L1. Development of high-resolution gas-phase separations for analysis of complex biological mixtures.

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During the last decade hybrid ion mobility spectrometry (IMS)/mass spectrometry (MS) techniques have emerged as a powerful approach for analyzing complex mixtures from biological sources. Our group has focused on the analysis of proteomes from tissue and plasma. This talk will describe efforts to develop high-throughput LC-IMS-MS methods for characterizing proteomes. The inclusion of an IMS separation increases peak capacity, leads to a reduction in interference associated with chemical noise, and provides a means of generating fragmentation data for mixtures of ions in a parallel fashion. Recently, we have worked toward the development of a circular drift tube. In theory, multiple passes should allow very long separation lengths to be realized. In some studies we have drifted ions for more than 50 m. The development of this technology has included a number of surprises, including a new type of mobility measurement that we call overtone mobility spectrometry. The emergence of these methods, current limitations, and future potential will be discussed.

L2. Using Chromatographic Intelligence to simplify LC/MS Peptide Map Analysis.

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LCMS peptide maps are primary tools for characterizing recombinant and purified proteins. The correct assignment of detected ions to peptide structures is critical to properly interpreting data from such studies. In theory, proteolytic digestion of a protein, separation by high resolution chromatography and detection by high resolution mass spectrometry should produce readily interpretable results. This is not the case. Even simple tryptic peptide maps can generate thousands of ions spanning the full dynamic range of detection of modern mass spectrometers. While many of the detected components arise from the imperfections of sample processing workflows, others are artifactually generated during the analysis itself. This presentation will discuss our efforts to understand the sources of such data complexity, detail experimental factors critical to obtaining high quality mapping results, and document ongoing efforts to automate processing of LCMS maps. In particular, the utility of recognizing chromatographic alignment of groups of ions will be explored as a means of improving and simplifying the interpretation of peptide mapping results.

L3. Ultrafast immunoextraction and microaffinity columns: Recent developments in the use of affinity-based separations for pharmaceutical and biomedical applications

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Affinity ligands have been used for decades for both the selective isolation and analysis of sample components in complex mixtures. The selectivity and strong binding of many biological agents that are used as affinity ligands, such as antibodies, have also made these ligands of great interest over the last decade for use in HPLC methods and, more recently, in microaffinity LC methods. This presentation will examine some recent developments in the creation and bioanalytical applications of affinity microcolumns. One set of methods that will be discussed are chromatographic-based immunoassay, in which antibodies or related agents have been used in a variety of detection formats for the rapid analysis or drugs, hormones, proteins, and other analytes. Affinity microcolumns are particularly valuable in the area of ultrafast immunoextraction, in which analytes are isolated from samples in the sub-second time domain. One unique application that will be discussed for this approach is in the analysis of free drug and drug and hormone fractions in blood. Several approaches for conducting these assays will also be examined, including a number of competitive or displacement assay formats and the use of either packed microcolumns or small affinity monoliths. Affinity microcolumns have been shown to be valuable in the studying the thermodynamics and kinetics of biological interactions. This type of application will be illustrated by using examples based on the binding of drugs with serum agents such as human serum albumin HSA, alpha 1-acid glycoprotein and lipoproteins. The benefits and limitations of using affinity microcolumns for this purpose will be examined and the use of this approach in the high-throughput screening of biological interactions will be discussed.

L4. Solubility Studies and Binding Capacity and Resolution Evaluation on HIC Resins for PEG Lysozyme

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For the purification of PEGylated proteins mainly Size-Exclusion and Cation-Exchange Chromatography are used. Applications of the Hydrophobic-Interaction Chromatography (HIC) are rather rare.

In this paper, the binding properties and resolution of PEG Lysozyme derivatives with varying molecular weights of PEG with 5 kDa, 10 kDa and 30 kDa are presented. To find the optimal range for the operating conditions, solubility studies were performed in 96 well plate format and optimal salt concentrations and pH value were determined. The solubility of PEG proteins was strongly influenced by the length of the PEG moiety. With increasing molecular weight of the PEG, higher deviations from the classical precipitation theory from Melander and Horvath [1] of proteins were found. Additionally, big differences for the solubilities of PEG Lysozyme in ammonium sulfate and sodium chloride were found.

The binding capacities for PEG Lysozyme to HIC resins are dependent on the salt type and molecular weight of the polymer. Binding capacity maxima of about 80 mg/ml for the 5 kDa mono-PEG Lysozyme were found in 1.2 M ammonium sulfate and more than 100 mg/ml in 4 M sodium chloride. For the 30 kDa PEG Lysozyme the binding capacities were lower due to restricted access to the pore volume because of the large molecule size.

In ammonium sulfate, the elution order for Lysozyme, Mono PEG Lysozyme and Oligo PEG Lysozyme separated on a TSKgel Butyl-NPR hydrophobic interaction column was found to be reversed to that obtained on a TSKgel SP-NPR cation-exchanger column. In contrast to the cation-exchanger column, resolution of the Mono PEGylated Isoforms was not possible with the HIC column in ammonium sullfate. Interestingly, the elution order reversed in 4 M sodium chloride solution again and same fine resolution as in cation exchange could be achieved. For these phenomena, a tentative explanation is given.

