P1. Expression, Purification, and crystallization of the Glycoprotein 1ba and von Willebrand Factor (vWF) Complex

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von Willebrand disease is a common congenital bleeding disorder that affects millions of people. The von Willebrand Factor (vWF) is a multimeric adhesive glycoprotein found in the blood plasma and endothelial cell lining of the blood vessel wall and binds to exposed connective tissue and the platelet surface receptors. The direct interaction of the A1 domain of the vWF and the platelet receptor glycoprotein 1ba (GpIba) mediates the addition of platelets to subendothelium of the injured blood vessel site under high shear conditions. The multimeric glycoprotein receptor complex GpIb-IX-V comprise of four transmembrane subunits, GpIba, GpIbß, GpIX, and GpV, and the binding site of the vWF A1 domain is in the extracellular domain of Gp1ba. In this study two constructs were prepared. The construct containing the residues 496-709 of vWF A1 domain was expressed in E.coli. The expressed protein was purified to homogeneity using Ni-NTA affinity chromatography, ion exchange, and gel filtration chromatography. The mutated Gp1ba domain encoding 288 residues lacking sites of N-linked gycosylation (N21D and N159D) were fused to the CH2-CH3 region of human IgG1 via an intervening enterokinase (EK) cleavage sequence. The protein was expressed in CHO cells and recovered from conditioned media by protein A sepharose chromatography. Monomeric Gp1ba domain was produced by digestion of the dimeric Fc constructs with EK. The heterogeneous Gp1ba proteins were further purified by gel filtration on Q sepharose column. The two peaks from the Q column corresponded to the predicted sizes of Gp1ba with two or three sulfated residues respectively. The Gp1ba was then purified to homogeneity by gel filtration chromatography over superdex-200. This homogeneous Gp1ba and vWF A1 domain were co-crystallized. The study of this crystal complex will enhance our knowledge in future research towards finding effective antagonists.

P2. Comparative study of L-lysine equilibrium sorption on imprinted carboxylic sorbents

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In our work the first stage of research was devoted to screening the chromatographic carrier which the most correspond to the specific peculiarities of L-lysine molecular. For that purpose, the radical copolymerization of the methacrylic acid monomers (M) with the ethyleneglycol dimethacrylate as crosslinker (X) in aqueous and isopropylic alcohol medium was realized. So series of non-imprinted polymers (NIP) with the different X/M ratio was obtained and L-lysine equilibrium sorption on these sorbents was studied. The L-lysine sorption selectivity versus X/M ration permitted to choice carrier for imprinting.
The next stage was devoted to elaboration of the L-lysine template (T) imprinting methods. For non-covalent imprinting, the optimal ratio of T/M was achieved empirically (saving the optimal X/M) by evaluating several polymers with different formulations with increasing template. Namely, series of the carboxylic cationic exchangers with the different concentrations of imprinted L-lysine were obtained. After removing template from polymeric matrix, the quantity of the L-lysine imprints (N) in the molecular imprinted polymers (MIP) was evaluated as 3; 6 and 9mol% and MIPs were named as MIP-3, MIP-6 and MIP-9 correspondently.
Since L-lysine is a zwitter-ion, it can change its ionic form in the wide pH range and also can form ionic and molecular associates. So the L-lysine equilibrium sorption by MIPs and initial NIP was studied in dependence on pH, sorptive concentration and the solution ionic strength.
The study showed that in the structurally segregated matrix of NIP the sorption capacity greatly depended on these physico-chemical factors. Non-specific intermolecular interactions could be realized as for positively, so for negatively charged amino acid. If the solution ionic strength and pH promoted the decreasing of the L-lysine associates forming, the NIP matrix permeability increased, as well as the sorption capacity increased.
Study of L-lysine equilibrium sorption by MIP-3 demonstrated the non-specific interactions in the sorption system. Simultaneously, in the pH range equal 10-11 the sorption capacity was independent of the solution ionic strength. That pointed at appearance of the affinity binding sites specifically interacted with L-lysine.
Increase of the imprinting degree in the MIPs led to leveling of the ionic strength and pH influence on the sorption capacity. Thus, for MIP-9 there wasn't observed any changes in the sorption capacity values in the wide pH and ionic strength range. That pointed at growth of affinity of the binding sites in the sorent matrix.

P3.A quantitative detection of clinically important pathogen based on capillary electrophoresis coupled with multiplex PCR

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Rapid diagnosis of bacterial infections has been strongly considered as important criteria for a patient management and an appropriate therapy of bacteria-induced disease in the early phase. Currently, several techniques have been developed and among them, capillary electrophoresis-based single-strand conformation polymorphism (CE-SSCP) combined with 16S rRNA gene-specific PCR has come into the spotlight due to its benefits such as high sensitivity, resolution and great reproducibility. Despite advantages of the CE-SSCP method, however, identification of CE peaks in SSCP is major bottle-neck to use this method because the influence of single-strand DNA's conformation on the mobility in CE is hardly predictable for analyzing pathogens as many as possible in a single run

In this study, we developed a novel technique for quantitative pathogen diagnosis by CE-SSCP coupled with multiplex PCR. PCR primers were designed for the optimal separation of their products in CE-SSCP using a stochastic model that leads to avoid experimental efforts for CE peak identification. PCR conditions were then optimized in order to obtain linear correlation between peak area and the concentrations of the target pathogens. Consequently, we developed a two-step CE-SSCP diagnosis method; first step for identification and the second step for quantification. As a model system, clinically important bacteria such as Bacillus anthracis , Candida albicans, Escherichia coli O157, Porphyromonas gingivalis, Borrelia burgdorferi, Mycoplasma genitalium G-37, Haemophilus ducreyi, Neisseria gonorrhoeae, Trichomonas vaginalis, Campylobacter jejuni and Clostridium perfringens were simultaneously identified and individually quantified. The results illustrated the potentials of this method on pathogen diagnosis and high-throughput drug discovery.

P4. Newly developed, high performance HILIC columns for use in pharmaceutical analysis by LC/MS

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Introduction:
TSK-GEL Amide-80, 3µm hydrophilic interaction liquid chromatography (HILIC) columns were developed for use in LC/MS applications, in particular for the analysis of active pharmaceutical ingredients and their metabolites. HILIC is known as one type of normal phase chromatography to retain hydrophilic compounds; such compounds are difficult to effectively retain by reversed phase chromatography (RPC). HILIC has been one of the essential tools for the separation of oligosaccharides/glycans in glycomics and recently it has become one of the essential tools in pharmaceutical analysis, along with RPC.

Preliminary Results:
TSK-GEL Amide-80, 3µm HILIC columns were prepared by reacting carbamoyl-containing silane reagents to the silica surface. The resulting phase retained polar/hydrophilic compounds that were either only moderately retained, or not at all retained, on reversed phase columns. A 4.6mm ID TSKgel Amide-80, 3µm column was found to show higher column efficiency for mannitol, even at higher linear velocity, when compared with a 4.6mm ID TSKgel Amide-80, 5µm column. As expected, using shorter length columns, faster separations of saccharides, along with higher resolution, were possible on the TSKgel Amide-80, 3µm column. In several glycomics applications, a 2mm ID TSKgel Amide-80, 3µm column showed higher resolution and reduced analysis time for sugar chains as a 2mm ID TSKgel Amide-80, 5µm column. Compared with other commercial HILIC columns, the TSK-GEL Amide-80, 3µm columns showed appropriate retention and good separation of sugar alcohols. Good durability with regards to column performance in repetitive injections was shown with these 3µm columns. The behavior of 2mm ID TSK-GEL Amide-80, 3µm HILIC columns in LC/MS applications was studied, including the effects of mobile phase components, buffer concentration and pH on retention, selectivity and column efficiency.

