotechnology-derived drugs.

With such second-generation products, it is possible prolonging intellectual property protection and controlling market erosion due to competition from biosimilar drugs. By improving a critical, continuous treatment, often plagued with side-effects, even companies detaining the market for α -interferons and insulin could increase their sales and contain the arrival of biosimilars.

Protein PEGylation, obtained by covalent binding of one or more hydrophilic and biocompatible poly(ethylene glycol) polymeric chain (PEG), is one of the main technology used for altering/improving in vivo behaviour of therapeutic proteins to get increased stability, improved PK/PD, and decreased immunogenicity. Different chemical strategies for coupling linear or branched PEG (with different molecular weight from 5 kDa to 40 kDa), have been used in order to obtain mono- or poly- conjugated proteins. More recently site specific conjugations are preferred and enzymatic approaches have been positively adopted.

The most important approved and marketed PEGylated biodrugs are PEGylated – Adenosine deaminase (Adagen[®]) Enzon; PEGylated – Asparaginase (Oncaspar[®]) Enzon; PEGylated - interferon α 2a (Pegasys[®]), Roche; PEGylated - interferon α 2b (Peg-Intron[®]), Schering-Plough; PEGylated - erythropoietin beta (Micera[®]) Roche; PEGylated – human growth hormone antagonist (Somavert[®])

Pfizer; PEGylated - G-CSF (Neulasta[®]), Amgen; PEGylated - anti TNF Fab (Cimzia[®]) UCB. These PEGylated drugs occupy a very strong position in the market and much more pegylated proteins are at various stages of clinical development. In this presentation, various aspects of the PEGylation technology will be treated, including physicochemical, pharmacokinetic, immunogenic and toxicological properties of PEG and PEG-conjugates. Moreover, general perspectives for future development of the most important PEGylated products are considered, describing examples of key PEGylated proteins, and reviewing the strategy, advantages and limitations of all aspects of drug PEGylation considering both the marketed biodrugs and those under development.

HESylation® technology - a technology platform for the enhancement of active pharmaceutical ingredients

Helmut Knoller, Fresenius Kabi, Bad Homburg, GER



Helmut Knoller joined Fresenius in 2000 where he started as R&D scientist in the area of pathogen inactivation of blood products. He went on to work on the new platform technology HESylation® Technology where he is presently Head of Scientific Management. His responsibilities include set up and management of several external co-operations. Additionally he cares for the broad IP portfolio, competitive intelligence and the performance of early preclinical studies. Helmut Knoller is co-inventor of several patent applications especially regarding HESylation® Technology.

Helmut Knoller received his diploma in polymer chemistry and his Ph.D. in molecular biology from the University of Ulm.

Technology description

HESylation® Technology allows a targeted modification of drugs and their characteristics by site-specific coupling to HES molecules. HES-coupling enables the modification of key pharmacological parameters such as absorption, metabolization, half-life, water solubility and safety.

HESylation® is based on the extensive expertise of Fresenius Kabi in the field of hydroxyethyl starch (“HES”) as the world’s largest producer of HES. HES is a modified natural polymer that has been widely used in clinical practice for decades as a plasma volume expander. Consequently, it has an impressive safety record.

The most important application areas for the HESylation® technology are biopharmaceuticals like proteins, peptides and oligonucleotides.

IP rights

Fresenius Kabi has a broad portfolio of patents and patent applications covering the proprietary technology.

Corporate description / mission

Fresenius Kabi’s Business Unit HESylation® Technology is a focused team of experienced professionals within the Kabi Innovation Centre. With access to customised HES species and dedicated fully equipped R&D and GMP facilities the support for pharmaceutical product developments are fulfilled at highest standards.

Workshop 1: Novel Therapeutics, Vaccines Diagnostics

Session 1a: Novel Therapeutics

Histidine residues as a tool for protein nanoparticle formation

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One of our recent research challenges has been exploitation of the active principle of protein aggregation, involving metal coordination of specifically designed protein analogues.