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L5. Chemical Proteomics: Beyond the Profiling of Proteins

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Chemical proteomics is a new branch of proteomics research with the goal of not only profiling, identifying and quantifying proteins in complex biological samples, but also to obtain information about protein activity (e.g. in case of enzymes) or protein-protein interactions. Chemical reagents designed to interact with the active site of a given class of enzymes, so-called affinity-based proteomic probes (ABPs), allow to probe the activity profile of this enzyme class in a quantitative manner1,2. Since enzymes, such as kinases or proteases, exert their function through their activity, this gives a better picture of their implication in given biological processes as compared to measuring their abundance or their respective mRNA profiles.

Notably proteases are involved in tissue destruction or the liberation of powerful signaling molecules from cellular membranes and must be tightly controlled in time and space to avoid pathologies due to excessive tissue destruction. In this lecture I will give an overview over various ABPs and their use to profile different protease families. While serine and cysteine proteases can be profiled with ABPs containing a reactive group that covalently modify the active site serine or cysteine residue, this strategy is not viable in case of metalloproteases, which use an activated water molecule in the active site for substrate cleavage3. I will describe approaches to generate photoactivatable ABPs to overcome this limitation.

Another promising approach to profile active proteases relies on immobilized, reversible protease inhibitors and affinity solid-phase extraction (A-SPE). A-SPE is particularly useful when profiling active proteases in body fluids, where they are at low concentration. Combining A-SPE with stable isotope labeled internal standards allows quantification by LC-MS. I will present first data about an integrated A-SPE-LC-MS system with a built-in trypsin reactor that allows quantification of MMP-12 down to sub-nM levels4.

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L6. On-column Refolding of Autoprotease Fusion Proteins Using N^{pro} Fusion Technology

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Efficient production of peptides and proteins with authentic N-terminus is still an unsolved problem. Our approach is the expression of fusion proteins using an autoprotease system. Advantage of this system is the high expression yield (15 g/l) and refolding at concentrations up to 10 mg/ml for small peptides. On-column refolding (OCR) is a potential option to overcome the limitations adherent with batch refolding techniques based on dilution. The N-terminal autoprotease Npro and the mutant EDDIE from classical swine fever virus are used as fusion tags for the expression of peptides and proteins as IBs. Target peptides and proteins respectively with an authentic N-terminus are generated by self cleavage by switching from chaotropic to kosmotropic conditions. Two different approaches have been followed using GFP and interferon as model proteins. In a one-step procedure the denatured fusion protein is loaded onto an ion exchange column and thereupon autoproteolytic cleavage and refolding of the target protein is immediately initiated by application of a refolding buffer with high ionic strength. Refolding takes place along with the elution procedure. The second technique investigated comprises a two-step procedure. After loading and washing, a refolding buffer with low ionic strength is applied whereupon only refolding and cleavage of the autoprotease takes place in the adsorbed state. Subsequently, a second buffer which is optimized with respect to refolding conditions of the target protein is applied. Examples for both techniques with different target proteins will be presented and compared to conventional refolding techniques. The developed OCR technique has high refolding yield and enables refolding at high concentrations.

L7. An Alternative to 2DGE: PEC-TLC for Peptidomics and Proteomics

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Planar electrochromatography (PEC) has been described in the literature for many years, as a possible alternative to thin layer chromatography (TLC) or gel electrophoresis (GE). It continues to be of interest today in several formats, especially under high pressures, where it performance is comparable to HPLC. We have studied using PEC with modern, commercial TLC plates, in 1D or 2D (PEC-TLC) formats, for separating individual peptides or for peptide mapping, especially for phosphopeptides, and in pursuing phosphoproteomics. Various detection schemes are possible, spray reagents, UV/FL, selective tagging for phosphopeptides alone, DIGE, and, of course, on-plate MALDI-TOFMS. We describe here the development of doing 2D separations using PEC-TLC, applications for phosphopeptides, and where this field could move in the future.

L8. Shotgun Proteomics has some holes

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Proteomics has generated considerable interest and many laboratories have invested in long-range and costly studies, notably in the area of biomarkers for the diagnosis, prognosis or prediction of disease. Although there is no dispute that existing approaches are powerful, many studies have failed to deliver what is anticipated; worse still, others have misdirected other research efforts.

By way of examples (e.g., biomarker discovery and validation, protein quantification, pathway elucidation, comprehensive peptide and characterization), the strengths and weakness of existing proteomic methods will be illustrated and discussed. Special attention will be direct at the shotgun strategies that make use of combined liquid chromatography and tandem mass spectrometry. The rationale for this approach will be critically examined, and the strengths and weaknesses objectively discussed.

L9. Antibodix Packings

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This paper will present recently developed non-porous PS/DVB packing materials for ion-exchange chromatography, specifically for monoclonal antibody separation. Their particle sizes are controlled at 1.7, 3, 5 and 10 um. Those PS/DVB particles are surface modified with a proprietary technology that has negligible non-specific interactions with monoclonal antibodies. The smaller particle size offered higher resolving power for the variants of monoclonal antibodies. A number of applications have been developed for monoclonal antibodies, pegylated antibodies, peptide modified antibodies and other monoclonal antibody derivatives.