P5. High sensitivity protein separations using a new size exclusion chromatography microcolumn for use in conjunction with MS

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Introduction
Recently, Tosoh Corporation introduced TSK-GEL SuperSW3000 columns in 1mm and 2mm ID micro-bore column format. Size exclusion chromatography (SEC) in an aqueous mobile phase is a powerful tool for analyzing biological polymers like peptides, proteins and DNA and TSK-GEL SW series SEC columns are routinely utilized for analyzing such biological samples. The ability to detect very small amounts of proteins is one requirement in proteomics. These new TSK-GEL SuperSW3000 micro-bore columns with increased resolution, excellent sensitivity and high recovery were developed to analyze trace amounts of proteins.

Preliminary Results:
The TSK-GEL SuperSW3000 4µm micro-columns were characterized by analyzing protein resolution, detection sensitivity, sample capacity, and column efficiency in comparison to conventional column sizes. A 5-fold increase in peak height of a standard protein mixture was obtained when using a 2mm ID x 30cm TSKgel SuperSW3000 column, compared to a 4.6mm ID x 30cm column. The same improvement in sensitivity was also evident when analyzing aggregate-containing IgG samples. Linear calibration curves confirmed that nonspecific adsorption on the stationary phase was minimal. The detection limit of IgG was 18ng using the 1mm ID TSKgel SuperSW3000 column while still being able to detect small amounts of IgG aggregates. Unlike larger particle size columns, four micron SuperSW3000 columns showed a smaller drop in efficiency when increasing flow rate. As with 1mm ID columns, we found that reducing the injection volume of IgG solution from 10µL to 1µL greatly improved efficiency of the 2mm ID column, although at constant injection volume, efficiency did not vary with IgG concentration in the range of 1-5g/L. Results showed that trace analysis of biological components was possible when the TSKgel SuperSW3000 1mm ID column was utilized with an off-line SELDI/TOF/MS.

P6. Achieving Highly Efficient Bioanalytical Separations at Moderate Pressures using Fused-Core ™ Particle Technology

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Recent advances in high-performance liquid chromatography (HPLC) columns have focused on various approaches to increase the speed of analyses. Monolithic columns, for example, were introduced for their potential for use at high mobile phase velocities due to decreased mass transfer effects over conventional fully porous particles. More recently, ultra-high performance chromatography on sub-2 um particles have provided increased speed and efficiencies over conventional particles at the expense of high backpressures and, at times, column ruggedness. Fused Core™ Technology is the next significant breakthrough in column technology aimed at reducing analyses time through increased efficiencies.

The fused core technology breakthrough is based on a solid 1.7 um core particle fused to a 0.5 um outer shell of porous silica. The reduced intraparticle flow path results in significantly less diffusion within the confines of the analytical column. The result is similar column efficiencies to columns packed with sub-2 um particles at about half the backpressure. Consequently, sub-2 um-like efficiencies can be realized using conventional LC-MS instrumentation. In addition to the kinetic advantages of the Fused Core particle, the larger overall particle size and narrow particle size distribution lead to improved packing methods and hardware configurations. These improvements then lead to greatly improved ruggedness.

In this report we introduce the Fused-Core Technology and discuss its advantages through fundamental kinetic discussions and bioanalytical-related chromatographic examples. Improved speed, sensitivity, overall separation power and ruggedness will be highlighted.

P7. Mamalian plasma membrane proteomics as possible biomarker candidates

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Plasma membrane proteins serve essential functions for cells, interacting with both cellular and extracellular components, structures and signaling molecules. Additionally, plasma membrane proteins comprise more then two-thirds of the known targets for existing drugs. Consequently, defining membrane proteomes is crucial to understanding the role of plasma membranes in fundamental biological processes in health and disease, and for finding new targets for action in drug development.
Mass spectrometry-based identification methods combined with chromatographic and traditional cell-biology techniques are powerful tools for proteomic mapping of proteins from organelles. However the separation and identification of plasma membrane proteins remains a challenge for proteomic technology because of their hydrophobicity and microhetergeneity. Creative approaches to solve these problems will be discussed. Recent developments in discovery of membrane proteins as potential biomarkers and their role in treatment of diseases, especially in cancer treatment were presented.

P8. The Importance of Porosity and Mass Transfer in Biomolecules Purification

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Commercial stationary phases for chromatographic purification are conceptually old materials, developed for the purification of small molecules. In recent years, the importance of biomolecules purification has rapidly increased. However, this was not followed by an equally rapid development of new stationary phases. Two are the main disadvantages of commercially available stationary phases: pore exclusion and slow mass transport. In fact, large biomolecules, as proteins, are characterized by large sizes and low diffusion rates. As a result, most of the available surface for adsorption is not accessible, whereas it is accessible to impurities, and mass transfer within the macropores of the beads can become very slow, so that resolution is lost and competition effects, which are beneficial for high loading purifications, are minimized.

This is true for the purification of proteins by ion exchange chromatograph. In this case, the different proteins in the medium have very different pore accessibility, so that competition is limited to only a part of the stationary phase. Moreover, transport is very slow and, thus, productivity during loading is low. Additional important effects, especially with modeling the behavior of such substrates, are the change in accessible porosity as a function of salt and protein concentration. These two effects not only are making difficult the prediction of the residence time and the estimation of the correct adsorption isotherm, but they are dramatically changing the mass transport rate within the bead pores. A large benefit to separation can derive from the reduction of the bead size. Similar effects are observed in the peptide purification by reversed phase chromatography. In these stationary phases, the main role is played by the organic modifier (e.g., acetonitrile), which is characterized by multilayer adsorption on the pore surface. As a result, not only the accessible porosity is changing, but it will be shown that organic modifier is the main responsible for peptide adsorption. Therefore, when pore accessibility is correctly taken into account, polymeric and silica based supports have almost identical adsorption isotherms. The effect of salt concentration is also important in determining the behavior of these supports. This is shielding the residual changes on the surface and plays an important role in both determining the pore accessibility and the adsorption isotherm. Similar effect is played by the peptide itself, which can also contribute in shielding the surface charges.

In this presentation, the importance all these effects will be discussed and supported by experimental results.

P9. Capillary Electrophoresis as a Nanoliter-Volume Reactor for Protein Enrichment, Tryptic Digestion, Separation and Desalting

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Protein sample preparation including enrichment, proteolysis and desalting, primarily involves the use of chromatographic solid phases. However, the complexity and limited reproducibility in the packing of these solid phases has limited their applications in multiplexed microfluidic devices. Alternatively, our research team has developed the use of a discontinuous buffer system to perform protein sample preparation in open capillaries. Specifically pH 4 acetate and pH 10 ammonium buffers are used to establish a pH junction within a capillary. Upon voltage application, the proteins migrate toward the interface of the two buffers and are trapped at their isoelectric points (pI) at this pH junction. The capillary inner wall was modified by phospholipids to suppress the electroosmotic flow, and thus allowing the pH junction to remain in the capillary for prolonged protein enrichment. The buffer combination of acetate and ammonium uniquely created an extremely sharp junction, where the pH change was confined within a few nanoliters. As a result, all of the protein molecules were compressed into this tiny region, and up to a 2000-fold concentration was recorded from 0.1 ng/μL myoglobin (Journal of Chromatography A, 2006, Vol. 1134, p 317-325).

Recently, the use of this pH junction setup has been extended to co-enrich proteins and trypsin for in-capillary proteolysis. The enrichment was very beneficial in promoting digestion of proteins present at low concentrations. Following the enrichment, the voltage application was suspended for two hours to facilitate the digestion. The pH junction then refocuses the resulting peptides prior to subsequent mass spectral (MS) analysis by MALDI. The data also revealed a partial separation of the peptides within the pH junction based on pI. The partial separation was useful in minimizing ionization suppression, and in addition, providing valuable pI information to confirm the peptide identification.

