ISPPP 2007 Lecture Abstracts

L1. Ultratrace LC/MS Analysis Using 10 µm i.d. PLOT Columns

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It is well known that electrospray ionization signals are substantially enhanced when liquid flow rates are reduced from typical nanoflows of 100 - 200 nL/min down to 10 - 20 nL/min. Our group has taken advantage of this behavior by developing porous layer open tubular (PLOT) columns of 10 µm i.d. The open structure of the column allows column lengths of 3 - 4 meters to be used with conventional LC pumping systems. We will demonstrate that such columns,using gradient elution, produce separations with high peak capacity and detection limits down to the subattomole level. Using a multi-valve design, we have produced an on-line, multidimensional trapping/separation system to handle small volume samples while minimizing sample loss. We will demonstrate the power of the approach in the proteomic analysis of roughly 1000 cells. We will conclude with a discussion of future areas of application of this ultranarrow bore column design.

1) Yue, G. Luo, Q., Zhang, J., Wu, S. And Karger B.L.,"Ultratrace LC/MS Proteomic Analysis Using 10 µm i.d. Porous Layer Open Tubular (PLOT) Poly(styrene-divinylbenzene) Capillary Columns", Anal. Chem, 79 (3), 938-946, 2007.

2) Luo, Q., Yue, G., Valaskovic, G., Gu, Y., Wu., S-L. and Karger, B.L. "On-Line 1D and 2D
PLOT/LC-ESI-MS Using 10 µm i.d. Poly(styrene-divinylbenzene) Porous Layer Open Tubular (PLOT) columns for Ultrasensitive Proteomic Analysis", Anal. Chem, ASAP Article; DOI: 10.1021/ac070583w

L2. UPLC Separation of Oligonucleotides: Method Development.

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A column peak capacity model was developed for oligonucleotides and utilized for the prediction of the conditions for a desirable separation. Predictions were compared to experimental data obtained for oligonucleotides (and partially for peptides and proteins). The optimal flow rate in terms of the van-Deemter curve minimum was found to be less than 0.15 mL/min (2.1 mm I.D. columns packed with a porous 1.7 µm C18 sorbent). However, the maximum peak capacity is achieved at flow rates between 0.15 – 1.0 mL/min, depending on the molecular weight of analyte. The rational method development for UPLC analysis of oligonucleotides is discussed in examples, including the impact of column packing particle size, gradient slope, flow rate and other parameters. A case study featuring 15-60mer oligonucleotides is presented. Baseline separation of 15-35mer oligonucleotides was achieved in less than five minutes. Using shallow gradient and carefully optimized initial gradient strength, it was possible to resolve a 30-60mer oligonucleotide ladder within seven minutes.

L3. New Packing Materials for Applications in Analysis of Cell Lysates

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This talk will be focused on the new packing materials we have recently developed for biological separation. From the most comprehensive Sepax bioseparation LC phases, a few of examples will be presented to demonstrate the problem-solving driven research and development of new packing materials, including size exclusion media with most wide range of pore size selection from 100 to 2000 Å, and high capacity and high efficiency non-porous ion-exchange resins that have achieved highest peak capacity for cell lysates. Those new packing materials will address the questions, such as “what are the problems and challenges associated with the current products for biological molecule separation?”, and “Do we really need 1.7 µm or even 1.0 µm ion-exchange resins?”. Do those columns really bring benefits for our research?”

L4. Theoretical and Practical Considerations in the Application of UPLC to the Separation of Peptides

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UltraPerformance LC® (UPLC®) separation techniques have been applied to the analytical problem of high resolution peptide mapping. This technology takes advantage of sub-2µm particles using an instrument optimized for such columns. UPLC improves resolution, speed, and sensitivity for many HPLC methods, and the same improvements are observed for peptide maps. To fully exploit this technique, it has proven useful to incorporate the theory of small particle separations into the experimental development of peptide maps.

The basis of UPLC is described in the van Deemter equation and is explicitly related to reduced diffusion distances. When the principle is applied to the range of molecular properties represented by a peptide digest, multiple physical mechanisms impact the separation and must be considered in somewhat more detail. Peptides exist over a wide range of sizes and chemical properties. Because peptides are larger molecules that diffuse more slowly, the effects of chromatographic operating parameters interact in unexpected ways. Optimizing separations requires, therefore, an understanding of the diffusion-related variables that affect peptide chromatography. This requires explicit development of theoretical models describing the interactive effects of particle size, pore size, linear velocity, and gradient slope. These predictive models can also be applied to the thermal effects within and around the column.

These principles have been tested with a fairly wide range of sample types, including glycopeptides. The separation of authentic, complex digests adds an additional dimension to the optimization framework. The chemical interactions between peptides of various properties and the surface chemistry of the packing are superimposed on the more purely physical models describing UPLC mechanisms.

The combination of these physical and chemical principles can lead to the development of more highly resolving systems for peptide mapping. In practice, this can be exploited to produce maps with greater resolution without regard to run time, maps with shorter runtimes that do not compromise resolution, and very fast maps targeted to specific characteristics of the protein. The use of smaller particles provides a mechanism for

obtaining peptide maps that combine higher resolution and sensitivity in the shortest run times.

L5. Novel capturing method for currently spread influenza viruses from cell cultures by affinity separation

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Influenza remains a major public health concern. Every year several million people are getting infected. Strategies to control influenza outbreaks are mainly focused on prophylactic vaccinations in conjunction with antiviral medications. Hence, every year large amounts of vaccines have to be produced. In the case of pandemic outbreaks these production processes have to be shifted to the appropriate pandemic influenza strain, reducing the production capacity of seasonal vaccines. Human influenza vaccines are traditionally produced in embryonated chicken eggs. This method has only a reduced possibility to be scaled up. Furthermore it potentially causes allergic reactions induced by egg proteins. Hence cell culture based vaccine production has been developed requiring new downstream processing strategies for virus purification.

Studies have shown that Euonymus europaeus lectin (EEL) is a suitable ligand for an affinity capture step to purify different human influenza virus strains derived from Madin Darby canine kidney (MDCK) cell cultures [1] including two topical virus strains (A/Wisconsin/67/2005 (subtype H3N2), B/Malaysia/2506/2004: both from 33016 MDCK PF, Novartis Behring, Marburg, Germany; A/Puerto Rico/8/34 (subtype H1N1)). The lectin EEL was immobilized on polymer beads and binds to terminal alpha-galactose of glycosylated viral envelope proteins such as the hemagglutinin. Characterization of the capturing potential demonstrated very good host cell DNA and protein reduction, while the majority of the virus (around 90 %) could be recovered as product. The method indicated excellent reproducibility between various tested influenza virus strains as well as different cultivation batches. In addition, the impact of the host cells on purification by EEL-affinity chromatography has been evaluated. Therefore, Influenza A/Puerto Rico/8/34 virus was propagated in MDCK and Vero cells and screened for ligand binding.
In a second part of the study different supports have been screened for EEL as ligand. This screening included stabilized reinforced cellulose membranes, polymer and porous glass particles, cellulose and agarose beads. Comparing virus binding abilities and product recoveries some of the tested materials indicated advantages for virus purification. The performance seems to be even superior to cellufine sulfate and heparin,

two alternative affinity matrices currently used for influenza virus purification. Most virus binding was achieved by the cellulose membranes and the polymer based adsorbent. In addition, reinforced cellulose membranes have a far higher binding capacity than other tested adsorbents.
With the tested virus strains our studies have shown that EEL-affinity chromatography is a valuable alternative to capture MDCK cell derived influenza viruses.
Capturing studies will be completed using a reverse genetics derived reassortant H5N1 virus. In addition, kinetic measurements are being currently performed to characterize the virus ligand binding in more detail by surface plasmon resonance technology.