L10. Temperature responsive ion-exchange chromatography resins

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Temperature responsive chromatographic resins have potential for the cost effective and environmentally benign extraction and purification of high value bio-molecules. This paper reports the development of novel temperature responsive resins using pH stable organic matrices and their suitability for large scale protein separation. Temperature responsive ion-exchange resins were manufactured by grafting various N-isopropylacrylamide copolymers onto organic support materials (e.g. agarose beads). Adsorption isotherms for various proteins, e.g. lactoferrin at 20 ºC and 50 ºC were obtained statically and the maximum adsorption capacities (Bmax) determined using a Langmuir isotherm model. In addition, the dynamic lactoferrin adsorption and desorption characteristics of lactoferrin with these resins were studied at 20 ºC and 50 ºC. There was a significant increase in static lactoferrin adsorption capacity at high temperature compared to that obtained at low temperature. Using dynamic conditions, protein adsorbed at high temperature could be desorbed by simply reducing the temperature of the mobile and stationary phases. Additional studies have demonstrated that these new

resins selectively adsorbed cationic proteins from a mixture of cationic and anionic proteins in solution. A model for the adsorption and desorption of proteins from these new resins is proposed. The temperature responsive resins offer a novel and environmentally-friendly separation process amenable to processing industrial feedstocks.

This investigation was funded by the Australian Research Council Special Research Centre and the CSIRO Food Futures National Research Flagship. P.M. is a postgraduate scholar supported by a Victorian Government Science, Technology and Innovation (STI) Grant and the Australian Research Council Special Research Centre.

L11. The Use of De Novo Designed Synthetic Peptide Mixtures to Develop Effective Reversed-Phase Materials for Separation and Purification of Peptides

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There is a high demand for new reversed-phase high-performance liquid chromatography (RP-HPLC) materials with unique selectivities that can be used effectively in the purification of synthetic peptides in order to meet FDA purity expectations. Recent developments in silica surface modification have yielded sorbents modified with polar groups which can provide alternative selectivity for reversed-phase matrices. In the present study, we have designed and synthesized four series of synthetic peptide standards with the sequences Gly-X-Leu-Gly-Leu-Ala-Leu-Gly-Gly-Leu-Lys-Lys-amide (where the N-terminal either contains a free a-amino group (Series 1) or is Na-acetylated (Series 2)) and Gly-Gly-Leu-Gly-Gly-Ala-Leu-Gly-X-Leu-Lys-Lys-amide (where the N-terminal either contains a free a-amino group (Series 3) or is Na-acetylated (Series 4)), where position X is substituted by Gly, Ala, Val, Ile, Phe, Tyr, Asp or Glu. Thus, these peptides represent series of peptides with single substitutions of n-alkyl (Gly<Ala<Val<Ile in order of increasing hydrophobicity), polar and acidic (Asp, Glu) or aromatic side-chains (Phe, Tyr). Note that peptide series 1 and 3 or 2 and 4, with the same amino acid substitution, represent peptides of the same composition and minimal sequence variation (SCMSV), with the substitution site, X, being towards the N- or C-terminal, albeit with identical adjacent residues (Gly-X-Leu) to maintain the same environment around position X.

These peptides would most likely be considered extremely difficult, if not impossible, to separate. The current work evaluates the chromatographic selectivity of silica-based stationary phases with various modifications (polar embedding and polar endcapping on C18 columns; ether-linked phenyl column with polar endcapping) compared to n-alkyl C18 and C8 and aromatic phenylhexyl columns. In addition, the current worldwide shortage of acetonitrile also underlines the importance of a fresh assessment of the general utility of alternative organic modifiers, such as methanol, for analytical and preparative peptide separations. Thus, peptide mixtures were run on all columns at pH 2 (linear gradients of acetonitrile or methanol in aqueous mobile phases). Our results clearly demonstrate the useful complementarity of different RP-HPLC packing materials. For instance, peptide mixtures showed profound selectivity differences between n-alkyl stationary phases and the polar embedded and polar endcapped C18 stationary phases. In addition, changing the polarity of the reversed-phase matrix in combination with changing the polarity of the mobile phase (acetonitrile versus methanol) is shown to be synergistic to maximize selectivity of peptide separations. Finally, our approach has enabled us to assign the type of side-chain that is affected by

the change in the type of reversed-phase column or change in the polarity of the mobile phase. This information is critical in the design of new packing materials as well as allowing the end user to choose the optimum packing material for specific applications.

L12. Use of peptide retention prediction model for investigation of RP-LC peptide separation selectivity

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Ultra high performance reversed-phase liquid chromatography provides great peak capacity, improving the prospects for separation of complex mixtures. However, the resolution of all critical peaks in complex mixtures is still difficult, e.g. in peptide mapping applications. The optimization of peptide separation is usually performed by modifying the concentration of ion-pairing agent, gradient slope, separation temperature or type of sorbent. Because the impact of these parameters on separation selectivity is not incompletely understood, the optimization could be a frustrating task.
In order to better understand the peptide separation selectivity, we have measured the retention behavior of set of tryptic peptides at various chromatographic conditions, and developed a robust retention prediction model. The model is based on summation of amino acid retention coefficients (AARC's). The AARC values at selected chromatographic conditions represent the contribution of given amino acid residue type to peptide retention. The AARC values and their changes at various conditions (e.g. ion-pairing reagent concentration) were helpful to elucidate the impact of separation conditions on the peptide retention and provide a rationale for changes of the separation selectivity. The preliminary conclusions are:

(1) The concentration of ion-pairing reagent predominantly affects the AARC values of charged amino acids (H, K, R). The co-eluting peptides differing in their charge can be resolved by adjusting the ion-pairing reagent concentration.

(2) The presence of P, V, I, and L aliphatic amino acid residues enhances the peptide retention at elevated temperatures. Y and W aromatic residues have the opposite effect. Retention contribution of H, K, and R residues also decreases with the temperature.

(3) Ion-pairing interaction is more pronounced at shallow gradients.