Finally, the discontinuous buffer setup was applied to remove non-volatile salts in the protein samples. During the protein enrichment, salt ions in the sample are slowly replaced by the ions of the discontinuous buffers, which are ammonium and acetate in this case. Proteins containing up to 0.1 M of NaCl was successfully enriched by the pH junction. During the application of constant potential, a gradual decrease in current was observed, which signified the removal of NaCl. Subsequent analysis by MALDI MS confirmed a significant improvement in both the signal-to-noise ratio and mass resolution.

P10. Separation and Characterization of Monoclonal Immunoglobulin IgG2 Antibody by Cation Exchange Chromatography (CEX), Capillary Isoelectric focusing Electrophoresis (cIEF), and LC-MS

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Cation exchange chromatography (CEX) and Capillary Isoelectric focusing Electrophoresis (cIEF) have been developed to separate and quantify monoclonal antibody N-terminal glutamine, C-terminal lysine, and acidic variants of one IgG2 antibody. Typically, CEX chromatography separates antibodies into a few peaks representing acidic variants and basic variants at the N-terminus and C-terminus or other chemical modifications. For this IgG2 molecule, doublets were observed for each peak from those charge variants in CEX chromatograms. These doublets are convertible in a redox process suggesting they are disulfide bond-related structural isoforms. CEX and capillary electrophoresis (CE-SDS) were used to separate the structural variants. Characterizations were performed by use of enzymatic digestion, peptide mapping, CE-SDS, oligomapping, sialic acid assay, and bioassay. Cell based bioassay shows that all variants have comparable potency which indicates that acidic variants, basic variants, and disulfide structural variants do not affect the potency of this IgG2 molecule.

P11. Iso-ASP Formation in IL-1R type II Correlates with Loss of Binding Activity in Accelerated Stability Samples

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Hydrophobic interaction chromatography (HIC) is used to resolve a protein IL-1R type II. The accelerated stability samples of IL-1R-II are resolved into three peaks. The inactive HIC peak I is shown to consist of molecules with "large" C-terminal truncations of 10-15 amino acids, whereas the active HIC peak II contains C-terminally full length and "small" C-terminal truncations of two amino acids. Peak III contains only high molecule weight (aggregated) species. By C-terminal LC-MS analysis (CNBr) of stressed samples, deamidation at ASN317 forms both ASP and iso-ASP. The iso-ASP formation occurs in HIC peak I and the ASP species in HIC peak II. The binding activity correlates with the percentage of HIC peak II. HIC is a stability-indicating assay, resolving into the inactive peak I molecules with "large" C-terminal truncations as well as molecules containing iso-ASP317. Formation of the iso-ASP317 form, but not the ASP317 form, results in altered retention time on HIC and is accompanied by loss of binding activity, presumably by introducing a significant conformational change.

P12. Improvement in Downstream Processing for Serum Protein Separation

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Separation technologies based on a high specific interaction between molecules are known as an effective means for the isolation of biomolecules. One promising tool employed for the recovery of new compounds is the specific affinity separation technique based on the interaction of lectins to glycoproteins. An increasing number of publications indicate their versatile applications. However most of them report about insertion in the last part of the downstream process. Lectin affinity separation is also applicable as the initial step in protein purification from crude feedstock with the purpose to enrich selected glycoproteins which reduces the complexity of the sample and facilitates the adjacent separation process.

Results will be presented of the isolation of glycosylated Acute Phase Proteins (APP) from the blood serum of seals. The blood concentration as well as the glycosylation pattern of APPs may serve as biomarkers in health assessment.

Different lectins were used for the separation, which provide also valuable information about the glycosylation pattern. Furthermore details about the fabrication of the lectin affinity adsorbents were provided by means of elucidating immobilization conditions, support material comparison and interaction investigations of the immobilized lectins.

P13. Analysis of the E. coli Proteome by Different 2D-LC-MS/MS Methods

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Introduction: Two dimensional (2D) separations are an effective way to separate complex peptide mixtures for analysis by tandem mass spectrometry (MS/MS). Recent publications demonstrate new 2D methods that have been developed, but few show a direct comparison of the resulting peptide and protein identifications between the different methods. In this study, a complex sample of peptides from E. coli lysate was used to evaluate several 2D-LC-MS/MS methods where either a strong cation exchange (SCX) column, reverse phase column with mobile phases at pH 10, or hydrophilic-interaction chromatography (HILIC) column was used in the first dimension of separation. The aim of this study is to compare differences in peptides and proteins identified between these 2D methods using high resolution off-line fraction collection.

Method: E. coli K-12 (ATCC # 700926) was grown to stationary phase. Pelleted cells were lysed with a Mini-BeadBeater. Soluble proteins were assayed, alkylated with iodoacetamide and digested overnight with trypsin at 37 °C in the presence of 0.1% RapiGest. During the first dimension of separation, off-line collection of fractions from either a SCX (PolyLC), HILIC (Tosoh Bioscience LLC), or reverse phase C18 (Waters) column at pH 10 was performed. For the second dimension, about 75 fractions per method were injected onto a Supelco C18 capillary column (at pH 2.6) which was online with a linear ion trap (Thermo Fisher Scientific). Tandem mass spectra were collected and analyzed with TurboSequest using the E. coli protein database (ftp://ftp.expasy.org/). Results were filtered to remove false positive identifications and exported to Microsoft Office Excel for further processing.

Results: Large datasets of E. coli peptides and proteins were generated from the three 2D-LC-MS/MS methods. For all methods combined, 22,227 unique peptides were identified from 1,884 unique proteins (87 are classified as membrane or lipoproteins). Analysis of the data from each 2D-LC-MS/MS method shows that the largest number of peptides and proteins were identified in the pH 10 method followed by the SCX and HILIC methods. While there was a large overlap of peptides and proteins identified between the methods, the pH 10 method had the greatest number of identifications not found by the others. Differences in the characteristics of the peptides unique to each method were also investigated by calculating the grand average of hydropathy (GRAVY), peptide length, number of semitryptic peptides and the distribution of observed charge states. Although the HILIC method had the lowest number of peptide and protein identifications, the peptides unique to this method had the largest percentage of

semitryptic peptides and the most positive (more hydrophobic) average GRAVY value. These results may be used to help select a method for future experiments and serve as a reference during evaluation of new 2D separation methods.

P14. Novel High Binding Capacity Ion Exchange Resins for Biochromatography

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Ion exchange chromatography is often used as capture or intermediate step in purification of recombinant proteins, especially for biopharmaceuticals and monoclonal antibodies. We developed novel high binding capacity ion exchangers, Toyopearl® GigaCap S-650M, GigaCap Q-650M, for these applications. These GigaCap series resins are based on the well proven methacrylic polymer backbone of Toyopearl® and TSKgel media and especially designed for protein purification in packed bed operation at process scale column chromatography. These resins have excellent pressure flow characteristics, superior dynamic binding capacities, and high protein recoveries.

GigaCap S-Type resin has a strong cationic exchange functionality, sulfo group. This S-Type resin is especially designed for purification of monoclonal antibodies and has dynamic binding capacity up to 140mg IgG per ml resin.

GigaCap Q-Type resin has a strong anion exchange functionality, trimethyl ammonium group. This Q-Type resin has also high dynamic binding capacity. For example, it has DBC up to 170mg BSA per ml resin and 100mg IgG per ml resin. Especially, it can maintain high binding capacity for large size protein such as Thyroglobulin.

We were compared these resins with some competitive medium about for binding capacity. GigaCap series resins show also high chemical stability. The pressure drop is extremely lower than competitive medium, so these resins are suitable for higher flow rate operation at process scale columns. This is accomplished by optimization of particle size and rigidity of methacrylate polymer backbone. All GigaCap series resins have 50-100 micron particle size.

We report here the physical properties and application on these new ion exchangers.

