Lecture L1

Monoliths Modified with Nanoparticles for Separation of Biomacromolecules

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Since the very beginning, porous polymer monoliths have proven to be an excellent stationary phase for the rapid separation of large molecules. Their well known advantages include ease of the preparation, robustness, high permeability to flow, mass transfer via convection, and a vast variety of chemistries. Traditionally, the surface chemistry of the polymer monoliths is controlled via copolymerization with monomers bearing the desired functionality, chemical modification of preformed monolith, and photografting of pore surface with polymer chains bearing the desired functionalities. Recently, we have introduced a novel approach involving nanochemistry. For example, we modify the pore surface within our monoliths with gold nanoparticles. This universal ligand can be both prepared *in situ* within the pores of the monolith or their dispersion is pumped through the monolith that contains thiol functionalities. This column then appears to be useful in the "fishing out" thiol containing peptides, a process useful in simplification of mixtures such as protein digests. However, the dynamic nature of the bond between gold and thiol group containing compound also facilitates replacement of this compound with a different thiol compound present in excess in surrounding solution. This unique feature can be then used for functionalization of gold nanoparticles with thiol compounds that in addition to the SH group contain another functionality. This process affords monoliths with a variety of interchangeable chemistries suitable for chromatography of large biomolecules while the strong gold-thiol interaction ensures the stability of this stationary phase. In another implementation, we prepared iron oxide nanoparticles and attached them to amine modified monolith. This column enabled isolation of phosphorylated peptides from complex protein digests.

We also used commercially available hydroxyapatite nanoparticles for the preparation of monoliths with designed selectivity. Since it is difficult to immobilize hydroxyapatite nanoparticles on the pore surface, we admixed hydroxyapatite to the polymerization mixture and prepared columns with entrapped particles. These columns also enable isolation of phosphorylated peptides. They can also be used for the separation of protein aggregates such as those formed from IgG and Protein A.

Lecture L2

New Core-Shell Packing Materials for High Performance HILIC Separations of Peptides and Glycopeptides

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During the past 5 years superficially porous silica particles have emerged as preferred materials for high efficiency and high speed separations in HPLC. Fused-Core® silica particles are designed with a 1.7 µm solid core and a 0.5 µm thick porous layer to yield 90 Å and 160 Å size pores. Columns packed with 2.7 µm diameter Fused-Core particles exhibit surprising efficiency. Reversed-phase separations using columns packed with Fused-Core 90 Å pore size or 160 Å Peptide ES-C18 bonded phase particles demonstrate performance comparable to efficient sub-2 µm particle diameter columns, but with less than ½ the column operating back pressure. Similarly high column efficiencies have been observed for the use of bare silica Fused-Core particle columns operated in hydrophilic interaction liquid chromatography separations (HILIC). Our objective has been to develop highly polar bonded-phase surface modifications of Fused-Core silica particles that maintain the high efficiencies previously observed with bare Fused-Core silica, but that exhibit the advantages of covalently-modified HILIC packing materials. To this end, a variety of highly polar covalent bonded-phases have been applied to Fused-Core Silica particles of 90 and 160 Å pore size. Novel hydroxylated bonded-phases are observed to be highly hydrophilic, exhibit typical HILIC retention properties, but with notably reduced ionic interactions. These polar bonded phases have been characterized for a variety of HILIC separations of small molecules, synthetic peptides, tryptic digests and small proteins. We compare the retention properties and column efficiencies obtained for these Fused-Core HILIC packing materials using small and larger pore materials. These novel HILIC column packing materials are shown to exhibit advantages for very rapid and high resolution separations of larger molecules, including polypeptides, glycopeptides and enzymatically-released protein N-glycans.