Specifically designed analogues of TNF-alpha with surface exposed clusters of histidines (LK801) and histidine tags attached to N-terminus (His10-TNF and H7dN6TNF) served as model proteins.

In the first approach, self-assembled protein nanostructures were formed using different polyfunctional biocompatible chelators in combination with histidine rich TNF-alpha analogues and zinc ions.

The other approach was formation of phosphate nanoparticles decorated with zinc ions, which were decorated with TNF-alpha analogues.

In all cases metal coordinative binding was found to be reversible, enabling controlled release of individual protein molecules upon suitably changed environmental conditions. Size, stability, in vitro biological activity and release profiles of different protein nanostructures were determined.

Nanoparticles containing TNF-alpha analogues with reduced biological activity (H7dN6TNF and His10-TNF) could be used in active principle of immunization. An increased immune response was achieved after administration to the testing animals. Enhanced formation of antibodies against TNF-alpha would be advantageous serving as a basis for developing new drugs for chronic diseases associated with pathogenically elevated levels of TNF-alpha (rheumatoid arthritis, Crohn's disease, etc.).

Nanoparticles containing TNF-alpha analogue LK801 could be used in anti-cancer therapy, where slow release of individual, active molecules from nanoparticles would presumably result in sustained anti-tumor activity with reduced side effects. That was confirmed in experiments on mice with implanted Sa-1 tumors, where self-assembled nanoparticles with LK801 showed lower toxicity, reduced side effects and better anti-tumor effects, meaning longer tumor growth delay.

In Nude Rats, Tumor Growth and Osteolytic Lesions induced by MDA-MB-231 human Breast Cancer Cells were effectively treated by a monoclonal Rat Antibody against Bone Sialoprotein II

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² Immundiagnostik, Bensheim, Germany

The SIBLING protein bone sialoprotein II (BSP II) may mediate the targeting and attachment of (normal and) metastasizing cells to the bone surface and has been found implicated in lytic skeletal metastasis. The aim of this study was to investigate the effect of a new rat monoclonal antibody against BSP II as measured by proliferation and osteolytic activity of breast cancer cells growing in nude rats.

Following inoculation of 1×10^5 MDA-MB-231^{luc} breast cancer cells into the femoral artery of male nude rats, lesions developed exclusively in the tibia, femur or fibula of the respective leg. Their development was monitored by light emission released by the luciferase reporter gene and was recorded at regular intervals.

Treatment with the anti-BSP antibody TGC-9 started when tumor bearing rats had shown stable tumor growth. Treated rats received 10mg TGC-9 per week for six weeks starting at four (late onset) or two (early onset) weeks after tumor cell implantation. Each mode included 10 treated and control rats, respectively, and was followed for up to six weeks.

All untreated tumor bearing rats showed tumor growth accompanied by lytic destruction of the femur and tibia of the respective hind leg. In contrast, rats treated with TGC-9 did not show a significant increase in light emission nor a clinical deterioration. In fact, 7 of 10 rats receiving the late onset therapy with TGC-9 didn't show any light emission after 4 to 6 weeks (70%; $p = 0.01$ versus control) as well as 8 of 10 rats receiving the early onset therapy with TGC-9 (80%; $p < 0.01$). Radiological and histological examination confirmed that animals without light emission were free of tumor growth, corresponding to a complete remission. In conclusion, the monoclonal antibody TGC-9 directed against BSP II is a powerful tool in treating experimental skeletal metastasis and warrants further development.

Workshop 2: Novel Technologies

Session 2a: Novel up- and downstream developments

Could be used downstream processes for increasing the antibiotics activity? Studies on Gentamicins separation

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The less active Gentamicin C_1 has been selectively removed from the biosynthetic mixture containing Gentamicins C_1 , C_{1a} , C_2 and C_{2a} by facilitated pertraction with D2EHPA dissolved in dichloromethane as the liquid membrane using an U-shaped pertraction cell. The pertraction has been analyzed by means of the initial and final mass flows, permeability and selectivity factors. The selectivity of the Gentamicins transport through liquid membrane is mainly controlled by the pH-gradient between the feed and stripping phases, mixing intensity of the aqueous phases and carrier concentration in the membrane phase. Using the proper levels of the factors influencing the separation process, the most active Gentamicins (Gentamicins C_{1a} , C_2 and C_{2a}) can be selectively pertracted from the initial mixture.