P15. Novel TOYOPEARL® Hydrophobic Interaction Chromatographic Resin for Antibody Purification

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Hydrophobic interaction chromatography (HIC) is an effective tool for the purification of biomolecules at analytical and preparative scales, especially in the capture/intermediate purification step for proteins and monoclonal antibodies (MAbs). In this mode, chromatographic performance such as binding capacity, recovery and selectivity is affected by various properties of the chromatographic resin, mobile phase variables (salt concentration, salt type and pH), and so on. The hydrophobicity and the pore size distribution of HIC resin are known to be the key points for determining the chromatographic features, especially binding capacity. On the basis of this knowledge, two TOYOPEARL® HIC resins (PPG-600M and Butyl-600M, Tosoh Corporation, Japan) with increased binding capacity for antibodies have been developed for the preparative chromatography. These resins are suitable for the development of purification methods amenable to a variety of MAb needs.

A high binding capacity HIC resin, TOYOPEARL® Phenyl-600M, was developed for the preparative chromatography. Phenyl-600M was prepared by optimizing a pore size of TOYOPEARL® resin for the binding of immunoglobulin G (IgG) and derivatizing with a phenyl ligand. TOYOPEARL® resin is a hydrophilic, macroporous spherical beads based on cross-linked polymethacrylate for medium pressure liquid chromatographic applications. Chromatographic properties of Phenyl-600M were characterized by evaluating binding capacity, selectivity and elution behavior for some kinds of IgG and protein samples, and then compared with them of commercially available other HIC resins.

As a result, IgG and protein binding capacities of Phenyl-600M were approximately 2.3 and 1.2 times higher than those of conventional TOYOPEARL® Phenyl-650M and phenyl-agarose resin (high sub), respectively. On Phenyl-600M, adsorbed proteins could be quantitatively eluted (>90%) with an aqueous buffer solution not containing organic solvent. In the separation of smaller proteins, Phenyl-600M exhibited better resolutions compared to the phenyl-agarose, while the resolutions on Phenyl-600M and Phenyl-650M were equivalent. Although Phenyl-600M was designed for IgG purification, these results suggest that Phenyl-600M can be one of the powerful tools for the protein purification as well as MAbs.

We report here the fundamental features of the new HIC resin and application on antibodies purification in comparison of other HIC resins.

P16. Method Development and Optimization for Characterization of Therapeutic Proteins Using Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS).

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Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the traditional method for the molecular weight estimation and purity analysis for the therapeutic proteins. However SDS-PAGE is labor intensive, quantitative indirectly, and requires use of toxic reagents. Compared to SDS-PAGE, capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) has shown great advantages on the automation, high-speed separation, enhanced resolution, and direct, on-line quantification using UV detection. This study is to evaluate the usage of CE-SDS technology for the analysis of therapeutic proteins. The method development and optimization of CE-SDS are highlighted in this presentation. The comparison of CE-SDS and SDS-PAGE for the analysis of therapeutic proteins is also studied. . The results of CE-SDS method demonstrate its potential application in characterization, in-process, and GMP sample testing for therapeutic proteins.

P17. New ProSwift monolith Reversed Phase and Ion Exchange columns and their Comparative Evaluation with other Bio-columns

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Monolith columns offer significant advantages over conventional porous columns. They include fast mass transfer, high loading capacity, improved resolution even at elevated flow rates with low back pressures and wide pH stability. These exclusive characteristics support versatile performance in a wide range of bio-molecule separations. Earlier, we introduced reversed phase, anion exchange and cation exchange phases of ProSwift monoliths (4.6 x 50 mm). Reverse phased columns (4.6 x 50 mm) include RP-1S, RP-2H and RP-3U versions, which differ in their pore structure and therefore in their use for various bio-separations. The low back pressure of the ProSwift RP columns supports fast separation of analytes at high flow rates resulting in increased productivity. ProSwift RP columns are rugged and exhibited excellent performance. ProSwift ion exchange phases (4.6 x 50 mm) include weak anion exchange (WAX-1S) and a strong anion exchange (SAX-1S) columns, as well as a weak cation exchange (WCX-1S) column for biomolecule separations. Recently, we introduced 1x50 mm format of WAX-1S and WCX-1S columns for high resolution micro analytical bio molecule separations. 1mm columns offer improved sensitivity and reduce solvent consumption. ProSwift ion exchange columns are rugged, high capacity columns and exhibited excellent performance. Using both reverse phase and ion exchange columns, we have developed various applications and compared with other competitor columns. These results will be presented.

P18. Characteristics of short gel filtration columns for rapid screening of aggregates and impurities

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Gel filtration (GF) is a gentle technique suitable for the study of aggregation and purity of a target protein. GF, however, is often time consuming and can also be held up due to the expense of sample and buffers. A new, small (3 ml) column format packed with Superdex™ 75 and Superdex 200 has been developed for rapid and reliable gel filtration enabling analyses in less than 6 min using only 4 µl of sample.

Results illustrating the resolution of a standard protein mixture, sensitivity, flow rate properties and influence of system dead volumes upon resolution will be shown. The short gel filtration columns will also be compared to the longer Superdex 75 and 200 10/300 GL columns.

P19. Grafting of monolithic columns for bioseparation

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The application of monolithic columns has increased during the recent years especially in the field of bioprocessing mainly due to their superior flow characteristics and dynamic capacities. These characteristics are especially important for the separation of very large bioproducts like DNA, virus, and even whole cells. As column materials several different chemistries can be employed of whom silica and methacrylate polymers are the most common ones. The application of cryopolymerisation of acrylamides has also been described recently and offers a convenient and simple approach for monolithic synthesis. However, in most cases these chromatographic phases have only limited capacity due to the large internal pore diameters. Therefore, several researchers have employed graft polymerization in order to increase the capacity of these columns. Here we present the synthesis of monolithic columns by cryopolymerisation. Stationary phases were designed as anion-exchanges and as IMAC columns respectively by derivatising the functional allyglycidylether or by integrating appropriate monomers into the polymeric network. These columns were compared to grafted monoliths made by graft polymerisation of methacrylate polymers on the polyacrylamide backbone of the monolith after activation with Cu(III). It could be shown that graft polymerisation in general increased the capacity of the monolithic columns for proteins as well as nucleic acids. While ungrafted monoliths only showed a capacity of 40 µg/mL of protein, grafted columns were able to bind up to 4 mg/mL. Different forms of grafting resulted in varying dynamic capacities which can be explained by the accessibility of the functional groups on the polymeric ligand by the target molecules.

P20. Extraction of nucleic acids using reverse micellar two-phase systems

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Plasmid DNA as an active pharmaceutical ingredient is gaining more and more importance. Plasmid DNA can be used in gene therapy as a vector for therapeutic genes or encoded antigenes in genetic vaccination. For the production of multigram quantities of this substance robust and scalable processes have to be designed following the guidelines set by regulatory authorities. One main challenge is the separation of plasmid DNA and RNA. In this study we investigated the distribution of plasmid DNA and RNA in reverse micellar two-phase systems comprising isooctane and the cationic surfactant methyltrioctylammoniumchloride (TOMAC). This method offers an alternative to conventional purification procedures since it only needs inexpensive chemicals, is highly scalable, and can easily be integrated into current purification schemes. The partitioning of biomolecules in these systems is influenced by ionic strength, nature of the organic phase, surfactant, and co-surfactant, resulting in a multitude of different extraction experiments. Anion concentrations at which the partitioning behaviour for nucleic acids is inverted were identified. Systems capable of separating RNA from plasmid DNA out of a bacterial lysate were analysed further and successfully tested. The capability of reverse micellar systems for plasmid form separation was also shown by capillary and agarose gel electrophoresis.

P21. Fast development of small molecule affinity ligands for hGH using Encoded Bead Technology

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Affinity-based separation utilizing specific interaction between the affinity ligand and the corresponding target is a very powerful method for separation of biomolecules. Traditionally, affinity separation of proteins use protein based ligands, which do have some limitations in chemical and biological stability.