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L6. Biothermodynamic studies of adsorption of monoclonal antibodies

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Ion Exchange Chromatography (IEC) and Hydrophobic Interaction Chromatography (HIC) are interesting techniques for downstream recovery of diagnostic and pharmaceutical proteins. Unfortunately, the design of separations with IEC or HIC is still based on trial and error, because of the large number of process parameters like the type of chromatographic resin, the pH value, and the type and quantity of salts. The present work contributes to overcoming these drawbacks by supplying basic thermodynamic data that give insight in the adsorption process. The adsorption of two different monoclonal antibodies, which were supplied by an industrial partner, was studied with Isothermal Titration Calorimetry (ITC) and by measuring equilibrium adsorption isotherms. Furthermore the antibody solutions were studied with Laser Light Scattering. All above mentioned parameters were systematically varied in these experiments. The ITC data and the adsorption isotherms allow determining the molar heat of adsorption of the antibody ?hads. The results depend on the chromatographic resin, the pH value as well as the type and quantity of salts, but they give a consistent overall picture: ?hads is not constant along the isotherm, the magnitude of ?hads always decreases with increasing loading, showing that mainly energetically less attractive positions remain available at higher loadings, while the most attractive positions are filled first. Furthermore, it was found that the magnitude of ?hads is closely correlated with the binding capacity. As expected, the adsorption on HIC resins is always endothermal, hence, the process is entropy driven. It was shown for the first time that IEC is not always exothermal but can also be endothermal, hence the process can be either enthalpy or entropy driven, depending on the conditions. Furthermore, the antibody-antibody interactions and aggregation were studied by laser light scattering measuring the second osmotic virial coefficient A2 and the average molecular weight of the antibodies. The results are closely correlated with the solubility of the antibodies. In the presentation, the background, experimental procedures and results will be discussed together with qualitative models that give insight in IEC and HIC of antibodies.

L7. Developments in membrane affinity chromatography for monoclonal antibody recovery

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The great number of process development for monoclonal antibodies, presently in development stage, has emphasized the capability limits of the biotech industry. The recent improvements of fermentation technology, allow also to achieve high titers of monoclonal antibody in the supernatant production, and the present bottleneck for MABs's production is associated to the downstream process required for the pure product recovery.
Bead-based affinity chromatography with Protein A is widely used in the primary capture stage. Membrane affinity chromatography has not yet experienced extensive application due to the lower capacity of membrane supports compared to chromatographic beads, yet it has several advantages deserving serious attention.
This work is focused on the purification of Immunoglobulin G (IgG) with affinity membranes. A new Protein A affinity membranes (Sartorius, Göettingen, Germany), as well as affinity membranes prepared with synthetic ligands have been characterized in detail in batch and dynamic experiments.
The membranes have been analysed by using pure solutions of polyclonal IgG, to determine their binding capacity, as well as a cell supernatant containing monoclonal IgG, to investigate their selectivity and general behavior. The influence of process conditions like flow rate and feed concentration on adsorption and elution have been studied to obtain indications for the optimal process conditions.
The affinity membrane purification process was also simulated with a mathematical model which was validated by using the experimental data obtained. The model can simulate adsorption, washing and elution steps by taking into account all the relevant transport phenomena.

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L8. New Glycomic and Glycoproteomic Tools and Methods for a Better Understanding of Human Diseases

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Biomolecular mass spectrometry (MS) has revolutionized the ways in which we study proteins in their most structural details. This includes posttranslational modifications, such as extensive glycosylation of certain asparagine, serine and threonine residues within the protein backbone. While glycoproteins are actively involved in a great number of biological functions, they have not been studied to appropriate extent because of the unique and difficult analytical problems. Coupling MS with a number of capillary separation techniques and sample derivation at microscale further enhance the scope of important structural information such as determination of sequences, sites of glycosylation, branching, and different linkage forms. The current trends in biology and medicines now endorse a yet more difficult analytical task: high-sensitivity structural determinations in complex protein mixtures such as tissue extracts and physiological fluids. This, in turn, mandates the development of powerful bioanalytical platforms for glycomic and glycoproteomic measurements and their bioinformatic counterparts for data evaluation and reduction. While quantitative glycomic profiling and appropriate chemometric evaluation show considerable promise toward diagnostic and prognostic utilization of these new analytical methodologies, the glycoproteomic platforms employing lectin chromatography and other enrichment steps together with routine quantitative LC-tandem MS could yield the ultimate information in discovery of disease-related biomarkers.

L9. Analytical and biological implications of dynamics of protein abundances, molecular isoforms and localizations

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The search for surrogate protein biomarkers from complex biological samples requires meticulous considerations regarding the choice of appropriate experimental strategies. Next to analytical challenges by the huge dynamic range, the sheer number and the chemical diversity of proteins, the biological dynamics of protein isoform distribution, localisation and life time have a major impact on experimental design. Usually posttranslational modifications and conformational changes which on the biological level provide a maximum of flexibility using a given reading frame are responsible for pitfalls and shortcomings on the analytical side.

Here we present and analyze some examples of successful identification and characterization of protein biomarkers, and show the prerequisites and necessary considerations while moving protein candidates from purely descriptive phenomena to a stage of validated surrogate biomarkers. This includes discussing requirements of resolution and statistics of differential quantification regarding initial separation techniques, but in particular addresses strategies imposed by proteins which dynamically change localisation from different intracellular compartments to extracellular forms. As an example the analytics of a single biomarker, annexin A3 in prostatic disease will be treated, which exists in a diversity of membrane-bound, cytosolic and extracellular forms, which each have distinct biological significance.

L10. Automated Metal-free Nanoscale HPLC System for Phosphoproteomic Analysis

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We have developed a series of high-efficiency LC methods that have been successfully used for proteomic analysis with an average protein identification rate by MS/MS that exceeded 10 protein/min., but the use of those systems for phosphopeptide identification was problematic due to the formation of phosphopeptide-metal ion complexes at all metal surfaces during LC/ES-MS.

Here we demonstrate an automated metal-free nanoscale RPLC system based-on our previous methods for phosphopeptide studies. Columns from 50um id x 30cm to 30um id x 50cm packed with 5 and 3-um porous (300-A surface pore size) silica-based C18 were used for LC operations at 1500-5000 psi. Initially integrated ESI tips were constructed with a laser puller after packing the column. Later a more robust etched tip coupled with New Objective Picoclear union was used on this system. A solid phase extraction (SPE) column (150 um id x 4cm, packed with 5 um C18 porous particles) with in-line frits was used for concentrated sample and on-line transferring of the loaded sample to the analytical nanoscale LC column.

A Shewanella oneidensis tryptic digest was used to evaluate the 50 um id x 30cm column system (1500 psi). A peak capacity of 400 was obtained and >4000 different peptides were identified using MS/MS identification on a linear ion trap (Finnegan LTQ) for 50 ng of the test sample in a singe 6-hour analysis. The linear dynamic range for proteome abundance was from 1 ng to 1000 ng measured using a conventional ion trap mass spectrometer (Finnigan LCQ). The system provided a limit of detection of 0.8 pg for phosphorylated peptides.

Applying this system for global analysis of the COS-7 cell line from African Green monkeys, a single LC-LTQ MS/MS analysis led to the identification of 4604 phosphopeptide candidates from ~60 ?g of the whole cell digests followed by IMAC enrichment. A targeted phosphoproteome analysis of ~2 mg of human T cells digest that was enriched by phosphotyrosine peptide immunoprecipitation identified 525 tyrosine phosphorylated peptides.