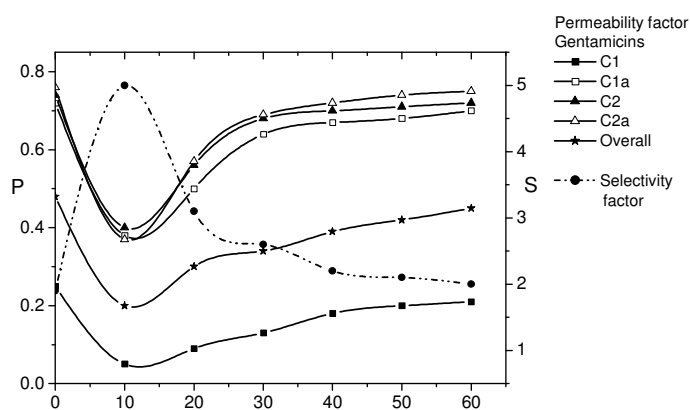


Figure 1. Influence of carrier concentration on mass flows of Gentamicins (pH of feed phase = 8, pH of stripping phase = 1.5, rotation speed = 500 rpm).

For the considered experimental conditions, the selectivity factor reached the highest values for pH of feed phase of 8, pH of stripping phase of 3, rotation speed of the feed and stripping phases below 100 rpm and carrier concentration of 10 g/l. Therefore, the removal by facilitated pertraction of Gentamicin C_1 , which has the lowest activity against the infections, from the biosynthetic mixture increases the therapeutic activity of the antibiotic.

Evaluation of residual moisture in lyocakes and corresponding lyophilization stoppers of different rubber formulations

Heike Kofler,

West Pharmaceutical Services, Eschweiler, Germany

One of the critical success factors for packaging lyophilized drugs is protection against product degradation caused by moisture. Moisture can be introduced into a lyophilized drug product cake from the elastomeric stopper and from the atmospheric headspace; it can also permeate through the stopper, a process known as moisture transmission.

Prior to packaging the drug product, stoppers are typically washed, steam sterilized and dried. Steam sterilization drives moisture into the stopper. If the drying conditions for the stopper are not optimized, residual moisture can transfer into the lyophilized drug product over time. In 2005, West began a three-year study observing the moisture content in stoppers and lyophilized cakes. The study, performed by West Analytical Services, was based on the assumption that residual moisture in elastomeric stoppers can cause degradation of lyophilized drug product. The evaluation should help manufacturers to choose the most suitable stopper for moisture-sensitive lyophilized drug products.

The investigation was done on stoppers made with chlorobutyl, bromobutyl and butyl, as well as fluoropolymer laminated stoppers. This study shows evidence that moisture will migrate from the environment to the stopper and consequently to the lyophilized product cake over time. The amount of residual moisture is dependent on the formulation as well as the drying time.

According to the results it is very important to optimize the drying time of stoppers to reduce residual moisture, it is also important to choose a stopper formulation that will reduce the transfer of retained moisture to the lyophilized cake.

FACS-Assisted Directed Evolution to obtain a CHOK1 host cell line growing in glutamine free medium

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² Unitargeting Research, Bergen, Norway

For reliable biopharmaceutical production it is essential to have cell lines with robust and reproducible behaviour under fermentation conditions. In addition to robustness against stress, a good cell line should be able to reach high cell densities fast and then to maintain viability under these conditions to ensure attainment of high product titers. In recent years recombinant CHO cell lines have been adapted to growth in protein-free media and have even been subjected to “Bioreactor Evolution” (Biotechnology Progress (2007) 23:2, 458-464) to improve process relevant properties. In addition to the mentioned goals, cell line and media optimisation also needs to ensure stable and high product quality. In this respect the use of glutamine as the preferred energy substrate of mammalian cells is problematic, as the released ammonium will decrease the terminal sialidation of glycoproteins, thus reducing their biological half-life.