In this presentation a bead based screening method is discussed, for the development of small molecule affinity ligands for hGH. This method utilizes a combination of biocompatible PEG based resins, combinatorial synthesis and fluorescence based single bead screening. In this way, ligand libraries with a large number of different chemical entities can be screened efficiently and can avoid the time consuming step in traditional library screening using different spectroscopic and chemical methods.

P22. Purification of Strep(II)-tagged proteins using new prepacked columns

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The Strep(II)-tag is ideal as an affinity tag due to the small size (8 aa, 1 kDa), often making removal of the tag unnecessary. The new StrepTrap™ HP 1 ml and 5 ml columns have been specially developed for high specificity towards the Strep(II)-tag, with the prepacked format facilitating fast and convenient purification. Purification is done under physiological conditions and the mild elution preserves the activity of the target protein. The small bead size of the medium (34 µm) allows elution in narrow peaks, minimizing the need for further concentration steps.

In this work, purifications of Strep(II)-tagged proteins on StrepTrap HP 1ml and 5 ml columns are presented. A dual-tagged fluorescent protein, (His)6-mCherry-Strep(II) expressed in E. coli, was purified in a purification procedure including two affinity columns, StrepTrap HP followed by HisTrap™ HP. The second affinity column was necessary for removal of a truncated variant of the target protein. The multi-step purification was performed fully automatically using ÄKTAxpress™. Another dual-tagged protein expressed in insect cells was purified to high purity in a single step using StrepTrap HP. The final purity was above 95% according to SDS-PAGE analysis. Finally, six repeated purifications of a Strep(II)-tagged protein expressed in E. coli were performed on the same StrepTrap HP column with 0.5 M NaOH regeneration between the runs. The final purity and yield of the target protein remained almost unchanged, with no tendency of decreasing values, demonstrating the high reproducibility during repeated use of StrepTrap HP.

In summary, the use of prepacked StrepTrap HP columns for convenient purification of Strep(II)tagged proteins results in high final purity and yield of the target protein. In addition the column is efficiently regenerated with 0.5 M NaOH.

P23. Purification of MBP-tagged proteins using new prepacked columns

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Maltose binding protein (MBP) is beneficial as an affinity tag due to its ability to increase expression level and solubility of the fusion protein. The new MBPTrap™ HP 1 ml and 5 ml columns have been specially developed for high specificity towards the MBP-tag and the prepacked format facilitate fast, convenient and reproducible purifications. Additionally, it can easily be regenerated using 0.5 M sodium hydroxide without losing binding capacity.

In this work, MBP-tagged proteins expressed in E.coli were purified on prepacked 1 ml or 5 ml MBPTrap HP columns. A stability study was performed with six repeated purification runs on the same column, each followed by regeneration with 0.5 M NaOH. The results showed no significant change in yield or purity. A purification of medium-chain acyl-CoA dehydrogenase (MCAD), a protein involved in metabolic disease, was performed. The total purification time was reduced due to elution of highly concentrated target protein, thereby eliminating the need for a concentration step and simplifying the whole purification procedure. Furthermore, an automated two step purification of Apoptin was performed on ÄKTAxpress™ where the affinity chromatography step on MBPTrap HP was followed by a gel filtration step.

In summary, the results show that prepacked MBPTrap HP columns decrease purification time and give high purity and reproducibility.

P24. Rapid screening of antibody aggregates and protein impurities using short gel filtration columns

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Gel filtration (GF) provides reliable information about size and the purity of a protein of interest, especially when it is in a multimeric state. Superdex™ 200 and Superdex 75 are excellent media for such analyses. To speed up analysis and keep sample and buffer consumption to a minimum, two prepacked short gel filtration columns (15 cm long and 3 ml bed volume) have been developed, Superdex™ 200 5/150 GL and Superdex 75 5/150 GL.

Results from rapid screening of optimal conditions for HIC purification of antibody to minimize dimer and higher aggregate content will be shown. An assessment of integrity of a recombinant protein and rapid purity check of two Strep(II)-tagged proteins will also be presented.

P25. Rapid protein analysis and screening of membrane protein buffer conditions by gel filtration

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Depending on parameters such as ionic strength, isoelectric point and hydrophobicity proteins are liable to aggregate into larger complexes or oligomers. Screening of different conditions is often needed to obtain a protocol resulting in pure and stable target proteins. Size-exclusion chromatography (SEC) is an excellent and gentle technique to study the state of aggregation of a protein as well as the purity of a target protein. This procedure is often time consuming, available sample might be limited and expensive chemicals, like detergents, might also be limiting factors.

Superdex™200 is a powerful media for size distribution analysis and in order to minimize the cycle time a new column format has been developed for rapid and reliable size-exclusion chromatography. The new Superdex™200 5/150 GL prepacked column has a length of only 150 mm and a total gel volume of 3 mL allowing small volumes of material (4-50 µl) to be analyzed in a short time (6-12 min/run).

Results from a homogeneity check of a purified membrane protein, a purity check of a soluble histidine-tagged protein and the study of formation of a protease-inhibitor complex using Superdex ™ 200 5/150 GL will be presented.

P26. Purification of Antibody Heteropolymer Targeting Staphylococcus Aureus

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A Heteropolymer (HP) is a unique dual antibody conjugate composed of specific, chemically cross-linked monoclonal antibodies (MAbs). One antibody recognizes human complement receptor type 1 (CR1) and a second antibody is directed against a blood-borne pathogen target. The HP conjugation process produced a complex mixture of molecular species. The HP reaction mixture included unconjugated monomers, dimers, trimers, tetramers and conjugates with a molecular size greater than 106 Dalton MW. Unconjugated monomers as well as species larger than or equal to tetramers must be removed during the purification process. The methods used to achieve this purification must be scalable to produce large batches of HP final product.

Affinity chromatography can not be used since MAbs and HP have a common origin. Similar net charges of HP and unreacted antibodies extremely complicate the development of HP purification processes using ion exchange chromatography for purification of HP.

Existing size exclusion chromatography (SEC) media available for the scaling up of protein purification processes does not provide adequate protein separation with molecular weights above 600kDa. They also have certain limitations in scalability. Therefore we explored the use of hydrophobic interaction chromatography for the HP purification process development because of limitations of other chromatographic techniques for the separation of non cross-linked antibodies from the conjugated ones. In this study we have demonstrated that HP targeting S.aureus can be purified using EMD Fractogel® Propyl (S) resin. Conjugated MAbs were successfully separated from unreacted antibody monomers with propyl resin using buffers with ammonium sulfate. Elution conditions were developed to separate HP product from high molecular weight MAbs conjugates. The propyl HIC purification of HP was successfully scaled-up to 400mg of total loaded protein.

P27. Analysis of serine proteases from marine sponges by two-dimensional zymography

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Proteolytic activities isolated from the marine demosponges Geodia cydonium and Suberites domuncula were analyzed by two-dimensional (2-D) zymography, a technique that combines IEF and zymography. After purification, a 200 kDa proteolytically active protein band from G. cydonium was obtained when analyzed in gelatin copolymerized 1-D zymograms. The enzymatic activity was quantified using (-N-benzoyl-d-arginine p-nitroanilide (BAPNA) as substrate and corresponded to a serine protease. The protease activity was resistant to urea and SDS. DTT and 2-mercaptoethanol (2-ME) did not significantly change protease activity, but induced a shift in molecular mass of the proteolytic band to lower Mr values as detected by zymography. Under mild denaturing conditions, lower-Mr bands (<200 kDa) were identified in 1-D zymograms, suggesting that the protease is composed of subunits which retain the catalytic activity. After 2-D zymography, the protease from G. cydonium revealed a pI of 8.0 and a Mr shift from 200 kDa to 66 kDa. To contrast these results, a cytosolic sample from S. domuncula was analyzed. The proteolytic activity of this sponge after 2-D zymography corresponded to a Mr of 40 kDa and a pI of 4.0. The biological function of both sponge proteases is not yet known. This study demonstrates that mild denaturing conditions required for IEF may alter the interpretation of the 2-D zymography and care must be taken during sample preparation.