L11. Innovative Mass Spectrometry Technology for the Study of Cell Signaling

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This lecture will focus on three different aspects of cell signaling and the mass spectrometry technologies developed to study them. Presented first will be results of studies to elucidate the "histone code", complex patterns of post-translational modifications on histone proteins that regulate gene expression, gene silencing, DNA damage repair, stem cell differentiation, and that block reprogramming of a somatic cell nucleus by an enucleated oocyte. The same modifications also facilitate heritable changes in phenotype that do not involve mutation of DNA (epigenetics). To study these post-translational modifications, we constructed the prototype for the commercially available, tandem linear ion trap/ FTMS instrument and also developed methodology that makes it possible to analyze intact proteins on a chromatographic times scale (1 protein/2-5 sec). Proteins are converted to gas-phase, positive ions by electrospray ionization and then allowed to react with fluoranthene radical anions. Electron transfer to the multiply charged protein promotes random fragmentation of amide bonds along the protein backbone. Multiply charged fragment ions are then de-protonated in a second ion/ion reaction with the carboxylate anion of benzoic acid. The m/z values for the resulting singly, doubly, and triply charged ions are used to read a sequence of 15-60 amino acids at both the N and C termini of the protein. This information, along with the measured mass of the intact protein, is used to identify unknown proteins, to confirm the amino acid sequence of a known protein, to detect post-translational modifications, and to determine the presence of possible splice variants.

Part two of the presentation will describe results of studies to define sites of phosphorylation that regulate the formation of focal adhesions involved in cell migration. For this work we employ immobilized metal affinity chromatography (IMAC) to identify phosphorylaton sites present at levels as low as 0.1% of the parent protein. Stable isotopes are employed to follow changes in site usage as a function of cellular perturbation. Further information on this topic is available at cellmigration.org (site guide, phosphoproteomics).

Part three of the presentation will focus on signaling between cancer cells and cytotoxic killer cells by the class I, antigen-processing pathway. Since signal transduction pathways in cancer cells are highly dysregulated, we hypothesized that this might manifest itself in the presentation of unique phosphopeptides to the immune system in association with Class I MHC molecules. By using a combination of IMAC, stable isotope labeling, and nano-flow HPLC-tandem mass spectrometry, we are now able to detect cancer specific phosphopeptides present at levels as low as 1-5 copies per/cell.

Results of studies to use these phosphopeptides as immunotherapeutics and/or vaccines against cancer will be discussed.

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Analysis of Intact Proteins on a Chromatographic Time Scale by Electron Transfer Dissociation Mass Spectrometry, Chi, A, Bai, DL, Geer, LY, Shabanowitz, J, and Hunt, DF, Int. J. Mass Spectrom. 2007, 259, 197-203.

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L12. Rapid identification of protein variants

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Protein variants and isoforms result from genetic modifications (e.g. multiple gene copies, alternative splicing, mutation, truncation, degradation) or from chemical or biological, pre-, co- or post-translational modifications of the expressed proteins. The availability of new procedures that allow the rapid, comprehensive, high-throughput identification of such variants and isoforms would enhance the understanding and identification of the structure and function of protein variations and their role in biological systems as well as advance the biomedical diagnostics of genetic diseases associated with protein variants. Existing methods to investigate protein variants complement two-dimensional gel electrophoresis with multi-dimensional liquid chromatography, whereby the proteolysis of several hundred proteins is performed before chromatographic separation, whereby the correct assignment of peptides containing a particular modification to their parental protein variants/isoforms may be extremely difficult and sometimes impossible.

We have developed methods that maintain the information linkage between a particular protein variant or isoform and the associated structural changes of the peptide fragment during the entire analytical procedure. It consists of an on-target proteolysis after either "separation free" sample preparation or after capillary liquid chromatographic separation and micro-deposition of intact protein variants and subsequent MALDI TOF MS analysis. Workflows are presented with examples from procedures developed on more than 15 haemoglobin variants, including haemoglobin C, E, and S of EDTA-treated human blood. Fast (< 3 min) surfactant-aided on-target proteolytic digestions generate peptide mass fingerprints with very high sequence coverage and allow the rapid identification of amino acid sequence aberrations with MALDI TOF MS. Green Chemistry principles apply due to the replacement of neurotoxic polyacrylamide-based gel electrophoresis with capillary-based separation techniques, the use of small amounts (< 5 mL/sample) of solvents, the choice of degradable surfactants and sample-economic on-probe enzymatic digestions of proteins which can be delivered as very small volumes of biofluids (= 200 nL).

These new techniques are expected to significantly contribute to the development of fast and efficient methods in biomedical diagnostics in which protein variant identification is crucial for disease identification and may find applications in the field of Process Analytical Technologies (PATs) for the real-time monitoring of recombinantly produced therapeutics.

L13. Monolithic columns for bioseparations: Present state-of-the-art and future trends

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After briefly summarizing the history of monolithic technologies in general, the lecture will focus on developments including (i) thin discs for rapid separations of proteins, (ii) analytical size columns for the "classical" HPLC, and (iii) capillary columns for both micro and nanoHPLC. The future trends including capillary columns with grafted chemistries for nanoHPLC, separations in microfluidic chips, and a variety of other applications both demonstrated and envisioned will also be presented.

L14. Macroporous Polymeric Monoliths by Reactive Gelation for Protein Purification

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The use of macroporous polymer supports for the chromatographic separation of biomolecules is old. Such supports are typically synthesized by suspension polymerization in presence of porogenic agents. Though remarkably simple, this process is not conceptually fully understood, so that its control remains difficult. The reactive gelation approach presented here mimics the mechanism of pore formation of suspension polymerization by a sequence of independent steps. Namely, crosslinked particles are first produced by emulsion polymerization and then gelated in controlled way, which determines size and type of porosity of the final polymer. The obtained gel is then re-polymerized to impart enough mechanical resistance to the material, so to obtain a porous polymeric monolith. A comprehensive analysis of the impact of the different parameters in the gelation and re-polymerization steps upon the properties of the final polymer is carried out, using different polymeric supports.
Monolithic supports prepared by this technique have some key advantages upon conventional polymeric macroporous supports. First, the formation of the monolith is involving the polymerization of a small fraction of the total polymer only and it carried out in water as continuous medium, so that heat removal is much more efficient and large monoliths for industrial applications can be obtained. Second, the monolith formation as a sequence of independent steps allows a precise control of the porosity. In particular, monoliths with large permeability and no size exclusion can be easily obtained. Finally, the use of emulsion particles as starting building block allows a precise and economic surface functionalization of the support.
Some chromatographic applications are presented where these features are exemplified. In particular, two kind of monolithic supports are discussed. First, hydrophobic (non-functionalized) supports, both made of crosslinked styrene and methyl methacrylate, have been synthesized. These are applied for the purification of peptides by reversed phase chromatography and for the purification of proteins by hydrophobic interaction chromatography. A second set of functionalized supports is presented. In the first one, the surface has been covered with poly-acrylic acid, so to carry out protein purification by weak cation exchange chromatography. In the second type, a more complex surface functionalization with controlled radical polymerization techniques (ATRP) in the presence of thermo-responsive polymers (PNIPAAM) is carried out. This will serve as example of “clean” chromatographic purification for biomolecules based on temperature changes only.