(4) Sorbents with different sorbent pore size (300Å versus 130Å) appears to have different selectivity, although AARC values suggest otherwise. The differences are caused by differences in available surface area. When scaling gradient volumes proportionally to the available surface area in the column, selectivity is essentially identical.

(5)Gradient slope changes affect relative retention of peptides with different molecular weight (length). Relative retention of peptides with similar length is not affected.

(6) Various types of reversed-phase stationary phases have been investigated. AARC values suggest the differences in selectivity (Phenyl vs. C18 vs. embedded polar group C12 vs. unendcapped C18).

The postulated hypotheses were tested with specifically designed sets of synthetic peptides.

L13. An alternative method for peptide mapping using mixed-mode stationary phases

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Pepsin-catalyzed hydrolysis of porcine haemoglobin revealed the presence of several interesting bioactive peptides in the mixture. A detailed knowledge of the different fragments is necessary in order to better understand bioactivity, and the separation steps must be optimized in order to ameliorate purification and quality control processes. In many cases the first chromatographic step for peptide separation is ion exchange chromatography, whereas reversed phase chromatography is usually applied for the last purification.

Mixed-mode silica-based chromatography materials developed in our laboratories combine both reversed phase (RP) and weak anion exchange (WAX) characteristics and offer a wide range of applicability for the separation of neutral, acidic, basic, or amphoteric compounds. Chromatographic runs can be performed in many different interaction modes such as reversed phase, hydrophilic and hydrophobic interaction, ion exclusion, ion exchange, or a combination of several of those modes.

In our study, we used the versatility of RP/WAX stationary phases to perform peptide separation in different modes just by varying modifier concentration and gradients. The use of different eluent systems on a single multimodal column lead to complementary elution profiles. Consequently, the use of such material for peptide mapping enables an increase in sequence resolution.

L14. High-resolution Separations of Polypeptides Using Fused-Core™ Particles

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Fused-core™ 2.7-µm silica particles with 90 Å pores previously have been shown to be highly efficient for separating small molecules in the range of up to about 2000 molecular weight (MW). Columns of such particles demonstrate efficiencies that are comparable to those for sub-2-µm totally porous particles, but with less than one-half of the operating back pressure. Wider-pore fused-core particles (140 Å) have been developed for rapid and high-resolution separations of polypeptide mixtures of up to about 20,000 MW. In addition to high efficiency, columns of these new particles demonstrate excellent peak shape and broad compatibility with desired mobile phase compositions and with relevant sample types. Separations typically are faster because of superior mass transfer properties of core-shell particles at higher mobile phase velocities. Gradient elution peak capacity studies for columns of the new fused-core particles demonstrate the high-resolution utility obtained with superficially porous particles, without disadvantages previously seen with non-porous particles. The fused-core particles show good sample loading properties, since the surface area per column is nearly equivalent to that in columns packed with comparable totally porous particles, and high retention, due to optimal bonded-phase selection and surface modification approaches. Highly stable bonded phases permit extended operation at temperatures up to at least 60 oC with the low pH mobile phases usually required for separating peptides and conducting LC-MS analyses.

L15. pISep, a multi-dimensional LC system for separating proteins using fully controllable pH gradients

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pISep is a major new advance in low ionic strength ion exchange chromatography. It enables the formation of externally controlled pH gradients over the very broad pH range from 2 to 12. The gradients can be generated on either cationic or anionic exchangers over arbitrary pH ranges wherein the stationary phases remain totally charged. Associated pISep software makes possible the calculation of either linear, nonlinear or combined, multi-step, multi-slope pH gradients. These highly reproducible pH gradients, while separating proteins and glycoproteins in the order of their electrophoretic pIs, provide superior chromatographic resolution compared to salt. The interesting elution properties of proteins in these controlled pH gradients stimulated us to formulate a statistical mechanical model for protein binding to ion exchange stationary phases that is an extension of the current electrostatic interaction theory for the general dependence of the chromatographic retention factor, k, on both salt and pH simultaneously. Further development of controlled pH gradients in the presence of salt, urea or acetonitrile has allowed us to develop the first true two dimensional liquid chromatography.

L16. Development of a Chromatofocusing Method for Charge Variant Determination of a Monoclonal Antibody

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In the production of monoclonal antibodies it is necessary to quantify and characterize antibody charge variants. Ion-exchange chromatography with a salt gradient is typically used for this purpose. This poster details the development an ion-exchange chromatography method for the analysis of the monoclonal antibody MAB1. Due to the near neutral pI of the MAB1 protein, both cation-exchange and anion-exchange chromatographic modes were studied and within each chromatographic mode, the separation of charge variants using either a salt gradient or a pH gradient (termed chromatofocusing) was assessed. The optimal method for the analysis of MAB1 was determined to be an anion-exchange chromatofocusing method. This method was shown to resolve multiple charge variants with excellent repeatability and was free of the chromatographic artifacts and lack of resolution observed in the other chromatographic methods studied. The chromatofocusing method is suitable for use as a characterization tool and for inclusion into the Quality Control System of MAB1.