In an attempt to establish a robust and optimised cell line for recombinant protein production we have adapted the original ATCC derived CHO K1 cell line to growth in chemically defined, protein-free medium. Today’s media formulations are sophisticated to a level that allows weaning of cells within two to three weeks. The adapted cells reached final cell densities of $\sim 4 \times 10^6$ /ml when grown in batch cultures using medium with 8mM glutamine. Reduction of glutamine to 4mM caused a decrease in final cell density to 2×10^6 /ml. To regain the original VCC, cells were subjected to an iterative evolutionary process that consisted of repeatedly seeding cells at high densities followed by sorting of viable cells when culture viability had dropped below 10%. The obtained cell line reached cell densities around $4\text{-}5 \times 10^6$ /ml and in addition was characterised by prolonged culture life, maintaining viabilities of above 90% for 10 or more days in batch culture. Subsequently glutamine concentration was further decreased to 2mM and the same procedure repeated. To document maintenance of the inherent production capacity of the cell line, transient transfections of antibody plasmids were performed. Finally, the 2mM Glutamine-cell line was successfully cultivated in glutamine free medium.

In summary, a robust cell line with high viability and high cell density was obtained by subjecting it to stress conditions similar to those encountered at the end of bioprocesses and by selecting rare cells that are exceptionally well able to survive this treatment.

MicroRNAs – small RNAs with big future in Chinese Hamster Ovary cell culture technology

Matthias Hackl, Nicole Borth

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With nearly 70% of all recombinant protein therapeutics being produced in Chinese hamster ovary (CHO) cells, this cell line represents the primarily used cell factory in industrial biotechnology. Consequently, much effort has been taken to increase the space-time yield of CHO cell-based processes, either by optimizing the bioreactor environment or by engineering the intracellular setup. For the latter approach microRNAs (miRNAs), which are approximately 22nt long non-coding RNAs acting as global post-transcriptional regulators of gene expression, offer new perspectives. Several studies attribute miRNAs fundamental roles in the regulation of cellular processes such as proliferation or apoptosis, and by overexpressing miRNAs it might be possible to orchestrate the expression of a large variety of genes while not competing with recombinant proteins for translational resources.

In order to identify miRNAs with potential for cell engineering (*engimiRs*) we previously established a microarray platform for cross-species profiling of CHO miRNA transcription. The next tasks are twofold: first sequencing of the expressed miRNAs of CHO cells to obtain an overview on conserved and species specific sequences. Second, miRNA transcription in different CHO cells needs to be characterised and correlated with cellular properties, to identify miRNAs for both diagnostics of cellular performance and cell engineering.

We have recently started a deep sequencing approach, using Illumina's Genome Analyzer, to characterize the CHO miRNome, by looking at miRNA sequence homology to other organisms as well as miRNA expression levels. Seven RNA libraries from several cell lines under various conditions were generated and sequenced yielding total of 100 million short sequence reads. As the Chinese hamster genome is not yet available, a bioinformatics pipeline was developed based on the Needleman-Wunsch algorithm for short sequence alignment to known miRNA sequences (miRBase v14.0). Thus, more than 200 orthologous CHO microRNAs and their respective miRNA* sequences were discovered as well as a broad range of other non coding small RNAs. The results of this next-generation sequencing approach allow deep insights into microRNA transcription and regulation in CHO cells and set the stage for identification as well as functional characterization of potential *engimiRs*.