L15. Purification of large plasmids on ion-exchange monolithic columns

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In recent years plasmids are intensively investigated for gene therapy purposes and genetic vaccination. The rapid evolution of gene therapy and DNA vaccines results in an increasing interest in producing large quantities of pharmaceutical grade plasmid DNA. Most current clinical trials involve plasmids of 10 kb or smaller, however future requirements for multigene vectors including extensive control regions may require the production of larger plasmids e.g. 20 kb and bigger [1].
Purified plasmid has to be predominantly in its intact, supercoiled conformation and might degrade due to its size during the purification process [2]. The rate of degradation increases with plasmid size and is also dependent on ionic strength of the plasmid environment [3].
Downstream processing of plasmid DNA usually comprises several chromatographic steps using different types of interactions. Since polynucleotides are negatively charged over a wide pH range anion-exchange chromatography seems to be the most suitable chromatographic method for purification of plasmid DNA. However, choice of chromatographic matrix is not so straightforward, since most of chromatographic resins used for biotechnological processes are optimized for purification of much smaller molecules, such as proteins, and therefore have very poor capacities for bigger molecules as plasmid DNA.
CIM anion-exchange monolithic columns have already been successfully used for the industrial scale purification of pharmaceutical grade small plasmid DNA [2,4]. Furthermore, preliminary tests showed that monolithic structure is also very convenient for the purification of very large plasmids i.e. even up to 93 kb [5].
The objective of our work was to investigate possibility to purify plasmids of sizes from 21 to 93 kb using CIM monolithic columns. Because of possible mechanical cleavage of plasmids during processing we studied the degradation of plasmid molecules under nonbinding conditions at various flow rates using monolithic columns with different pore size. Effect of adsorption was tested using different mobile phases and surface ligand densities of the monolithic columns. Furthermore, due to high plasmid concentration during the elution permeability decrease and potential clogging of the column was examined. Finally, we investigated the impact of contact time and mobile phase ionic strength on the capacity and recovery of supercoiled plasmid DNA of different sizes.

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L16. Separation of viruses by monolithic columns

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For large-scale separation of viruses ultrafiltration, precipitation with polymers preferably PEG and density gradient separation methods are employed. These methods are well established and meet the requirements for manufacturing of vaccines. Although the methods were not very selective sufficient amounts of active virus can be produced. Purity of such a virus preparations is not very high, but often not requested. Currently therapeutic applications of viruses go beyond vaccination. Viruses are also used as therapeutic agents for cancer treatment in case of onco-viruses and as delivery vehicles for gene therapy. Moreover viruses are produced by mammalian cell culture system and thus the level of purity-requirements has been elevated. It is possible to obtain highly purified viruses by conventional methods, but low recovery has to be taken into account. Chromatography is an alternative method to purify viruses. Chromatographic methods have been described in the past, but they have not been applied in large-scale separation. Chromatography media have been designed for separation of proteins. Thus the average pore size is smaller than 100 nm. Viruses cannot exploit the whole surface area of such chromatography media, even, if they have a pore size of 300 nm. Still hindered transport into the pores occurs. Monoliths are continuous stationary phases cast as a homogenous column in a single piece prepared in various dimensions with agglomeration-type or fibrous microstructures. They are considered as the latest type of stationary phases for separation of biomolecules of all kind. Monoliths became very popular for the separation of biopolymers such proteins, peptides, oligonucleotides, pDNA, gDNA and viruses. Monoliths with different surface functionalities can be stacked into a single column. Thus multidimensional chromatography is possible without column switching. Monoliths with macropores do not show mass transfer resistancy. Peak dispersion is dominated by hydrodynamic contributions. Separation of large proteins, pDNA and viruses will be shown as prime examples for application of monoliths in bioseparation. To complete the whole picture of the possibilities of monoliths, scale up scenarios will be described and the future of separation viruses will be discussed. Chromatographic purification processes of virus of different sizes and shapes will be described.

L17. High Speed Separation of Peptides Using Columns of "Fused-Core" Particles

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Columns of unique "fused-core" particles are useful for the very fast reversed-phase separation of complex peptide mixtures. These silica-based particles are 2.7-µm in overall diameter with a 1.7-µm solid core and a 0.5-µm-thick shell of ~12 nm pores. The thin outer porous shell has superior mass transfer (kinetic) properties as a result of the shorter diffusion distance required for interaction with the stationary phase, relative to that for conventional totally porous particles. As a result, higher mobile phase velocities can be used to achieve short analysis times with minimal loss in column efficiency and separation resolution. Gradient elution separation of peptides with short columns provides unusually high peak capacities (in excess of 400) because of the unusual efficiency of the "fused-core" particles. The unusually high efficiency of "fused-core" particle columns is a result of the extremely narrow particle size distribution (~ 5% one sigma) and the higher particle density, compared to conventional totally porous particles. The very high silica purity of the "fused-core" particles results in superior peak shape and high yields for compounds such as peptides and related structures. Columns of these new "fused-core" particles appear well suited for the rapid, high-resolution separation of complex peptide mixtures and other complex samples of small organic compounds.

L18. Comparison of the Performance of some Modern HPLC Columns in the Gradient Elution of a few Protein Digests

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For the last few years, there has been a rapid evolution in column technology. Monolithic columns, developed in the 1990s, were commercialized after 2000. They have very attractive properties and have become popular. Yet, few monolithic columns made of either silica or polymers are commercially available and can provide results that are reproducible from one laboratory to another. Columns packed with many different fine particles, in the 1 to 3 μm range, are now commercially available, together with the instruments needed to operate these at high flow velocities. In principle, the use of fine particles allows a considerable improvement in column performance, with a tunable balance between the gains made in analysis time and in column efficiency. Finally, there is renewed interest in the shell particle concept, these particles being made of a solid core surrounded with a layer of porous adsorbent. A new brand exhibits impressive properties. Since there is much competition between these different approaches, we can expect further progress in the years ahead.

Experimental data on the mass transfer kinetics of a few selected peptides (e.g., Bradykinin) on a series of columns packed with some of these new materials will be reported. Together with the separation of the trypsine digests of myoglobin, bovine serum albumin and a few other proteins under gradient elution conditions these data permit a comparison between the performance of these columns for the separation of complex peptide mixtures.

L19. Efficient Purification and Trace Impurity Analysis of Crude Synthetic Peptides Using Reversed-Phase Displacement Chromatography and New RP Displacers

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New displacer molecules have been designed for the purification of crude synthetic peptides (50 to 75% initial purity) by reversed-phase displacement chromatography (RPDC). In one displacement-chromatographic step operated at high column loadings (60 % of peptide breakthrough), high purities (96-99%), and high recoveries (80-90%) are routinely obtained starting from the crude peptide. The new displacers produce excellent displacement trains on most reversed-phase matrices, and only low levels (0-10%) of organic solvents are needed during a displacement experiment which operates without the use of gradients. In addition to the recovery of the purified peptide, most impurity components are greatly enriched and displaced before or after the main product band. These effects of the RPDC experiment allow for detection and measurement of many low-level impurities not detected by other analytical methods. The purification of crude Angiotensin-I yielded the desired peptide with 99.3% purity at 89% recovery while a total of 85 impurity peptides were also detected. Using the same RP column, an analytical HPLC run of the same crude Angiotensin-I showed only 35 impurities. Similar data will be presented for crude a-Melanocyte Stimulating Hormone (a-MSH). A variety of pure displacer molecules with a range of affinities for stationary phases allow for easy optimization of the chromatographic protocols. The new displacer molecules possess chromophores for convenient monitoring. Straightforward cleaning methods and column regeneration protocols allow for complete removal the displacers and preparation of the RP columns for subsequent reuse.

L20. Novel Separation and Quantification Strategies for the Characterization of Glycopeptides from Complex Biological Mixtures

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The glycomics field is currently focused on the structural characterization of released glycans. Typically, glycans are released from glycoproteins then analyzed by MS either in their native state or following chemical modification. However, this procedure results in a complete loss of information regarding the glycoproteins from which the glycans originated. To analyze glycoprotein expression, lectin affinity chromatography (LAC) has been employed to separate the glycopeptides from the non glycosylated species, thus simplifying further analyses by MS/MS. Unfortunately; the specificity of the lectin however, does not facilitate the global isolation of glycoproteins or glycopeptides with a diverse population of glycan structures.