Lecture L3

New Insights on Separation Characteristics of Proteins and Peptides on Core-Shell Based HPLC Columns

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The introduction of next-generation chromatography instruments and columns (commonly known as UHPLC or UPLC) have allowed for better separation of peptides from closely related impurities. Such increases in resolution have allowed for better purity of peptides as well as better quantitation of peptides and any modified forms of such peptides. Recent developments in UHPLC column technology have included the introduction of core-shell chromatography media which allow for UHPLC separations at system backpressure amenable to operation on standard HPLC systems. Protein, peptide and peptide mapping applications were performed on several different offerings of core-shell media and results were compared in regards to average peak width, peak capacity, as well as resolution of closely eluting species. Results suggest that critical parameters for separation for small peptides differ from large peptide/ small proteins. Particle size and surface area of a chromatographic media are major factors in the increased resolution of small peptides. This is contrast with large peptides/small proteins where shell thickness and pore accessibility play a much larger role in increasing resolution and minimizing peak width. Optimizing separations based on the size and complexity of specific peptide (and peptide maps) will be discussed.

Lecture L4

Surface energetics of protein adsorption onto Ceramic hydroxyapatite

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Protein adsorption behavior during chromatography is studied by surface thermodynamics approach. Ceramic hydroxyapatite (CHT) is widely utilized in demanding downstream bio-processing. In this effort, the interaction between model proteins and an adsorbent (Ceramic hydroxyapatite Type I, Biorad) was studied via extended DLVO (XDLVO) calculations. The surface free energy and charge determination of proteins and adsorbent were quantitatively determined to access the surface properties. In Ceramic hydroxyapatite chromatography, protein adsorption usually takes place under mixed mode chromatographic conditions i.e., cationic exchange (P-site) and calcium chelation (C-site) interaction. Sample is loaded in 5 mM phosphate and eluted at increasing phosphate gradient (upto 500 mM) in the mobile phase. The XDLVO approach could calculate the free energy of interaction vs. distance profiles between the interacting surfaces at the time of elution. The calculations were compared with the actual elution behavior of the studied proteins. The correlations revealed that all the proteins under study showed the minimum binding energies i.e., $|0.005| \pm 0.002\ kT$ at the time of elution conditions. These energy values are considered at a margin between the retaining and non-retaining conditions. Energy values higher than it ($|0.005|\ kT$) will explain binding and lower will explain elution conditions.

The knowledge generated from these studies will assist a better understanding of real downstream bioprocess behavior which could facilitate process design and optimization.

Lecture L5

Advances in Mixed-Mode Hydrophilic Interaction/Cation-Exchange Chromatography of Peptides

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The term hydrophilic interaction chromatography (HILIC) was originally introduced to describe separations based on solute hydrophilicity, with the presence of a high organic modifier (generally acetonitrile) promoting hydrophilic interactions between the solutes and a hydrophilic stationary phase. Solutes are then eluted in order of increasing hydrophilicity, in direct contrast to reversed-phase high-performance liquid chromatography (RP-HPLC), where solutes are eluted from a hydrophobic stationary phase in order of increasing hydrophobicity. This laboratory took the concept of HILIC a stage further by taking advantage of the inherent hydrophilic character of ion-exchange, specifically strong cation-exchange (CEX), by subjecting peptide mixtures to linear salt gradients in the presence of high acetonitrile concentrations. This approach, termed mixed-mode HILIC/CEX, takes simultaneous advantage of both the charged character of peptides as well as any hydrophilic/hydrophobic properties they possess. This presentation compares the relative merits of these three HPLC modes for peptide separations, both as individual separation modes and as complementary approaches to separations of complex peptide mixtures. We have always believed that a rigorous assessment of HPLC modes for peptide separations, as well as different stationary phases and conditions within an individual separation mode, is best effected by the use of *de novo*-designed peptide standards. Thus, we assessed the performance of a RP-HPLC column (HALO Peptide ES-C18), a HILIC column (HALO Penta HILIC) and an ion-exchange column (PolySULFOETHYL A) operated in mixed-mode (HILIC/CEX) through separations of four sets of synthetic peptide standards: (1) five random coil standards with a small progression of overall hydrophobicity; (2) four random coil standards with increasing net positive charge (+1 to +4); (3) three groups of nine random coil standards (+1, +2 and +3 groups), with very subtle progressions of hydrophobicity within the three groups; and (4) two groups of highly charged amphipathic α-helical standards (+7 and +11 groups), with subtle progressions of overall hydrophobicity within the two groups. We believe our results serve to inform the researcher as to the most suitable HPLC mode for specific requirements as well aid in choosing the most promising approach to utilizing a specific column or combination of columns for peptide analysis.