L17. A Glycoprotein Approach to Biomarker Discovery

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We have developed affinity-based methodology for the selection of glycoproteins from serum. This affinity methodology is sensitive to specific structural glycan moieties particular to different stages and grades of cancer, and will be utilized for the production of glycoarrays to study cancer-specific changes in glycan structure using lectins. There will be two complementary strategies used to discover markers. We will use a lectin array to select the lectins that show the largest differential change between disease groups for extracting the glycoproteins from the serum samples. The first method will then use the glycoarray/lectin strategy for detection where glycoproteins extracted from serum will be separated and printed as a glycoarray on glass slides. Various fluorescently-labeled lectins will be hybrized against the slides to determine the response of each protein to the lectin. The lectins reveal changes in the structure of the glycoproteins during the course of the cancer. In a second strategy we will use glycoprotein extraction by a lectin column, isotope labeling, digestion and LC-MS/MS to identify markers. This method is a mass spec based method to identify changes in glycan level. The two methods will identify a set of complementary and overlapping markers. A lectin blot will then be used to confirm the lectin response to these markers. An antibody/glycoprotein/lectin sandwich assay using a bead based method is then demonstrated as a means to further confirm these markers in a high throughput manner. This bead based assay would be the basis for future work in clinical validation. We will demonstrate this marker development to pancreatic cancer for detection of pancreatic cancer against pancreatitis, diabetes and normal controls; colon cancer for colon adenocarcinoma, versus adenomas and normals; and hepatocellular cancer versus cirrhosis. We will show the improved specificity and sensitivity for these methods versus current markers used in the clinic.

L18. Beyond Protein Identification, Making Sense of Proteomic Data

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Proteomics has emerged as a key technology in the post-genomic era, and has gained widespread use in numerous fields. The mass spectrometers used for proteomics have become extraordinarily powerful, and can produce data at a rate that was unimaginable only a few years ago. This has led proteomic researchers to a multiple dilemmas – like what do I do with all of the data and what does it all mean? Automated database search strategies appeared to answer this problem, but recently it has come to light that these can yield a large number of false positives, which lead researchers to waste months of time in a futile effort to validate these incorrect assignments. This conundrum is further exacerbated by the large proteomic efforts that are typically employed for biomarker identification, where tens to hundreds of different samples are analyzed. In addition, proteomic experiments generate large lists of proteins, often exceeding 1,000, each of which needs to be evaluated from a biological perspective so that the research can piece together the meaning of the experiments that have been performed. Consequently, it has become relatively easy to obtain large amounts of proteomic data, however, the tools associated with interpreting this data have not kept pace. The focus of this talk will be to highlight techniques for this next step in proteomic data handling, specifically, focusing on new methods to statistically evaluate proteomic data and to place this data into its biological context using systems biology approaches.

L19. New Proteomic Tools in Protein Characterization and in Optimization of Process Production.

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Over the past several years, the need for comprehensive, rapid and reliable characterization of post-translationally modified proteins during their production by either mammalian cell culture procedures or fermentation methods has become ever more pressing. As a consequence, considerable interest has been generated in this and other laboratories to enable this goal to be realized. A central driving consideration behind this work has been the need to utilize experimental tools that can accommodate samples of considerably complexity and with the various components exhibiting differences in abundances extending over many orders of magnitude, yet the methods should be highly selective and compatible with highly sensitive methods of detection.

Here, we describe new ways to carry out LCxLCxLC/MSxMS and LCxLCxCE/MSxMS in the capillary modes, utilizing novel sample handling procedures and methods of quantitation via the use internal standards that allow recoveries and precise structural features to be determined. Particular attention has been place on optimizing the capillary separation steps prior to carrying out the mass spectroscopy with the objective to allow target protein or other bio-molecule characterization to be rapidly carried out on parallel with host cell protein (HCP) and cell metabolite determinations. Arising from these studies has thus been the opportunity to interface proteomic-like tools into a suite of methodologies necessary to achieve the optimization of the production cycle for recombinant proteins via process analytical technologies. Moreover, such approaches offer considerable scope in the discovery of new classes of biological effectors, such as novel oligosaccharide and lipid compounds with pronounced biological activities.

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L20. On-line HPLC as a PAT for Controlling Product Collection from Process Scale Chromatography Columns

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Process scale chromatography is a widely used unit operation in peptide and protein manufacturing to isolate the product(s) of interest with the required purity. Variability in the elution pattern from large scale purification columns makes it difficult to know precisely where to start and stop collecting the peak of interest. To account for this variability, companies typically collect fractions which are assayed off-line to determine which material to include in the mainstream pool for further processing. This off-line analysis approach adds a considerable amount of time to the overall process cycle time which negatively impacts throughput and process equipment utilization. Use of an on-line HPLC based analyzer capable of automatically sampling and analyzing the process column eluent eliminates the delays associated with off-line analysis and provides near real-time analysis of product purity. The availability of product purity information in near real-time enables the process scale chromatography process to be automatically controlled and eliminates all fraction collection. A process scale chromatography step in a commercial peptide manufacturing process will be presented to demonstrate the application of on-line HPLC as a PAT to enable increased product yield, decreased process variability, and decreased process cycle time.

L21. Continuous affinity purification of proteins by simulated moving bed (SMB) chromatography

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Simulated moving bed chromatography (SMBC), has been successfully applied to small and large-scale binary separations of hydrocarbons, sugars, and enantiomers but has rarely been used for affinity purifications. SMBC emulates counter current separation where the mobile phase flows in the opposite direction of the solid phase. The solid phase is represented by individual columns connected in series, and the mobile phase by inlet streams of feed and eluent and outlet streams of raffinate and product. Valves between the columns are successively switched open or closed at timed intervals to introduce the inlet streams and withdraw the outlet streams. The stepwise advance of the streams in the direction of fluid flow simulates counter current movement of the columns. Stable, highly, efficient zones of separation created by SMBC enable continuous peak shaving of the target molecule for extremely high purity and recovery. The presentation will demonstrate SMBC for continuous affinity purification of histidine-tagged proteins and monoclonal antibodies. A bench-scale SMBC instrument was employed to investigate immobilized metal and protein A affinity purification methods, respectively. Advantages of SMBC over standard linear or batch processes include dramatically increased productivity, purity, and efficiencies in chromatography media and buffer utilization. Affinity tag-affinity ligand interactions simplify the fractionation behavior of complex biological feed streams to binary mixtures for purposes of SMBC protein purification and increase the attractiveness of this continuous technique for bioprocessing.