The goal of this work was to develop methodologies to identify the glycan compositions and glycosylation sites on glycoproteins from complex mixtures. This glycoproteomic approach facilitates characterization of the intact glycopeptides, rather than analyzing the glycans and peptides separately which would result in the loss of information on the glycan structures present at each glycoprotein. The first step in this approach was the development of a normal phase chromatography (NPLC) based glycopeptide enrichment procedure. NPLC is routinely used for glycan separations and here we show that glycopeptides can be effectively separated from their non-glycosylated counterparts using this approach. Deglycosylation of the enriched glycopeptides following by RPLC-MS/MS analysis allowed the identification of 70 unique glycopeptides mapping to 37 unique glycoproteins at a 1% false discovery rate. The serum glycoprotein identifications in these experiments have greater than a four order of magnitude range in concentration, thus indicating the NPLC method is highly effective at enriching low abundance glycopeptides.

L21. 2-D Liquid Separations, Microarrays and Microproteomics for Mapping Changes in Disease States

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The use of a 2-D liquid separations technique for mapping the protein content of cells and bio-fluids will be described. This involves fractionation of intact proteins using chromatofocusing as a pI based separation in the first dimension followed by nonporous RP-HPLC. This method can produce a protein map from human cells where nearly 2500 bands can be observed. On-line ESI-TOF MS can be used to measure the molecular weight value of the intact proteins and protein collection with subsequent tryptic digestion and MALDI-MS/MS can be used to identify the bands. A natural protein microarray approach can be used to search for posttranslational modifications. The collected protein bands are spotted onto nitrocellulose coated glass slides to produce a microarray which can be hybridized against PTM indicator dyes or antibodies. A phospho-specific dye can be used to locate changes in phosphorylation and LC-ESI-MS/MS can be used to locate phospho-sites for these selected proteins. Alternatively, a lectin column can be used to pre-concentrate glycoproteins which can be detected by a glyco-dye or by lectin fluorescent probes. Different lectins can be used to probe different glycan structures in the proteins. These methods can be applied to tissues or plasma/serum samples and applications to various types of cancers and oxidative damage in diabetes and radiation damage will be discussed. Applications to urine will also be presented where strategies for pre-concentrating low levels of proteins using a lectin column will be described. Ultimately, recent attempts to produce a micro-proteomics version of these methods will be shown. The use of micro-proteomics for analysis of small amounts of tissue <10 ug in applications to tumor stem cells and mouse tumors will be presented.

L22. A Critical Comparison of the Peak Capacity and Information Content of One-Dimensional and Two-Dimensional Liquid Chromatography

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Two-dimensional liquid chromatography (2DLC) has become a mainstay of proteomics research due to its higher peak capacity compared to one-dimensional LC (1DLC). Historically this type of work has been associated with very long analysis times, often several hours to tens of hours per analysis. Because of the long analysis times and its use primarily in proteomics applications, 2DLC in the context of HPLC in general has been regarded as a niche technique compared to the far more common isocratic and gradient elution 1DLC. A logical next step in the development of 2DLC is to consider using its higher resolving power to reduce the analysis time of rather "simpler" mixtures, in the range of tens to hundreds of chemical constituents. It is intuitively obvious that at very short analysis times, the resolving power of 1DLC separations will be superior to 2DLC separations because of a variety of factors, the most serious being the deleterious effect of undersampling peaks as they elute from the first dimension column. The objective of this presentation is to provide guidance to practitioners that need to decide whether a 1D or 2DLC method will provide a superior separation in a given analysis time.
Peak capacities are predicted for optimized 1D and 2DLC separations of a low molecular weight extract of corn seed at 15, 30, and 60 minute analysis times using a model based on the chromatographic properties of real representative compounds. Corrections to the ideal peak capacity of 2DLC separations are made to account for incomplete usage of the separation space and the serious effect of first dimension undersampling; this allows, we believe for the first time, a fair comparison of the resolving power of 1D and 2DLC. Then, the predicted optimum conditions are used to carry out experimental separations of the low molecular weight corn seed extract, and the peaks are counted in each 1D and 2DLC chromatogram. Based on comparisons of both the predicted peak capacities and number of peaks observed in experimental chromatograms, we conclude that 2DLC becomes superior to fully optimized gradient 1DLC for separations lasting more than about fifteen minutes. This transition time is much shorter than expected, and we believe it will have a major impact on the role of 2DLC in liquid phase separations in general.

L23. Process Analytical Technology: Two-Dimensional Chromatography on-Line with Mass Spectrometry (2D-LC/MS) for in-Process Analysis of a Recombinant Protein Concentration and Glycosylation

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The physicochemical characterization of recombinant protein biopharmaceuticals plays a critical role not only for product release but also during the biopharmaceutical process development. The integration of different analytical methodologies enables identification and characterization of complex biologics in order to meet requirements of Food and Drug Administration (FDA) for a consistent drug production process and product quality. One of the challenges for Process Analytical Technology (PAT) in biopharmaceutical industry is the ability not only to assess manufacturing process parameters, but also to monitor critical quality attributes of a biomolecule at different stages of the process. Frequently, accurate real time information on quantity and quality of in-process intermediates is essential for process efficiency and yield, and quality of the final product. However, identification of different sources of a recombinant protein heterogeneity in complex sample matrices in a timely and quantitative manner remains a significant challenge. In this presentation, a novel approach to quantitative and qualitative assessment of a recombinant glycoprotein in conditioned media samples is described. This rapid and automated analytical method involves 2-dimensional liquid chromatography on line with UV and mass spectrometric (MS) detection. This approach provides important information enabling timely and efficient control and optimization of protein production process. The method employs ion exchange chromatography followed by rpHPLC with UV detection providing quantitative information. On-line electrospray ionization time of flight mass spectrometer (ESI-ToF-MS) provides critical information on protein glycosylation. In this presentation, the method development is discussed and it's application as a PAT tool is demonstrated.

L24. Fractionation and Characterization of Crude Protein Mixtures for Accelerating Bioseparation Process Design

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The development of numerous biomolecules for pharmaceutical application requires rapid development of their downstream processes. In order to speed-up bioseparation process development, an integrated approach is proposed in which process sequence is generated based on the rigorous thermodynamic modeling and high-throughput experimentation (HTE) [1]. HTE provides the necessary physicochemical and thermodynamic properties of the components present in a crude protein mixture. In this study, a novel two-dimensional (2-D) liquid chromatography (LC) system was developed for the fractionation and characterization of crude protein mixtures, in which a pH-gradient anion-exchange chromatography (AEC) was followed by a salt-gradient hydrophobic-interaction chromatography (HIC).
In pH-gradient AEC, acidic (pI >8) and basic (pI <6) proteins eluted roughly at their pI, but neutral proteins (pI 6-8) eluted at pH much remarkably higher than their pI. Because of the flat nature of protein titration curves from pH ~6 to ~9, neutral proteins indeed exhibit nearly zero net charge at pH ~9. Therefore, the elution-pH in pH-gradient IEC or the titration curve, but not the pI, is the parameter of interest in the design and optimization of an IEC process in a fast and rational way. When pH-gradient AEC was applied for fractionation of an E. coli lysate, more than 40 well distinguished peaks were identified in a single chromatogram, representing the major components present in the crude mixture. Therefore, the pH-gradient ion-exchange chromatography (IEC) was found to be an excellent analytical tool not only for the fractionation of crude protein mixtures but also for design and optimization of IEC [2].
Fractions obtained by pH-gradient AEC were further fractionated by salt-gradient HIC, in which proteins eluted according their hydrophobicity in the second dimension. This 2-D-LC fractionation system provided a complete profile of the major components present in a crude protein mixture and made them available in pure form for further analytics. Fractionated proteins were then identified by peptide mass fingerprinting. Once the identities of major components were known, their molecular mass and titration curve were obtained from databases. In addition, the elution-pH and the hydrophobicity of the components were readily available from the 2-D-LC fractionation system, which are essential parameters for fast development of the their downstream processes.