Efficient Removal of Aggregates from Impure Pharmaceutical Active Antibodies

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Aggregates are persistent and frequent process related impurities which have to be removed during downstream processing of protein biopharmaceuticals, since aggregates have the capability to trigger an immune response directed against the monomeric form of the protein *in vivo*. Due to the increased hydrophobicity of aggregates, they can efficiently be depleted by Hydrophobic Interaction Chromatography (HIC). Different conditions for removing aggregates using a special HIC membrane adsorber as stationary phase will be presented for the purification of recombinant antibodies resulting in a reduction of the aggregate level from 12 % to only 0.5 %.

Terminal radiation sterilization (25kGy) and long time storage of biopharmaceuticals enabled by stabilizing nanocoating technology

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Leukocare, Munich, Germany

Due to denaturation the terminal sterilization of biopharmaceuticals by radiation has not been routinely applied. Therefore, these products have to be produced under aseptic conditions, resulting in a sterility assurance level (SAL) of only $10E-3$. Furthermore costly downprocessing technologies have to be validated.

In order to allow terminal sterilization of proteins like antibodies and growth factors, we developed a nanocoating technology for the three-dimensional stabilization of proteins. This protective coating preserves the biological function of biopharmaceuticals during sterilization and subsequent storage, resulting in reduced production costs and higher product safety (SAL $10E-6$). The protecting nanocoating consists of water soluble pharmaceutical grade substances.

The nanocoating has been shown to protect even very large proteins like immunoglobulin M molecules during beta or gamma irradiation with >25 kGy and even ethylene oxide gas sterilization.

Basically, almost the same stabilizers can be used that we have developed for biologic-device combination products, which currently are in clinical evaluation.

This stabilizing nanocoating technology is expected to boost the market of biopharmaceuticals by enabling the manufacturers to use standard sterilization technologies instead of costly aseptic production environments resulting in lower validation and regulatory burden while at the same time increasing product safety.

LEUKOCARE is happy to share this technology with interested biopharmaceutical companies on a technology development and license basis.

Session 2b: Novel biopharmaceutical drug formats and pharmaceutical developments

Novel Excipients Stabilizing Salmon Calcitonin against Aggregation

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¹ University of Geneva, Switzerland

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During all stages of protein drug development, aggregation is one of the most often encountered problems compromising the drug's safety and efficacy. Protein aggregation, being a very complex process, may depend on a multitude of environmental factors (e.g., temperature, pH, ionic strength or protein concentration), applied stresses (e.g., shaking, freezing or thawing), and the structure of the protein itself.

For many proteins, a nucleation-dependent mechanism has been described, during which aggregation-prone folding/unfolding intermediates are formed by conformational changes. Those favour protein/protein interactions, and thus initiate aggregation, by liberation and approximation of hydrophobic patches, which are usually buried inside the intact protein.

PEGylation, the covalent attachment of poly(ethylene glycol) to therapeutics, is known, besides others, to reduce aggregation due to shielding of hydrophobic patches on protein surfaces. A major drawback of PEGylation is the conjugation step for which most often chemical strategies are employed.

Here we present a new method of stabilizing salmon calcitonin against aggregation. Several polymers, consisting of a hydrophobic headgroup, in this case a dansyl-moiety, attached to PEG-polymers of different molecular weight were synthesized and characterized via NMR, FTIR, MALDI-TOF and different spectroscopy techniques.

We were able to show that these polymers still possess environmental sensitivity. Subsequently, their influence on the aggregation propensity of salmon calcitonin in various buffer systems was tested. Depending on the molecular weight of the PEG-polymer, the onset of aggregation was prolonged and the final degree of aggregation reduced, while representing no perturbances on the peptide's stability in a stable formulation.

To conclude, the aggregational behaviour of salmon calcitonin is changed in presence of our polymers, leading to a decelerated process of aggregation and a less aggregated state of the peptide drug.