P28. Microheterogeneous monoclonal antibody subspecies with differential hepatitis C virus core antigen binding properties identified by SEC-HPLC

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A monoclonal antibody directed against the core protein of hepatitis C virus was characterized for its utility in a sandwich antigen immunoassay wherein the mAb was used as the conjugate. Analysis of unconjugated and acridinium-conjugated monoclonal IgG using a silica-based HPLC size exclusion column revealed the existence of a single peak. Subsequent analysis of unconjugated IgG using a methacrylate-based HPLC size exclusion column revealed the presence of two species of IgG but only by using a low ionic strength mobile phase buffer. Independent conjugation and testing of the two species showed significant differential reactivity towards HCV antigen. Isoelectric focusing gels indicated subtle differences in the subspecies composition. Measurement of target peptide binding constants using fluorescence correlation spectroscopy indicated that the two HPLC column fractions had different Kd values at low salt concentration. Differences in Kd disappeared in high salt buffer. Mass spec analysis of the fractionated peaks indicated masses were identical though both contained a mixture of two heavy chains species differing by only 160 mass units. Sequence analysis of heavy and lights chain cDNAs revealed the presence of a single potential N-linked glycosylation site in the heavy chain variable region CDR2. These results suggest that this monoclonal antibody consists of microheterogeneous subspecies that exhibit different antigen binding properties possibly due to differences in post-translational modification of the heavy chain variable region glycosylation pattern. The choice of size exclusion column matrix and buffer composition was critical to the identification of these monoclonal IgG subspecies.

P29. Evaluation of protein A immobilized resin for the capture of monoclonal antibodies

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Therapeutic antibodies have become the major product in the biopharmaceutical industry. Most of antibodies are purified by protein A affinity chromatography as a capture step due to the unique selectivity of the protein A. Two types of protein A resins, agarose and glass based, are frequently used for industrial purifications. Both resins have high dynamic binding capacity and can be used up to 200 cycles. However, agarose based resin can not be operated at high flow rates when the resin is packed in a larger column due to its lower mechanical strength, while glass based resins are labile to caustic mobile phases, a common and very effective for CIP solution.
To overcome such drawbacks, we have developed a new Toyopearl based protein A resin. Toyopearl is a hydrophilic polymer based resin with improved flow properties compared to agarose based resin and better chemical stability compared to glass based resins. Recombinant protein A was immobilized onto HW-65 which has a large, 1000 angstrom pore size.
In this poster, the basic properties of the experimental Toyopearl based protein A resin, such as flow-rate dependence on dynamic binding capacity, purification of monoclonal antibody, and a CIP study with caustic solutions will be presented.

P30. Application of Analytical CEX Chromatography for the Isolation and Characterization of Deamidated Species of a Kunitz Domain Protein.

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Deamidation is a common post-translational protein modification and is known to be a major source of protein degradation often occurring during purification and storage under mild conditions. This gives rise to increased protein heterogeneity often resulting in a loss of purity and stability. This modification is also known to result in conformational changes within the polypeptide backbone which may ultimately result in a loss of biological activity. Therefore, in the development and manufacture of a well-characterized protein therapeutic, it is necessary to carefully monitor these changes as well as to isolate these deamidated species for characterization. Since the rate and extent of deamidation is typically slow and low in the final drug substance/product, detection, isolation and subsequent characterization of the various closely-related deamidated species is often very challenging.

Here, we describe the approach taken to generate, isolate and characterize deamidated species of a Kunitz Domain Protein (KDP) produced by Pichia Pastoris fermentation. Deamidated species were isolated by Cation Exchange (CEX) Chromatography from a stressed sample of KDP using a silica-based analytical CEX-HPLC column from Tosoh Bioscience. A shallow pH gradient using 50 mM citrate from pH 4.8 to 5.2 in 20 column volumes was developed and used to isolate several different forms of deamidated KDP.

Further analyses by CEX-HPLC, gel-IEF, iCE280, RP-HPLC, SDS-PAGE, and peptide mapping with LC-MS and LC-MS/MS were used to characterize purity, isoelectric point (pI) and to confirm exact mass, site(s), and extent of modification. An enzymatic-based RP-HPLC method for isoaspartate detection was used to monitor relative levels of deamidation (Isoquant, Promega Corporation). Biological activity was also determined by measuring inhibition constant (Ki) and potency (specific activity).

Based on these analyses, we were able to successfully isolate and characterize several deamidated forms of known product-related species (PRS). The purified deamidated samples showed substantially reduced biological activity relative to the reference standard. Ion-exchange chromatography has proven to be a powerful technique for the

identification and isolation of closely-related deamidated variants of KDP. Additionally, the CEX-HPLC method may be a useful analytical tool for the quantitation and characterization of deamidated species of both KDP and non-KDP proteins.

P31. A Dual Column Method for Detecting Free Thiol Species in HCCF.

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Harvested cell culture fluid (HCCF) is a complex mixture of various molecules ranging from small molecules to large complex proteins, as well as cellular debris. Searching for a specific chemical moiety (e.g. free thiols) in this mixture is challenging and requires separation techniques that can look at the entire range of a specific characteristic – in this case hydrophobicity. This poster describes separating harvested cell culture fluid by two-dimensional off-line liquid chromatography, with sizing as the first dimension followed by either a hydrophobic-based separation (reversed-phase HPLC) or a hydrophilic-based separation (normal-phase HPLC). Fluorescent tagging and mass spectrometry aided in the detection and identification of all free thiol containing molecules found in the cell culture mixture by these complementary modes of separation. Additionally, experiments were conducted to designate responses for standard expected free thiol species (e.g. cysteine, glutathione, DTT and free light chain).

P32. Predominant Acidic Forms of a Recombinant Monoclonal Antibody

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Ion exchange chromatography can be used to separate the acidic and basic forms of recombinant monoclonal antibodies. In an effort to better understand the acidic variants, material was collected and characterized to determine the major species in the region. Acidic variant material was generated via scaled-up ion exchange separation. The initial separation was developed and optimized on an analytical (4.6x250 mm) column. The method was then scaled for use on a semi-preparative (9.0x250 mm) and preparative column (22x250 mm). Once the material was generated, the predominant acidic forms were characterized using size exclusion chromatography, capillary electrophoresis (CE-SDS, cIEF), peptide mapping, and potency.

P33. Characterization of Interferons and Their Variants by High Performance Liquid Chromatography

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Interferons (IFNs) are one of the most frequently prescribed therapeutic proteins in the treatment of variety of diseases such as hepatitis, multiple sclerosis, and leukemia. Like all other therapeutic proteins, however, IFNs are susceptible to a number of chemical and/or conformational changes during preparation, formulation and storage. These modifications include misfolding, aggregation, oxidation, disulfide bond scrambling, deamidation or isomerization. As a result, regulatory agencies routinely require that manufacturers extensively characterize and strictly limit product-related variants in each preparation of a therapeutic protein. In addition, once approved, manufacturing protocols must be adhered to in order to minimize the potential for further structural alterations of the product. Major concerns relate to the loss of therapeutic efficacy and on the potential for unwanted immune reactions. In recent studies, however, cytotoxic effects have been observed with some biotherapeutic proteins including IFNs. Some of these events are associated with denaturation or chemical alterations of the native proteins.

The ability to reliably detect and characterize various preparations of therapeutic interferons is crucial. In this study, high-performance liquid chromatography (HPLC) methods were developed for evaluating the stability of human IFNs. Native, aggregated and denatured IFNs were separated and characterized using size exclusion chromatography (SE-HPLC) coupled with intrinsic fluorescence detection. A rapid and easy-to-use reversed phase chromatography (RP-HPLC) method was developed to characterize IFNs and their chemically modified variants. These chromatographic methods, which quantitatively measure physical and chemical changes taking place in solution formulations, were found to be suitable to monitor IFN stability. In addition, IFN-induced toxicity and the effects of chemically and conformationally modified IFN variants were investigated. Preliminary bioassay results showed significant decrease of potency for altered IFNs variants compared to the native, unmodified IFNs. Results of assay tests on the effect of different interferon preparations on the expression of hepatic genes responsible for hepatotoxicity will also be discussed.