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L25. ISPPP 2007 Lifetime achievement award address

In Search of Separation Excellence: The Future Role of HPLC in Modern Life Sciences

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There is hardly any doubt that HPLC has played its role during the last 25 plus years in the development of life sciences. This series of symposia is a living proof of this fact. It has probably enabled more viable contributions to life sciences than any other field. However, considering the scope of unsolved questions and unmet needs in academia, industry and society the question comes to mind ; " Has HPLC released all its power and potentials yet , or is there more, maybe much more to exploit in the quest for better information, identification and isolation ? "
In this contribution we would like to critically address the achievements in the past, the challenges at present and discuss some maybe underutilized potentials of HPLC in life sciences.

The late A.V. Kiselev, Moscow, Russia, being one of the pioneers of column liquid chromatography, already recognized during the sixties the molecular recognition power of LC via the analyte retention coefficients to elucidate structural information of the separated species. This research direction, however, was never properly exploited later due to the success of the hyphenation of LC with powerful detection tools such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. The rapid development of MS was the main driver for the expansion of LC into the life sciences, particular in the OMICS approaches.

One should not forget that the quality and reliability of the structural and functional data obtained by LC/MS and LC/NMR, respectively. is highly dependent on the selectivity and reproducibility of the columns and on the validation of the systems employed. Furthermore the statistical approaches as well as bioinformatics play a significant role to elucidate solid data and information.

Up to now LC applied as multidimensional LC/MS (MS) has not yet fully mastered the current problems in proteomics such as structural complexity, abundance, high throughput, automation , scale up and process monitoring.

In conclusion we are still lacking a proper set or mix of high resolution platforms from analytical to prep and process scale to be applied to life sciences and to be utilized to understand transport mechanisms and speed in living organisms as well as separation, identification and purification mechanisms in organs such as liver and kidney.

L26. Towards Prediction of the Dynamic Binding Capacity of Proteins

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The dynamic binding capacity (DBC) is widely used as a measure of the effectiveness of stationary phases for preparative chromatography of proteins. Although it represents a straightforward concept, the DBC is mechanistically quite complex in that it captures the collective effects of the static binding capacity and transport processes (especially intraparticle). Transport effects are the most problematic in that different intraparticle transport mechanisms have been identified for different systems and under different conditions. Therefore, although efforts have been made to correlate the DBC with protein and stationary-phase properties, fully predictive models have been elusive. This presentation will discuss efforts to develop an approach to predicting the DBC using a minimal amount of experimental information to describe the stationary-phase structure and protein adsorption on it. The approach is based on independent modeling of the protein adsorption isotherm and of intraparticle transport, and their combination in a standard model of protein retention. The isotherm modeling is based on relations reflecting protein-surface and protein-protein interactions, and allows prediction of isotherms at different salt concentrations based on isocratic retention data, isotherms on an arbitrary adsorbent, and stationary-phase structural information. Intraparticle transport is modeled as a parallel transport model in which the relative contributions vary according to a "hopping" model related also to isocratic retention data. The different components of the overall model are discussed and predicted breakthrough fronts are compared with experimental ones.

L27. Investigation of protein – salt – interactions: Impact on dynamic binding capacity in chromatography with human monoclonal antibodies and their stability

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Downstream processing of diagnostic and pharmaceutical proteins often is the most cost-intensive step in their production. Therefore, the widely used separation steps cation-exchange chromatography (CEX) and hydrophobic interaction chromatography (HIC) have to be performed at maximal dynamic binding capacity (DBC) to save time and resources. By the large number of process parameters like pH, type and molarity of salt, the screening of conditions for maximal DBC by trial and error approach is long-winded. The aim of these studies is to optimize the determination time and - quality of conditions for maximal DBC in CEX and HIC with human monoclonal antibodies, one step to a generic strategy for the fast development of purification processes. Furthermore protein stabilizing conditions should be found by investigation of protein – salt – interactions to minimize product loss, e.g. aggregation, during purification processes.
The investigation of protein – salt – interactions was performed via zeta potential measurements. The zeta potential of a protein in solution is the potential existing on the moving particle. It depends on buffer salt, buffer quantity, pH value and the protein itself. The measurement is based on laser Doppler velocimetry. For the determination of the optimal process parameters for CEX and HIC the zeta potential of human monoclonal antibodies was measured in different buffer systems and different pH values. The isoionic points of zeta potential titration curves were taken as characteristic points to describe protein – salt – interactions. The isoionic points differ from the isoelectric points caused by the involvement of the current buffer conditions. In CEX the zeta potential data allow the determination of the conditions for maximum DBC by simple calculation. In HIC DBC depends on the salt concentration, salt type and pH value. The choice of the salt type for maximum DBC can be made out of zeta potential data. Additionally the investigation of protein – salt – interactions could throw some light on the fact, that the use of salt compositions allows higher total salt concentration without protein precipitation. This phenomenon can be used to achieve much higher DBC in HIC than in single salt applications. Furthermore specific protein stabilizing conditions could be detected by the investigation of protein – salt – interactions via zeta potential measurements based on the DLVO-theory.

L28. **Mixed-matrix membrane adsorber technology for the separation of therapeutic proteins.**

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"Time to market" is for the biotechnology industry an important milestone. A crucial part of the biotechnology processes is the development of purification procedures for therapeutic proteins (biologicals). Therefore, new and more sophisticated techniques are needed in relation to current chromatographic separation methods such as the conventional chromatographic resins, precipitation methods, the more sophisticated membrane adsorbers or other protein A mimics. These abstract deals with a new matrix technology for the capturing of therapeutic proteins.
The Mosaic Systems technology embeds resin particles in polymeric fibres that as such function as a matrix. The resin particles will keep full functionality so that proteins are able to bind in a similar way as observed with the chromatographic resins alone. The benefits of this technique are focused on the use of smaller beads, increasing overall binding capacity and flow rate without large increases in pressure in relation to the other separation methods. In this way it is achievable to scale down the column size for large scale production.
Results of therapeutic protein purifications will be presented by both static and dynamic studies.

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L29. Biomarker Discovery in Body Fluids by LC-MS

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Aim: Most diseases manifest themselves by more or less severe changes in human physiology. This forms the basis for clinical chemistry and its value in helping to diagnose disease correctly and in following therapeutic interventions. Presently, many biochemical and cellular parameters are routinely measured in plasma, serum or urine and the results of these measurements support decision making by clinicians. Here we present data on the comparative analysis of serum, urine or cerebrospinal fluid (CSF) in an effort to find discriminatory peptide or protein biomarkers.
Methods: Methods based on LC-MS were developed that combine sample preparation with separation and mass spectrometry. Focused on serum (cervical cancer) and urine analysis (proteinuria), we will highlight the workflow from sample preparation and separation to mass spectrometry, data processing and statistical analysis. New approaches like chipLC-MS will be described.
Results: To analyze serum samples from cervical cancer patients at various levels of disease, we developed sample preparation procedures based on the depletion of multiple high-abundance proteins followed by tryptic digestion and reversed-phase LC-MS (1,2). Optionally glycoproteins were enriched, deglycosylated and analyzed by LC-MS following tryptic digestion. Recently we have scaled this method down by a factor of appr. 30-fold and tested the robustness of a newly introduced microfluidics chipLC-MS system for comparative serum analysis (3). Urine samples from healthy and proteinuric patients were analyzed by another validated reversed-phase LC-MS method followed by supervised classification, statistical validation and non-supervised multivariate statistical analysis (4). Finally, we will report initial data from the analysis of CSF from multiple sclerosis patients.
Conclusions: LC-MS is a powerful, although somewhat time-consuming, analytical method for comparative biomarker profiling. LC-MS of complex samples generates datasets that require careful data processing and statistical analysis. Novel approaches based on time alignment, peak picking and matching as well as supervised classification in combination with non-supervised multivariate statistics show that discriminatory markers can be selected.