Lecture L6

Hydrophobic contribution of amino acids into RP-HPLC peptide retention: sequence-specific retention prediction based on proteomics measurements

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The ability to predict a peptide's RP LC retention time offers significant advantages for data analysis and method development in proteomics. Conversely, proteomic measurements provide a virtually unlimited source of peptide retention information, which can be used to better understand the peptide retention mechanisms through the development of retention prediction models. Our Sequence Specific Retention Calculator (SSRCalc) model combines knowledge acquired over years of studying peptide's RP LC, and attempts to better understand the role of ion-pairing mechanisms in peptide retention using novel multi-parametric optimization procedures and massive proteomics-derived data.

Recently we compared the hydrophobic contributions of individual amino acids under two approaches: values determined by a classical "designed peptide" collection, and those from a proteomics-derived "random peptide collection" for the SSRCalc model optimization. Strikingly, the retention coefficients determined by SSRCalc optimization [1] were found to be virtually identical to the intrinsic hydrophobicity values determined by designed peptide method (Hodges and co-workers [2]) for all residues except those charged at acidic pH- Arg, Lys, His. The high degree of coefficients similarity for neutral residues confirms the correctness of SSRCalc's accounting for nearest-neighbor and conformational effects in peptide retention. The unique behavior of the basic residues is related to the dualistic character of the RP peptide retention mechanism, where both hydrophobic and ion-pairing interactions are involved. Our findings suggest that the hydrophobicity/hydrophilicity of charged residues cannot be assigned correctly without taking into account overall peptide hydrophobicity.

The hydrophobicity values obtained as a result of predictive model optimization using typical tryptic peptides correctly describe amino acid's contribution for a "random coil" peptide conformation. Being transferred into the constrained environment of interactions between C18 phase and amphipathic helical peptides, amino acids demonstrate significant differences in behavior. The hydrophobic contributions of amino acids in helical conformation are determined by their position (hydrophilic or hydrophobic face) and the overall stability of the helical structure upon the interactions. For example, the simple substitution of Ala for Val could provide either an increase and decrease in RPLC retention. The designed peptide approach is preferable for detailed study of the interactions of amphipathic helical structures, while random peptide datasets shows greater variability in helical structures. Further developments in peptide retention prediction will require detailed studies incorporating the best practices of random peptide dataset and designed peptide approaches.

1. O.V. Krokhin, Anal Chem 78 (2006) 7785.

2. C.T. Mant, et al., Biopolymers 92 (2009) 573.

Lecture L7

Affecting Reversed-Phase Chromatography of Peptides Under MS-Compatible Conditions

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Peak elution of peptides on silica-based reversed-phase alkyl phases under the common MS-compatible condition of dilute formic acid is generally not as symmetrical or efficient as with the traditional UV-based methods with dilute trifluoroacetic acid (TFA). This is a phenomenon that may involve charge-charge interactions between the peptide analytes as well as between the peptide and silica surface. Therefore, such interactions may be mitigated by pH and/or cations in solution that may function as counterions in an ion-exchange process. We elucidate the chromatographic behavior of reversed-phase peptide separations as a function of pH and counterion concentration. The impetus to explore this is driven by rapid growth in peptide drug candidates and peptide-based active pharmaceutical ingredients.

Peptides of various basicities were chromatographed, and peak efficiency and symmetry recorded as a function of acidic modifier, pH and/or counterion concentration. Data analysis deciphered mechanistic reasons for poorer peak shape of peptides with formic acid (as compared to traditional methods with TFA. Peak shape of basic peptides was affected by the concentration of the acidic ion-pair reagent (TFA or formate), which in turn is controlled by pH relative to pKa of the ion-pairing anion. Concentration of the cation counterion, independent of pH, did mitigate against poor peak shape. Control of mobile phase pH not only effects peptide peak shape, but can be very significant in affecting selectivity as well.