L22. Structure-Based Modeling of Transport in Monoliths

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Monolithic ion-exchange media have emerged in the past decade as efficient, high-performance alternatives to traditional packed beds, particularly for preparative scale purification of large bioparticles such as viruses and plasmids. The most notable advantage of monoliths is that mass transfer is dominated by convection and is therefore fast, even for large bioparticles. However, some noteworthy aspects of chromatographic performance in monoliths have been observed, including significant tailing in breakthrough curves and convective entrapment of large bioparticles that is reversible by a reduction in flow rate. These phenomena may be attributable to possible mass-transfer limitations due to slow diffusive transport despite the convective dominance. This warrants further investigation by appropriate modeling, but most attempts at predictive modeling have been limited to simplified or reduced geometries. Because the complex, irregular structure of the monoliths results in an equally heterogeneous flow field, a thorough analysis of performance necessitates an explicit, high-resolution representation of the geometry and the corresponding velocity distribution. Such direct analyses have previously been successfully employed for analysis of flow and dispersion in packed beds.

This presentation will outline aspects of experimentally observed behavior in chromatography in monoliths, and how they may be explained by aspects of transport in these media. We will also present a modeling approach based on a realistic three-dimensional structural reconstruction of a polymeric monolith that was obtained by serial sectioning and imaging by scanning electron microscopy. Various characteristics of the monolith such as porosity and pore size distribution were obtained by image analysis and compared with existing literature values. The microscopic flow field was obtained for the entire sample block using a three-dimensional Lattice-Boltzmann method and a stochastic mass-transfer simulation was implemented to model the dispersion behavior of solute in the monolith. The calculated permeability falls within the range of published experimental values. Point velocities indicate considerable variation in local Peclet

numbers, suggesting varying degrees of contribution from diffusion and convection. The stochastic dispersion simulations further establish the complexity of mass transfer in these media, showing that overall transport is significantly influenced by various factors such as solute size, local geometry and the average column velocities. Extension of the model to account for adsorption behavior will also be discussed.

L23. Membrane adsorbers to capture cell culture derived virus particles

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Membrane processes have been used for bioprocesses long before modern membrane industry has been emerged. Their increasing application in chromatography is mainly triggered by the fact that they are not compressible compared to conventional bead supports and eliminate diffusion limitations. Over the last decades, new membrane systems have been developed to meet the requirements of the pharmaceutical industry to purify cell culture-derived viral particles and viral vectors. One focus is the application of highly specific affinity membrane adsorbers. For these systems economic capturing of large biomolecules is mainly limited by the properties of the affinity ligands. Particular attention has to be paid to the ligand interaction kinetics and the desorption conditions. Naturally, availability, stability, applicability in pharmaceutical production processes and ligand costs are also crucial points to be considered in the development of economic capturing processes.

Our study gives a comprehensive overview on applications of membrane adsorption chromatography to capture cell culture-derived influenza and vaccinia virus particles and compares these methods to bead based resins.
Madin-Darby canine kidney (MDCK) cell culture-derived influenza virus particles (A/Wisconsin/67/2005, A/Puerto Rico/8/34, B/Malaysia/2506/2004) have been captured by anion exchange and affinity membrane adsorbers. The applied affinity and pseudo-affinity ligands were lectins, sulfated carbohydrates (e.g. heparin, sulfated glucose polymers) and specific peptides. The cost effectiveness of the process is heavily influenced by the selection of the specific, but in the case of influenza virus particles, substrain independent ligands. One possibility are peptide ligands e.g. against the sialic acid binding pocket of hemagglutinin, the major immunogenic envelope protein of influenza viruses. Identification of such ligands from synthetic libraries is the focus of current experiments.

The pseudo-affinity ligands employed for the capturing of primary chicken embryo fibroblast (CEF) culture-produced Modified Vaccinia Ankara (MVA) virus were

sulfated carbohydrates. Here, in particular we compared the commercially available bead based resin Cellufine® sulfate with sulfated reinforced cellulose membranes. Furthermore, we compared the noticeable DNA depletion capabilities of this technology with classical ion exchange adsorbers.
Capturing both influenza and vaccinia virus particles the ligand specificity of affinity membrane adsorbers afforded a remarkable high degree of contaminant depletion. Up to 90% of the host cell DNA was eliminated compared to the starting content. Host cell proteins could be reduced to less than 5% of the initial amount, whereas an overall product yield of 80 to 90% could be achieved. In some applications host cell DNA and protein concentrations are close to meet regulatory requirements for vaccine products after a single unit operation. Hence, affinity membrane adsorption of cell culture-derived viral particles enables the capturing of viral particles at an excellent purity by a high volumetric throughput – representing an economic tool for vaccine production processes.