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L30. Quantitative proteomic analysis of influenza A virus infected mammalian cells: Elucidation of virus / host cell interactions with respect to the vaccine production process

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Influenza viruses, major agents of respiratory diseases, are responsible for epidemics resulting in high mortality and morbidity every year. Mammalian cell culture processes are in increasing demand for influenza vaccine production because they enable faster upscaling and higher yields of high quality vaccines compared to classical production in chicken eggs. Therefore, a better understanding of the process is necessary. A deeper insight of virus/host interactions on a cellular level may help to explain the differences of viral strains, concerning virulence and pathogenesis and might help to optimize virus yields and antigen quality in upstream processing.
The aim of this work was the characterization of host cell protein expression changes, comparing Madine Darby canine kidney cells (MDCK) and human epithelial lung carcinoma cells (A549) as response of the infection with human influenza A/PR/8/34 (H1N1). Host cell proteins from different post infection time points were analyzed using two-dimensional differential gel electrophoresis (2D-DIGE). Differences in relative protein expression were quantified, in order to trace time-dependent changes of the cellular proteom composition. Differentially expressed proteins, were digested enzymatically and identified via online coupling of nanoHPLC to tandem mass spectrometry (nanoHPLC-MS/MS) utilizing the MASCOT protein identification system. This strategy enables monitoring of the host cell response to viral infection on protein level.
The identified regulated Proteins, are reported to be involved in a wide spectrum of cellular functions and host defense mechanisms including: e.g. apoptosis induction, cytoskeletal rearrangement or protein synthesis and degradation. Distinct proteome patterns with differentially regulated proteins over time showed dynamic host cell response mechanisms in early and late phase of infection. Notably, our data indicate that influenza A virus induces different host defense mechanisms comparing human and canine cells. Surprisingly, significant differences in host cell response were observed comparing results of the same virus strain (H1N1) from different suppliers (RKI, Germany; NIBSC, UK).

L31.Proteomic investigation of some plasma-derived therapeutic proteins: How well characterized are "well characterized biologicals?"

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So-called "well characterized biologicals" such as plasma derived clotting factor VIII (FVIII), clotting factor IX and prothrombin complex concentrate are on the market since more than 25 years. Some of these therapeutic preparations, if not well controlled, can use severe side reactions.
We analyzed several preparation of plasma-derived therapeutic protein concentrates of inter-alpha inhibitor proteins (IaIp) and FVIII/vWF by use of proteomic methods.
We investigated several pilot batches of IaIp isolated for pre-clinical investigations by LC-ESI-MS/MS. After separation by SDS-PAGE or 2-D e;ectrophoresis, polypeptide bands were excised and tryptic peptides were analyzed by both MS methods. All three bands contain mainly IaIp and heavy chains (HC) H3, HC H1 and HC H2 of these very coplex proteins. However, in the 80 kDa band that is the main contamination during purification process, some vitamin K-dependent clotting factors and inhibitors and other plasma proteins were found. To ensure the safety of the product, especially to prevent its thrombogenicity, this fraction has to be removed from the final product.
Several plasma-derived FVIII were also investigated by LC-MS/MS after chromatographic and/or electrophoretic separation. The main components of the investigated concentrates were FVIII and its carrier protein von Willebrand factor (vWF). However, relatively high amount of other plasma proteins were also found. The main contaminant in some plasma-derived FVIII concentrates is a protein group with apparent molecular weight in SE-HPLC of about 250 kDa. The main proteins in this fraction were IaIp. However, in some FVIII samples, additional proteins that may be associated with IaIp were detected. To our surprise, clotting factor II (prothrombin) and prekallikrein were also detected. In active form, these impurities can cause harmful side reactions.
Several F VIII preparations were quantitatively compared by use of iTRAQ labeling. This method allows direct comparison between several batches to follow up batch-to-batch variations. Different FVIII preparations can also be compared regarding to their purity and content of active components, F VIII and vWF.

L32. Investigation of chemical selective displacers using robotic high throughput screening, SPR, NMR and MD simulations.

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High throughput screening was employed in concert with several analytical techniques to identify and evaluate the behavior of chemically selective displacers for protein purification in ion exchange systems. A robotic liquid handling system was adapted to efficiently carry out this parallel batch screen of selective displacers on multiple protein pairs. The results identified potential selective displacers and important functional group chemistries and also indicated that this selectivity was due primarily to the selective binding between the displacer and targeted proteins. Nuclear Magnetic Resonance was then conducted on several protein/displacer mixtures verifying the binding of the selective displacers to targeted proteins and the location of the binding event. Surface plasmon resonance experiments and molecular dynamic simulations were also carried out to corroborate the NMR results. This proof of concept study shows that more specific selectivities may also be possible by utilizing affinity based selective displacers for explicit protein systems.

L33. Investigation of the Stability of Human Serum Albumin and its Aggregates Using High Performance Size Exclusion Chromatography

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Human serum albumin (HSA) is used as a lyoprotectant in a lyophilized formulation of Interferon ?-2b, which is a therapeutic protein drug for Hepatitis C. Small amounts of HSA aggregates (dimer and high MW oligomer) is suspected to be present in HSA lyophilized Interferon ?-2b. The aims of this study are to develop a High Performance Size Exclusion Chromatography (HPSEC) method for separation and estimation of HSA aggregates from HSA and from each other in the drug and to investigate the effect of HSA sample preparation procedures and HPSEC chromatographic conditions on the stability of HSA and its aggregates in the protein drug.

An HPSEC method was successfully developed that is capable to separate the aggregates from HSA and from each other. The HPSEC method employs an YMC-Pack Diol-200, 5µm, 200Å, 500 x 8.0 mm ID (YMC Europe GmbH) with ultraviolet detection at 214 nm. The mobile phase consists of 0.1 M Phosphate buffer at pH 7.0 with 0.1 M Sodium sulfate. The method is capable to estimate HSA aggregates in HSA lyophilized Interferon ?-2b. By varying column pressure from 20 bar to 100 bar and by collecting HSA fractions and injecting back onto column, we demonstrate that there is no significant on-column HSA aggregation. Sample dilution should be avoided if possible because it will break down some of the native physically aggregated dimer and high MW oligomer. Inappropriate homogenization induces artificial formation of HSA aggregates. Excessive shear of protein solutions produced by vigorous vortexing should be avoided as it can cause protein aggregation. Appropriate homogenization method generates no artificial aggregates and the solution is stable for a reasonable period of time. Aspiration/dispensing has no impact on the stability of HSA standard solution. Gentle vortexing has no impact on the stability of thawed HSA sample solution. Gentle Swirling has no impact on the stability of the reconstituted lyophilized Interferon ?-2b powder. The kinetics of HSA degradation (measured as aggregation) was affected by homogenization method such as vortexing or aspirating and dispensing method.