Conversion of a Therapeutic Cytokine into Protein-coated Microcrystals Enabling Powder-based Dosage Forms

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Boehringer Ingelheim, Biberach, Germany

Protein-coated microcrystals (PCMCs) are formed, if an aqueous buffered solution of a protein in which a carrier material is dissolved is introduced rapidly into a water-miscible organic solvent such as propan-2-ol or 2-methyl-2,4-pentanediol. The rapid removal of the water causes the protein to precipitate in amorphous form in combination with other solutes dissolved in the aqueous phase as fine particles, since for kinetic reasons no crystal growth can occur after nucleation. After the precipitation process the PCMCs can be separated from the aqueous solvent mixture and subsequently either be dried or resuspended in another nonsolvent mixture.

In the presented study, the applicability of this technology to a nonglycosylated cytokine was to be tested, whilst earlier studies had focussed on glycosylated antibodies and proteins [1]. Seven formulations of the cytokine were chosen that differed in their carrier composition based on amino acids, sugars, buffers and salts. After supercritical fluid extraction drying, the powder was filled into vials and (double chamber) cartridges which serve as primary packaging when applying PCMCs to drug product for human use.

The analytical focus was placed on the protein integrity analyzed via SEC and turbidity testing after reconstitution of the PCMCs as well as on the particle size and morphology of the microcrystals. Overall, the protein integrity in terms of the monomeric status was preserved after the PCMC precipitation process.

For PCMCs with aspartic acid as main carrier component a dependence of the particle size on the pH of the aqueous phase prior to the precipitation was seen.

Thus the PCMC precipitation process could be successfully applied to a cytokine. Particle size and shape as well as protein stability can be controlled by the formulation.

[1] König, Corinna; Development of a pilot scale process for manufacture of protein-coated microcrystals, Thesis 2009

A new approach for preparing pegylated pharmaceutically interesting Proteins

Tatjana Milunović, Tina Maver, Simon Caserman, Vladka Gaberc Poreka

National Institute of Chemistry, Ljubljana, Slovenia

Pegylation has become the most widely used approach for improving physicochemical properties and biomedical efficacy of therapeutic proteins, however only highly purified proteins are usually subjected to pegylation reaction, thus requiring another purification cycle to obtain the final purified pegylated product. The aim of this work was to shorten the whole procedure by pegylating the pharmaceutical protein at the stage of solubilized inclusion bodies and purify only the target pegylated protein, which would increase the yield and lower the production costs. A solution of non-classical inclusion bodies with a high containment of recombinant granulocyte colony stimulating factor (G-CSF) was prepared and, prior to any separation, pegylated by a 10 kDa PEG-NHS reagent. A combination of immobilized metal affinity chromatography (IMAC) and ion exchange chromatography (IEC) was used to separate the desired pegylation products from undesired ones. Pegylation with a linear 10 kDa PEG-NHS reagent resulted in conjugates that only partially hindered the binding of monopegylated G-CSF via naturally exposed histidine residues to the IMAC column (Zn-IDA carrier), while binding of polypegylated and abnormally folded forms of G-CSF was prevented. The binding of the native, correctly folded G-CSF was not hindered. Using IMAC as a capture and main separation step we were able to purify and isolate the correctly folded nonpegylated and monopegylated G-CSF, which were partly separated, from the complex pegylation mixture also containing *E. coli* proteins, aggregated and irregularly folded G-CSF and higher pegylated G-CSF forms. For the final purification ion exchange chromatography was used. In this step, the monopegylated G-CSF was successfully separated from the non-pegylated G-CSF. The monopegylated G-CSF retained 50% of in vitro potency related to native G-CSF.

Correlation of the osmotic second virial coefficient with protein solubility and colloidal stability

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The purpose of the presented study is to understand the physicochemical properties of proteins in aqueous solutions in order to identify solution conditions with reduced protein-protein interactions, in order to avoid the formation of protein aggregates and protein precipitates and to increase protein solubility.

This is assessed by analyzing the osmotic second virial coefficient (OSVC), a parameter of solution non-ideality, which is obtained using self-interaction chromatography. The protein (antibody) is covalently bound to chromatography particles and the retention volume of aqueous protein solutions injected in the chromatography column is measured. The retention volume of the protein depends on the interaction of the free protein in solution and the protein bound to the chromatography particles. OSVC is calculated using the retention volume.