P34. Development of a polishing step for a glycoprotein expressed at low titer

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Approximately 70 % of biopharmaceuticals in clinical or preclinical development1) are glycosylated proteins. Mammalian cells are capable of producing the target molecule with human like glycan structures required for enhanced pharmacokinetics 2). Downstream processing of glycoproteins other than monoclonal Antibodies is challenging because expression titers are usually inferior compared to microbial expression systems and a highly efficient affinity column rarely exists.
Additionally, the composition of desired glycan species has to be adjusted during down stream purification since the oligosaccharide chain structure after fermentation is inhomogeneous and activity of the target molecule is often altered depending on the glycosylation pattern.

A down stream process for the purification of a glycoprotein expressed in CHO cell culture at low titer (20µg/ml) was developed and the polishing step using an HIC column is described in detail. Additionally to the common approach towards development of a purification step such as resin screening, determination of dynamic binding capacity and reproducibility studies, two methodologies were included:
Isocratic pulse experiments were performed in order to determine the minimum salt concentration required for binding. Once process parameters were defined, process optimization was carried out using a fractional factorial design of experiment approach. It could further been shown that the target glycosylation can be achieved by setting appropriate pooling criteria.
Although the SDS-Page of the load material showed more than 8 non-product related impurities, the polishing step was able to deliver a product of desired quality in terms of SDS Page band pattern, Isoform distribution and glycosylation pattern in small, medium and pilot scale with reasonable yield.

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2) Ahrere, K., Jungbauer, A. J. Chromatogr B 841 2006, 110-122

P35. **Optimizing Chromatographic Media and Separation Conditions for the Analysis and Isolation of Synthetic Peptides**

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Peptides are used for both basic research and for the development of biopharmaceuticals. In these applications, synthetic peptides are preferred to isolated natural products. A peptide that is heterogeneous at even a trace level can compromise the results of other biological or chemical studies. The range of possible contaminating sequences and side products can be large so it is essential to use the highest possible resolution for both analysis and purification. Chromatography must be adjusted to the properties of each peptide. The large range of peptide properties requires efficient method development and techniques that work well for as many samples as possible. The factors that influence purification protocols include the column, the mobile phase, and the operating parameters. The surface chemistry of the BEH Technology™ has proven advantageous for peptide separations. Columns are available with both 130? and with 300Å pores in a range of particle sizes. Good peak shape is observed for a wide range of samples. A library of synthetic peptides representing a range of properties including size, hydrophobicity, and isoelectric point was used for chromatographic evaluation. The same packing material gave good peak shape for isolectric points ranging from 3 to higher than 10. Little difference between 130Å and 300Å was observed for peptides up to 40 residues. Both formic acid and TFA gace useful retention, peak shape, and selectivity. Classes of peptides with extreme or special solubility properties were best separated with additional mobile phase manipulation. Elevated temperature, high pH, and/or alternative solvents were useful for peptides that are very difficult to dissolve in common chromatographic solvents. Scaling of the optimized separation to larger sample loads could be accomplished with simple geometric rules using columns with same chromatographic material, even when larger particles are chosen for economic reasons.

P36. Practical Considerations in the Application of UPLC to LC/MS Peptide Mapping

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The analysis of protein structure requires the the discrimination of small chemical changes in a very large molecule. Such an analysis has lead to the routine use of enzymatic digestion of the protein to more manageable fragments. The separation of these fragments, peptide mapping, has long been a fundamental tool for structural analysis. Reversed phase HPLC has become the preferred tool for peptide mapping because the separation is sensitive to to the chemical differences that are found in protein structure. When the chromatographic separation is coupled to mass spectral detection, the chromatographic peaks can identified. The LC-MS combination synergistically increases the information available. The analysis of real protein samples, is, however, more complicated because essentially all samples are mixtures of native and slightly modified or damaged proteins. Low abundance peptides, representing these trace modifications, may co-elute with major components. The trace peptides can be obscured by the more intense signal representing the native structure. The analysis benefits therefore from the use of the most highly resolving chromatographic techniques. UPLC® peptide mapping is a part of this approach since it yields higher resolution than is possible with HPLC.

Protein digests were separated on an ACQUITY UPLC® using both standard HPLC columns and Peptide Separation Technology UPLC® columns. Separations were monitored with UV coupled to LCT Premier oa ToF instruments. (All from Waters, Milford, MA) Gradient slope and temperature were varied to produce high resolution, high coverage maps for the test proteins. Samples of the proteins were stressed to contain oxidation sites. The separations were optimized for analysis of the peptides representing the specific modifications.

Chromatographic peaks were identified as specific sequences with exact mass measurement. That technique permits the identification of modified peptides in the stressed samples. This experiment can be used to assess the suitability of the peptide map for detecting variants of the native protein structure. The analytical method was also optimized to give a very rapid assay focused on the measurement of a specific site of modification. The systematically optimized chromatography gave improved limits on sensitivity and accuracy of MS characterization of modified proteins.

P37. Development of a Reversed-Phase HPLC Assay For Quantitation of Methionine Oxidation in a Recombinant Protein Pharmaceutical Candidate

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Protein oxidation is a covalent modification that may impact the structure and/or function of the protein. The oxidation of methionine to methionine sulfoxide has been shown to occur in a wide range of recombinant proteins as a consequence of storage or processing. A protein pharmaceutical currently under development (protein X) was found to be highly susceptible to methionine oxidation. Chemically-induced methionine oxidation with low levels of peracetic acid was found to reduce the biological activity of protein X. Therefore, a reversed-phase HPLC assay was developed to quantitatively monitor the level of methionine oxidation in the drug substance. Optimization of various method parameters will be presented, including sample preparation and sample stability. Prevention of method-induced oxidation or artificial oxidation will also be discussed, including the effect of metal contamination, and the effect of mobile phase age. The RP-HPLC method was used to correlate the extent of oxidation of protein X with its biological activity.

P38. A methodology for testing the removal of a polydimethylsiloxane based antifoam from biopharmaceutical products

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Chemical agents known as antifoams or defoamers are routinely used to limit foam accumulation in fermentation bioreactor processes. Dow Corning® Medical Antifoam C, a water-dilutable emulsion with the active ingredient of polydimethylsiloxane (PDMS), has limited aqueous solubility, complicating direct detection in a formulated active pharmaceutical ingredient (API). Following extraction with an acetonitrile and toluene solution, PDMS may be recovered in a volatile homogenous solution for analysis. If no other source of silicon is used during the manufacturing process, Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) may be used to detect silicon. The method was used to demonstrate clearance of PDMS post-filtration with cellulose acetate and polyvinylidene fluoride (PVDF) membranes.

P39. Expression and purification of human keratinocyte growth factor 2 with SUMO fusion in Escherichia coli

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The fibroblast growth factor (FGF) family consists of at least 22 members and 4 FGF-related growth factor members. Among them, keratinocyte growth factor (KGF) (FGF-7 and FGF-10) acts as a growth factor exclusively through the FGF receptor-2IIIb variant expressed by epithelial cells and hence can protect these cells from various insults, such as anti-cancer drugs and skin or organ injuries. SUMO (small ubiquitin-related modifier), a small protein with 100 amino acids, modulates protein structure and function by covalent modification of target proteins. SUMO fusion system that facilitates efficient expression of recombinant proteins in E. coli has recently been described. In this study, the fusion DNA fragment composed of SUMO gene and human KGF-2 gene was amplified by PCR and inserted into the expression vector pET28a to construct the recombinant plasmid, pET28a-SUMO-hKGF-2. The fusion protein was expressed in E. coli and recovered by Ni-NTA immobilized metal affinity chromatography. The expression level of the fusion protein was up to 30% of the total cellular protein. The fusion protein was then hydrolyzed by the SUMO proteases to get the recombinant hKGF-2 and further purified by gel filtration chromatography with the purity of higher than 95%., The mitogenicity assay showed that the recombinant hKGF-2 could significantly promote the proliferation of normal rat kidney epithelial (NRK52E) cells.