Human Serum Albumin Aggregation could be artificially induced by inappropriate sample preparation. This demands us to carefully investigate sample handling in order to be able to reach comprise between complete mixing, good sample stability and minimized aggregates formation.

L34. Lysozyme binding orientation on different adsorber materials with varying pH and ionic strength

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The substantial understanding of basic mechanisms in chromatography becomes more relevant, especially when it comes to designing adsorber materials on a structural level. It is well known that only some areas on protein surfaces are involved in the interaction with the adsorber surface. We used a rather simple experimental setup to identify the protein/adsorber interface by chemically modifying those parts of the protein surface that are still accessible after binding. As a model system lysozyme with six lysines on the surface that can chemically react with a fluorescent probe was chosen. To differentiate between the differently labelled forms we established a method to separate and collect all six differently modified lysozyme isoforms and combined mass-spectrometry analysis and electrostatic calculations to identify the surface location of the modification of each isoform. To determine the orientation of bound lysozyme on any given ion exchange adsorber, bound lysozyme was chemically modified under the conditions of interest. The protein is then eluted from the adsorber surface and the generated isoform distribution is quantified using ion exchange chromatography. The results indicate, that the chemical structure of the adsorber base matrix as well as the ligands on the adsorber surface greatly influence the binding orientation. For Source 15S material consisting of a relatively hydrophobic polystyrene base matrix, we found only one binding interface made up of a mixture of positively charged amino acids and a hydrophobic patch. In contrast to that, SP Sepharose FF revealed an additional binding site. Besides the base matrix, the chemical structure of the ligands has an impact on the binding mechanism. For the grafted adsorber EMD Fractogel SO3, we found a multi-point interaction with different patches on the surface of lysozyme. With increasing protein density on the surface, we also found a re-orientation of the protein molecule towards a less space consuming end-on orientation. For investigation of the effect of ionic strength and pH, we chose two adsorbers with the same base matrix (Sepharose FF and XL). Variation of the ionic strength up to 100 mM during adsorption did not show significant changes in the binding orientation of lysozyme, where as changes in the pH showed, that for Sepharose FF, one of the two identified binding orientations is slightly favoured at pH values closer to the pI of lysozyme.

L35. High throughput chromatographic separations at small scale in a liquid handling workstation

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A commercially available liquid handling workstation was equipped with a holder for minicolumn arrays in standard 96 microplate format and a stacker module, including a specific microplate transport mechanism. This system was used for small scale chromatographic separations in a high throughput mode, by running each eight columns of individual rows on the column array in parallel. Liquid transport through the columns was achieved by positive pressure liquid displacement. The connection between columns and liquid dispensing system was reversibly made up by the fixed tips of an eight-channel liquid dispensing head introduced into the sealing needle ports at the column inlets. Using the automated transport mechanism of the stacker module, fractions from step elution were collected in standard microplates and submitted to automated analysis in a respective UV plate reader.
Pseudo chromatograms obtained from the fractions of individual columns showed high reproducibility of the simultaneously carried out separations. Examples include protein separations on ion exchange media as well as fast desalting of small volumes of protein samples. The new system was also successfully used for rapid optimisation of protein separations with two-dimensional parameter variation.
The new high throughput chromatography system is useful in a broad range of applications in the field of bio-separations, including resin screening, parameter optimisation and in process monitoring of bio-reactors.

L36. Utilization of Macrocyclic Glycopeptide Stationary Phases for the Separation of Peptides

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Abstract:
Macrocyclic glycopeptide stationary phases are well known for their use in achieving variety enantiomeric separations. In 2004, Armstrong et. al., demonstrated that these same phases could be used for superb resolution of many classes of peptides, including single amino acid polymorphs (SAAP).[1] Resolution of closely related peptides is important for many areas of research including: synthesis of new drug candidates, biological activity studies and protein sequencing.

In this study several macrocyclic glycopeptide stationary phases are applied to difficult peptide separation challenges. Both reversed-phase conditions and those approaching hydrophilic interaction chromatography (HILIC) conditions are exploited. Interactions leading to resolution are investigated and compared to retention behavior observed in traditional reversed-phase systems.

[1] B. Zhang, R. Soukup, D.W. Armstrong, Journal of Chromatography A 1053 (2004) 89.

L37. How High Resolution Analytical Separation Methods can be Integrated into Recombinant Protein Production: From PAT to Process Intensification

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Following identification and cloning of the gene that corresponds to a specific protein, its manufacture by recombinant DNA methods involves the application of a large number of unit operations, typically in excess of 20. At each stage, complementary analytical methods are required to validate the integrity and levels of expression of the product by the host cell system, to determine product authenticity at various stages of recovery and purification and to document process efficiencies. In parallel, the requirement exists to progressively link these capabilities with the up-scaling (and occasionally the down-scaling) of the recovery process, in order to allow the product to be obtained in an economically viable manner. Careful analysis of the nature and impact of these various steps, which form the basis of process intensification in manufacture, reveals that advantage can increasingly be taken of the use of generic or platform technologies that manifest common attributes, and which exploit from the analytical to the laboratory scale and through pilot to process scale conditions some common molecular features.

Central to these requirements is thus the emerging need to have robust process analytical technologies (PATs), which can be integrated, preferably with on-line real-time capabilities or alternatively with rapid off-line analysis cycles, with efficient process scale production technologies. To obtain this outcome and to achieve optimal gains in selectivity and separation capability, technologies that exploit at specific analytical and process stage the same molecular recognition properties of host-guest systems offer considerable scope. As part of the research program of this Centre, investigations have thus been undertaken into the development of new procedures, which better capture the potential of chemical libraries of immobilised ligands, derived in part from the application of combinatorial synthesis methods, and their cognate partners, which can be incorporated by genetically engineering procedures into recombinantly derived proteins as fusion handle (tag) technologies. Arising from this work, we describe in this presentation several aspects of the concepts behind such integrated separation systems approaches, and illustrate how these methods can lead to new whole-of-cycle strategies --- from the PAT stage to the preparative scale --- that address concomitantly the dual requirements of rapid, efficient and selective purification and analysis of genetically engineered proteins.

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L38. An Integrated Field Portable Biochemical and Instrument Platform for the Detection of Biological Threats

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For a variety of applications, the ability to detect biological threat agents in the theater of operation is a significant goal. Examples of the application of such fielded detection technologies include detecting biological warfare agents in the security sector (bioterrorism), infectious agents detection for veterinary applications (livestock and herd management), and for clinical application (near-patient, or point-of-care). The boundaries between these applications blur, when considering infectious agents known to be, or which could be, transmissible between species, or which can be readily spread by contact, or by aerosols transfer, between animals or people. Smiths Detection has developed a field portable platform for biological detection of such biological threats, the BioSeeq handheld PCR platform. The BioSeeq platform supplies the ability to collect, process, and detect biological threat agents, through the use of unique nucleic acid isolation, amplification and hybridization technologies. The instrument platform is a ruggedized, portable, battery operated, fluorescence thermocycler, with sensitivity of detection comparable to laboratory-based systems. The device has multiple independently controlled thermocyclers, allowing for highly multiplexed reactions, and the device is water-tight, which allows for decontamination at point of use by complete submersion. The sample preparation and assay reagents are contained in a single disposable consumable that is driven by the instrument after the sample is loaded by the user. This system utilizes a novel amplification methodology that enables multiplexed detection of 10 – 20 target organisms in a single sample reaction. Sample handling by the BioSeeq family is integrated in the detection consumable, managing the purification and amplification of target nucleic acids from samples that range from simple powders (white powder incidents), to highly complex biological matrixes. This presentation will describe the biochemical, materials science, and instrument features of the integrated detection platforms, as well as applications to biothreat detection.