The osmotic second virial coefficient is correlated with protein solubility, and the data show that OSVC is a useful parameter for the identification of solution conditions maximizing protein solubility. The influence of various solution parameters upon OSVC was investigated: protonation degree, ionic strength, pharmaceutical relevant excipients and combinations thereof. For the tested proteins it was observed, that under acidic solution conditions OSVC is positive, favoring protein repulsion. A similar trend is observed for the variation of NaCl concentration, showing that with increasing the ionic strength protein attraction is more likely. OSVC decreases and becomes negative. Thus solutions conditions are obtained favoring protein-protein interactions.

Self-interaction chromatography for assessing the osmotic second virial coefficient allows the identification of solution conditions reducing protein-protein interactions. Such solution conditions are favorable for increasing the physical protein stability in solution.

Development of a pilot-scale process for protein-coated microcrystals

(PCMC)

- mixing and precipitation - part I

- solvent reduction - part II

- final drying - part III

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Boehringer Ingelheim, Biberach, Germany

Protein coated microcrystals PCMC form when a solution containing protein and carriers are transferred into a water-miscible solvent in which the carrier material and protein are not soluble. This precipitation technology can be applied to a great variety of biomolecules, ranging from small peptides to full-size antibodies or nucleic acids. Suitable carrier materials are e.g. amino acids, peptides, sugars, organic and inorganic salts. Suitable manufacturing procedures for application at pilot scale and under cGMP have been developed, which consist of the primary step mixing/precipitation in the continuous mode, solvent reduction as well as supercritical drying of the dispersed concentrate.

In this context double and quadruple jet impingement micromixers have been established since they enable fast mixing and thus fast formation of PCMC at turbulent flow conditions. The favourable mixing performance was verified through computational fluid dynamic simulations as well as by the Villermaux Dushman parallel reaction. For the concentration of the suspension before the final drying process a gentle modified candle filtration was developed, which left the physisorptive forces between protein layer and the carrier material uncompromised contrary to Tangential Flow Filtration TFF which exerted more shear stress upon the PCMC as indicated by a decreased protein recovery. After the final drying step by supercritical extraction a well flowing powder was obtained. Various proteins were successfully tested with the developed pilot scale process, such as Trypsin as a model protein for small proteins or a full-size therapeutic monoclonal antibody.

Session 2c: Novel analytical tools

CHO Host Cell Lines Optimised by Cell Sorter Assisted Directed Evolution

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Cell populations in culture, even when genetically identical, differ in their gene expression pattern due to natural, regulatory and/or epigenetically controlled variations. In general, this variation in cellular state is an advantage for the population, as it allows a faster adaptation to changing environmental conditions. On the one hand, this heterogeneity is the reason for variations in the outcome of bioprocess runs, leading to a high requirement for sophisticated process monitoring and PAT technologies. On the other hand, it may be taken advantage of to identify cells with just the right process relevant properties. Within this context, we have recently isolated several optimised CHO-K1 or CHO-S derived host cell lines, obtained by cell sorter assisted directed evolution. The general approach consists of subjecting a cell population to a stress condition under which the cells are required to perform better. Those cells able to handle the stress condition in an optimal way are sorted out of the population and expanded. In our experience, a single such sorting round is not sufficient to identify cells that have a stably altered phenotype from the naturally occurring variation in cell behaviour. However, with three rounds of bulk sorting followed by an additional subcloning sort we were able to isolate host cell lines 1) that grow to higher final cell densities (IVC+), 2) that grow in glutamine free medium with enhanced viability (GLN-) and 3) that have a threefold higher specific productivity in transient transfection experiments. Typically, these optimised properties are stable for approximately four months, unless maintaining pressure is applied as in the case of the GLN- phenotype. In this presentation we will compare these optimised subclones with their respective parents with regard to growth, production behaviour and stress resistance.