L24. Chromatographic Purification of Replication-Defective Influenza Virus Vaccine: Enabling Fast and Efficient Vaccine Production

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The past years have shown an increase in the demand for influenza vaccine and the inability to produce sufficient supply has emphasized the drawbacks of the existing production processes- embryonated chicken eggs as a substrate for influenza virus propagation. The inflexibility of these processes and new approaches to vaccinations have stimulated the development of cell culture based production techniques. Existing down stream processes were developed primarily for egg derived and inactivated influenza vaccines. With these new cell based vaccines, the need for alternative, efficient and scalable purification methods has arisen. Chromatography is becoming a method of choice for virus purification and the relevance of this technique was recently demonstrated for influenza viruses.

This presentation will deal with the development and optimization of a chromatography based influenza virus purification process. The presentation of different methods with an emphasis on anion and cation exchange monolithic supports and size exclusion chromatography will be presented. The robustness of the methods has been tested with diverse subtypes of influenza A and influenza B viruses and virus yields were estimated in terms of infectious virus particles and total virus particles. Purity was assessed in terms of host cell protein and host cell DNA. Various combinations of the methods will be presented and the process used for preparation of clinical grade material will be described in details. During the down stream process of more that 99% of host

cell DNA and proteins were removed and up to 50% of infectious virus was recovered. In addition, chromatographic in process control methods for influenza virus will be presented.

L25. Polymeric Monolithic Ion-exchange Stationary Phases for the Separation and Purity Profiling of Biopharmaceuticals

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Biopharmaceuticals have become an important part of the portfolio of many pharmaceutical companies and their importance is still growing. Biopharmaceuticals include for example oligonucleotides and proteins (e.g. monoclonal antibodies) and have found their use both in diagnostics and therapeutics. The identity and purity of biopharmaceuticals is of great importance in their development and for their end use in humans. Therefore, the characterization and efficient purification from closely related impurities is important. Maintaining the integrity and function of the biopharmaceutical throughout a purification or characterization scheme is also important and increases the demands put on such schemes. One example of a soft separation technique which enables the preservation of function is ion exchange chromatography (IEX).

Historically IEX stationary phases have been mostly developed for the efficient separation of inorganic or small organic ions. In the last 10-15 years the development of monolithic stationary phases, in particular porous polymer monoliths has extended this application to the high-throughput and efficient separation of proteins and large organic molecules due largely to the benefits of convective rather than diffusive mass transport. However, current IEX stationary phases are more suited to the separation of relatively small proteins and do not provide adequate selectivity, binding capacity or resolution power to characterize charge variants for biopharmaceutical compounds. Also, the retention behaviour of many biomolecules by IEX stationary phases is not well understood in terms of mixed mode interactions. Due to their size and complexity, characterization of their charge variants by IEX is far more challenging than other smaller size proteins. Currently, there is a great demand for high selective, efficient IEX stationary phase to characterize monoclonal antibodies as well as other biopharmaceutical compounds.

This presentation will describe our current work in the development of new polymeric monolithic stationary phases for separation and purity profiling of therapeutic proteins and oligonucleotides. A range of approaches for imparting suitable surface

chemistry will be demonstrated including co-polymerization, post-polymerization grafting or coating the monolith with suitable functional nanoparticles, and the separation capabilities compared. Comparison of a range of chemical functionalities will also be made, including cation, anion and zwitterionic ion-exchange materials. We will also demonstrate how careful optimization of the separation conditions (type and amount of salt, temperature, organic modifier) can be used to further improve separation performance and to elucidate retention mechanisms.

L26. Innovative high throughput protein purification in 96-array format

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A new platform technology has been developed which enables 96 array format column chromatography. The design allows the user to select any chromatographic material which is packed with due consideration to individual material compression requirements. Bed containment between two filter frits ensures high efficiency and peak symmetry similar to that of preparative and process separation columns, and distinguishes the system from the current filter based systems for simple on/off sample equilibration operation.

Quality packed MiniColumns allow high performance separations to be achieved with minimal use of mobile phase and extremely low sample volumes and mass.
Liquid flow in the columns (CV 50 to 600µl) was driven with positive displacement fluid transfer systems, thus mimicking the situation in columns individually connected to a one channel stand-alone chromatography system.

Fractions from step elution were collected into standard microplates, utilizing an automated microplate transport system and subsequently submitted to analysis in a UV plate reader or other analytical methods (ELISA, MS or HPLC) .
The combined robotic system (Atoll MiniColumn and Tecan Freedom EVO) allowed to perform automated high throughput small scale bio-chromatographic separations of protein samples by running up to eight individual columns simultaneously.
Application examples shown, include protein separations by step gradient elution after binding the samples to affinity chromatography media, followed in a second dimension by de-salting under isocratic conditions.

These applications were successfully implemented in industry for parameter elucidation and optimization in process development of therapeutic protein production, in-process monitoring of fermentation broth for mAb-production and sample preparation for mass spectrometry analysis in antibody screening.

The result was to establish fully automated, walk-away procedures with a significant reduction in processing time and increased process security.

L27. Evaluation of the Impact of the Variables Related to Thermal Melt Temperature Determination by UV Spectroscopy

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DNA melting is the process by which dsDNA unwinds and separates. For multiple copies of DNA molecules, the melting temperature (Tm) is defined as the temperature at which half of the DNA strands are in the double-helical state and half are in the "random-coil" states. The melting temperature depends on both the length and the nucleotide sequence, but can be impacted by several measurement technique variables. By understanding the variables affecting Tm determination and controlling them, more accurate Tm values can result. This paper investigates the impact of the variables related to this technique on the accurate determination of thermal melt.