P40. Intact Protein Separation Strategies for Complex Biological Samples through the use of a High Recovery Superficially Macroporous Column Material .

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Identification of proteins, in a vastly complex biological mixture, presents a formidable challenge for analysis that no one separation protocol can satisfy. As a result, there is a need for sample specific separation methods to achieve high-resolution and high-recovery results for the downstream analysis of these samples. Currently the standard technique for resolving protein mixtures, prior to LC/MS/MS analysis, is two dimensional polyacrylamide gel electrophoresis (2D PAGE). However, this technique offers poor recoveries, relatively limited resolution and sensitivity with only mid to high abundant proteins detectable. As an alternate approach, separation of complex mixtures by liquid chromatography has been explored. Although chromatographic methods for protein analyses are well documented and particularly well understood, comprehensive separation strategies for challenging biological samples, has been very limited. One way to address the complexity issue of these mixtures is through pre-fractionation of the intact proteins prior to other analytical techniques downstream. Chromatographic pre-fractionation of intact proteins has important advantages compared with strategies that digest proteins at an early step. These advantages include the ability to quantitate and recover proteins as well as reduce sample complexity for facilitating protein identification in critical research areas, such as biomarker discovery, protein research, new drug development and disease diagnosis.

In this study, we present a novel HPLC column and optimized conditions for separating intact proteins in highly complex biological samples, and have applied them to a workflow for increasing protein identifications. Specifically, we have developed a superficially macroporous column material (mRP column) and optimized reversed-phase conditions for the separation of membrane lipid rafts, HeLa cell membranes, E. coli and an entire HeLa cell lysate. With use of the macroporous column and optimized conditions determined by sample type, we have demonstrated enhanced peak resolution, achieved high recoveries >98%, obtained high run-to-run reproducibility and permitted higher column load tolerances than traditional porous column materials. The fractionated protein samples were further evaluated by SDS-PAGE for separation efficiency and chip based nano-LC/MS for protein identity. By use of this column and preferred fractionating conditions, in combination with sample selective separation protocols for intact proteins, we present a robust methodology for permitting a higher certainty for protein identifications in complex biological mixtures.

P41. Combination of Affinity Depletion of Abundant Proteins and Reversed-phase Fractionation in Proteomic Analysis of Human Plasma/Serum

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Serum and plasma represent the most complex sample of the human proteome, composed of the homeostatic blood proteins as well as tissue leakage proteins. It is the most utilized clinical sample that can be readily obtained. The tremendous complexity of this biofluids proteome presents extreme analytical challenges in comprehensive analysis due to the wide dynamic range of protein concentrations (spanning over 10 orders of magnitude). Consequently, robust sample preparation methods remain one of the important steps in the proteome characterization workflow.

Depletion of high-abundant proteins in serum and plasma has become an essential, routine and accepted technique. These high-abundant protein components interfere with identification and characterization of important low-abundant proteins by limiting the dynamic range for mass spectral and electrophoretic analyses. We are presenting the results on a new column for the specific depletion of 14 high-abundant proteins from serum and plasma. Through depletion of the 14 high-abundant proteins we are removing ~94% of the total protein mass. The depletion process is robust, easily automated and highly efficient (30 min.). The column depletes the 14 targeted proteins reproducibly during 200 runs and has excellent depletion efficiency as determined by ELISA. Results on the identification of the bound proteins indicate specific removal of the targeted proteins.

We have depleted plasma of 14 high-abundant proteins and performed a subsequent fractionation using a high-recovery superficially macroporous column (mRP) under optimized reversed phase conditions. The chromatographic conditions and methods enabled high protein recovery while permitting robust and reproducible fractionation. The collected column fractions were trypsin-digested and analyzed on a microfluidic HPLC-Chip/MS system, providing a reliable and fast peptide separation combined with ease of use, robust ionization and fast data acquisition.

High-abundant protein depletion and RP fractionation of plasma showed an improved dynamic range for proteomic analysis resulting in reduced ion suppression in electrospray MS. The multi-dimensional workflow approach presented here allowed the identification of low-abundant plasma proteins.

P42. 2-D DIGE to facilitate therapeutic protein process development

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2-D Differential Gel Electrophoresis (DIGE) is an established method for studying protein expressions levels, identifying of biomarkers, designing novel drug targets and monitoring of therapeutic processes. We applied 2-D DIGE for downstream process development of a recombinant antibody; the anti-Rh(D) IgG1 expressed in CHO cells. Cell culture supernatant of two different clones named m250-9, at low viability (~ 40%) and m500-11 at low (~ 40%) and high (~ 90%) viability were purified employing Protein A affinity chromatography as capture step. For clone m500-11 at high viability a second purification step including cation exchange chromatography using either Source30S or CM Sepharose FF was applied. Due to SDS-PAGE and 2-D DIGE results the culture supernatant shows different protein pattern depends on the cell viability. Clones m500-11 and m250-9 at low viability shows much higher host cell protein content, than the culture supernatant of m500-11 at high viability. Additional the clone m500-11 at low viability presents higher degradation of IgG than other samples. It could be a reason for different elution of pH 3.5 on the Protein A column, that occurred as a broad and tailing elution peak. In contrast for the clone m500-11 at high viability and m250-9 even for 40% viability a sharp elution was obtained. Further purification by Source30S and CM Sepharose FF column did not improve impurity. Protein bands detected by SDS-PAGE were identified as IgG and IgGdegradation products using western blot. 2-D DIGE confirmed the similarity of protein composition of the Protein A and cation exchange chromatography elution pools. Additional more details about isoform pattern of IgG were observed. DIGE allowed tracking of impurities by direct comparison of culture supernatant and purified product. Visualization of host cell proteins removal and evidence of product purity are further advantages of this method.

P43.A new ultra high purity silica for large scale purification of peptides

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PharmPrep is a new, ultra high purity silica gel designed for large scale purifications. The manufacturing process being developed to specifically address large scale manufacturing of ultra high purity silica gel. The resulting material has a narrow pore size distribution centered at 100 Angstroms and exhibits high mechanical stability. It has highly predictable scale up behavior and results of this behavior are demonstrated by the development of methods for the purification of 20 and 30mer peptides from standard solid phase synthesis columns. It is currently available in 10 micron particle size as Silica and C18 bonded phases. Due to the extremely high purity of the base material no end-capping is necessary as demonstrated by the separations shown.

P44. Proteomic Investigations into the toxicity of aristolochic acid

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Ingestion of extracts from Aristolochia species is associated with the development of a progressive renal disease designated Aristolochic Acid Nephropathy (AAN). AAN is characterized by chronic renal failure, tubulointerstitial fibrosis and eventually by urothelial cancer.

Aristolochia extracts contain a mixture of structurally similar components, the most abundant being aristolochic acid I (AA I), and aristolochic acid II (AA II). In animal studies both AA I, and AA II have been shown to form DNA adducts and are mutagenic (genotoxic). However, nephrotoxicity is only associated with exposure to AA I. We followed specific alternations to the kidney proteome in sensitive mice after treatment with AA I and AA II. Quantitative difference in proteome of treated and non-treated animals was determined by MS/MS after iTRAQ labeling.

Proteins identified in the urine of AA I, and AA II treated mice serve as candidate biomarkers for the diagnosis of AAN. It could be shown that the iTRAQ MS/MS and quantitation is a reliable and reproducible method for determining relative protein levels in complex samples such as whole cell lysates.

P45. 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.