Analysis of product comparability: using a novel risk based approach

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The current FDA initiative „Pharmaceutical cGMPs for the 21st Century – A Risk Based Approach“ establishes and demands documented risk management processes as a main tool for ensuring biopharmaceutical product quality. As a central new concept, Risk management is applicable to the whole product lifecycle. It should start in the planning and design phase, go on during product development and manufacturing as well as product release procedures, and comparability studies. Whereas different risk assessment tools are published in the literature and the regulatory GMP guidelines, the selection and application of the appropriate method is a major challenge. The selection depends on the product and process under investigation, as well as the philosophy of the respective company. Protagen AG has recognized these challenges early on and has established an innovative concept for a well documented workflow for risk assessment. We have developed a systematic approach supported by an Excel-based tool that guides the user through risk and project management and offers a transparent and traceable documentation. This approach can be easily adapted and customized to meet regulatory requirements. Here we show how this concept is applied to a comparability analysis and present the results.

Improved Primary Structure Characterization of Therapeutic Proteins by Mass Spectrometry combined with massive permutations of potential modifications

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Primary structure characterization of therapeutic proteins within development and production phase has to reveal even smallest changes in protein structure which may have an important influence on biological activity and product safety. In principle, mass spectrometry based protein analysis techniques give a rapid access to excellent peptide and protein fragmentation spectra and are a valuable tool to obtain extensive primary structure information. However, manual evaluation of the resulting large amount of data is impossible and commonly used mass spectrometry analysis software is primarily focused on protein identification (with low sequence coverage) rather than completing sequence information of targeted proteins. To overcome this limitation we applied the in-house developed software tool Modiro™ which is dedicated to full primary structure annotation of target proteins by mass spectrometry data. Focusing on predicted therapeutic protein sequence data and permutations thereof, acquired peptide fragmentation spectra are automatically screened for possible modifications, amino acid substitutions and mass spectrometry artifacts. Using Modiro™ is not necessary to search complete sequence databases (as it is common in other software), instead the search is restricted to one or more user defined proteins. A strong advantage of Modiro™ is the ability to cluster peptide spectra on the amino acid level, which gives the user rapid access to all spectra that contain information on a desired amino acid position within the therapeutic protein. As result manual evaluation time is reduced. Modiro™ increases the sensitivity in the detection of low level modification of therapeutic proteins as well as it allows more efficient elucidation of small product heterogeneities (e.g. deamidation / oxidation) compared to commonly used standard software.

MS based Primary Structure Mapping vs. Complete MS Data Interpretation for Characterization of Therapeutic Proteins

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The aim of the primary structure characterization of therapeutic proteins within development and production phase is to reveal that there are no unexpected changes in protein structure and no impurities which may have an important influence on biological activity and efficacy. A very good strategy for primary structure characterization is a mass spectrometry based full peptide mapping. This is commonly done by digesting the protein with several enzymes and acquiring LC/MS/MS runs. The MS/MS spectra of these runs are then searched against a sequence database containing the targeted protein sequence using standard software. Ideally, 100% sequence can be covered with this method. In this standard set-up however it is almost always the case that not all acquired spectra can be explained by this approach. Therefore this approach does not establish full comparability between production batches especially as product heterogeneities and impurities hidden in unexplained spectra may not be discovered. Best comparison of production batches requires explanation of all acquired mass spectrometry data above a defined signal threshold. In order to improve the comparability, we propose that instead of identifying spectra until full sequence coverage is reached, we analyzed all signals in LC-ESI MS data set regardless of the protein identification. This massive data analysis procedure becomes feasible by in-house developed dedicated software for automatic generation and subtraction of extracted ion chromatogram data from total ion current data. We were able to detect product heterogeneities in glycosylation, disulfide linkage, and other modifications. Mass spectrometry related artifacts (e.g. caused by skimmer nozzle fragmentation) as well as artifacts generated from sample preparation (e.g. caused by enzymatic digest) were detected as well, and were treated specifically. Nearly complete coverage of the chromatogram can be obtained leaving no major components of the LC run unexplained.



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Science to Market 2010
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