L28. Metabolic profiling of urine and tissues of laboratory animals after treatment by traditional by traditional herbal medicine

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In this paper, analytical methods based on nuclear magnetic resonance spectroscopy (NMR), liquid chromatography/mass spectrometry (LC/MS), and gas chromatography/mass spectrometry (GC/MS) in combination with pattern recognition tools such as principal component analysis (PCA) were used to study the metabolic profiles of rats after the administration of alkaloids. From the normalized peak areas obtained from LC/MS and GC/MS, and peak heights from NMR, statistical analyses were performed in the identification of potential biomarkers from urine samples and liver extracts.

The results from non-targeted NMR data processing proved the reliability and accuracy of targeted LC/MS analysis. The proposed approach provided a comprehensive picture of metabolic changes after administration of herbal medicines in rat model. The

results show that combination of multiple analytical approaches with pattern recognition tools is a useful platform for metabolomics.

L29. Adsorption properties of proteins on hydrophobic surfaces

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Hydrophobic interaction chromatography is an orthogonal method for separation of recombinant proteins. Partial unfolding of proteins upon adsorption on hydrophobic surfaces is a common problem and often may limit application of HIC. The surface acts as catalyst for partial unfolding, which is also known as spreading. The fraction of partial unfolded protein is increasing with length of the alkyl chain and thus with the hydrophobicity of the surface. It also increases with the concentration of the kosmotropic salt but decreases with loading of the protein Unfolding upon adsorption on hydrophobic interaction chromatography surfaces was confirmed by in-situ ATR FTIR measurements. With increasing residence time influences the unfolding progresses. This can be used to determine the activation energy assuming a certain ration rate and temperature dependence of the rate of adsorption. For set of references proteins the activation energy of the unfolding process has been determined in the range of 50-100 kJ/mol by isothermal titration calorimetry and. From temperature dependence of adsorption we calculated the thermodynamic quantities. We postulate a simple three state model describing this process. Mechanisms of protein adsorption and DNA will then discussed

L30. Purification of native proteins simultaneously satisfying with high resolution, high speed, high sample loading

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A very fast separation of intact proteins in the"top-down MS" strategy in proteomics and the fast purification of native proteins in laboratory and industry are required. Both require a high enough mass and purity. However, the protein purification by liquid chromatography (LC) in analytical scale has advantage of high resolution, but only operates small mass and slow process, while LC in preparative scale can fast purify proteins in a large sample but has low resolution. The purpose of the presentation is to accomplish the native protein purification simultaneously satifying with high resolution, high speed under high sample loading ("three H" purpose) by employing a "five S" strategy. The "five S" strategy is defined as the integration of a short column of very large diameter is packed with very small particles for protein separation under small overloading per unit cross-sectional area of the packed bed at a suitably low linear velocity. A series of chromatographic cake (cake) having very short length (10 mm) and large diameter (5~500 mm) can be employed for accomplishing the "three H" purpose in both analyltical and large scales. The "five S" strategy was tested with standard proteins and compared by a cake (10mm×20mm I.D.) and a conventionally long column (250 mm × 4.0 mm I. D.) packed with the packings of hydrophobic interaction chromatography (HIC) with very small particle (3μm, pore diameter 30nm). Although both have the same packed bed volume, with a comparable resolution of seven standard proteins, 2 mg running for 5 min could be done from of the cake, but only 0.04 mg running for 25 min from the long column. Additionally, the re-life time for the cake only requires 1 min (flow rate 60 mL/min) and 60 min for the long column due to its very slow rate. The advantage of the "five S" strategy is to have all advantages of protein separation in both analytical an preparative scales and overcomes all of their disadvantages. A industrial cake (10mm×300mm I.D.) was also employed for the renaturation with simultaneous purification of the recombinant human interferon-? under 120mL/min of flow rate to obtain purity =95% with only one step in 4h.

L31. Displacement Chromatography of Peptides Comes of Age

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Though displacement chromatography (DC) of peptides has been studied for over 30 years, its practical use has been limited. We find that new, high-purity, reversed-phase (RP) displacers coupled with proper protocols allow RP-columns to be operated efficiently in preparative displacement mode. We have compared a polymer-based C18 column, a porous polystyrene/divinylbenzene column and a silica-based C18 column using various operating conditions. Partial optimization studies were carried out at ambient temperature (23oC). Relevant operation parameters will be discussed: RP-matrix, column dimensions, flow-rates, temperature, pH & choice of buffer, ion-pairing (IP) agent & concentration, loading amount & loading concentration, type & amount of organic solvent, choice of displacer, displacer concentration.

Generally speaking, in one purification step starting with crude synthetic material, IP-RP-DC of peptides consistently gives good purities (98.5-99.5%) with good isolated yields (80-90%) and high column loadings (40-80g/L) regardless of the initial peptide purity (50-90%) or its sequence. Actual operational purity is primarily determined by the number and amounts of a few co-displacing peptide impurities. Similar purifications to those from small columns (4.6 mm x 250 mm, 5?m) are obtained using larger column sizes (10 x 500mm, 25 x 600 mm) and larger bead sizes (10 ?m, 15 ?m).

IP-RP-DC requires a suitable displacer in a pure form. Contamination of the product by the displacer is rarely a problem but contamination by displacer impurities can be troublesome. These issues have been addressed by using high purity (>99.9%), single-component displacers and by implementing suitable cleanup protocols (diafiltration, crystallization, precipitation).

Owing to the concentrating effect of DC, many impurity peptides, 100-300 in number, are observed in a typical synthesis mixture (15-20mer). Many of these impurities originate from impurities in the protected amino acids as well as from coupling failures during synthesis (deletions and double couplings).