Optimal mobile phase conditions for reversed-phase LC-MS peptide chromatography depends in part, on the nature of the peptides being analyzed. Not only is peak shape and selectivity varied by relatively minor changes in pH, but the results are also affected by the peptide basicity.

Lecture L8

A new approach for mAb aggregate characterization using HI- and SE-HPLC in combination with FL and MALS detection

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A growing therapeutic and diagnostic demand for monoclonal antibodies (mAb) has led to higher concentrated fed stocks. Such high titer fed stocks from mammalian cell culture may contain high aggregate levels. Aggregates may cause severe side-effects to a patient, therefore it is crucial to establish a sensitive and reliable method to quantify these possible aggregates. A standard QC method is SE-HPLC (Size Exclusion HPLC) in line with a UV-detector and now a day to a greater extent MALS as a second detector.

In our study we developed a method using Hydrophobic Interaction Chromatography (HIC) and fluorescence (FL) detection to increase sensitivity and peak resolution of mAb (IgG1) aggregates. A salt mixture of citrate and NaCl for HIC showed an improved resolution compared to Size Exclusion Chromatography (SEC). In addition the use of fluorescence gave us the possibility of a sensitive analytic with a very good Signal/Noise ratio. Also we compare the minimum amount of protein needed for detection with SEC-MALS analysis.

Further we were interested to learn more about the spatial form of aggregates. Using our new developed method and additional SEC with viscosimetry we were able to propose an approximate structure for the mAb aggregates.

Lecture L9

Antibody Solution Kit for Separation and Characterization of Monoclonal Antibodies

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Monoclonal antibodies have been increasingly becoming drug candidates for disease therapeutics. Due to the molecular complexity of monoclonal antibodies, the characterization remains a challenge and required step throughout the development and manufacturing process. In order to determine the efficacy of the molecules, aggregation, heterogeneity such as charge variants, C-terminal lysine processing, deamidation, glycosylation must be screened for their structural and biological changes. Antibody solution kit offers a complete separation solution for monoclonal antibody analysis and characterization. In the kit, the Zenix™-300 size exclusion chromatographic (SEC) column is designed for high efficiency and resolution separation of monoclonal antibody monomers, aggregates, fragments, heavy and light chain subunits. With its uniform, hydrophilic, and neutral nanometer thick films chemically bonded on high purity silica, the non-specific interaction between proteins and column surface is minimized to result in high resolution of monoclonal antibody separation. The antibody solution kit provides Antibodix™ NP3 weak cation exchange column to separate the charge variants. Multiple mobile phase systems were investigated for optimum charge variant separations. With its non-porous polymer bead, the Antibodix™ WCX is suitable for resolving slightly different structures of monoclonal antibodies within a wide pH range of 2-12. For studying more detailed structure of monoclonal antibodies, a reversed phase Bio-C8 column in the kit offers the high resolution separation of Fab, Fc fragments after the papain cleavage of the whole antibody, and heavy, light chains with organic mobile phase system. Bio-C18 reversed phase column provides a solution to study primary structure of monoclonal antibodies and peptide variants. With Zenix™-300 SEC, Antibodix NP3, Bio-C8 and Bio-C18, antibody solution kit offers a complete set of tools for monoclonal antibody analysis.

Lecture L10

Developing Next Generation IMS-MS Techniques for Analyzing Biological Mixtures

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This talk will describe recent efforts to develop high resolution ion mobility/mass spectrometry techniques for the analysis of complex biological mixtures. Several years ago, our group developed a nested ion mobility/time of flight mass spectrometry method that is useful for characterizing complex protein and metabolite mixtures. This approach couples naturally to condensed-phase chromatographic techniques. One drawback of the approach is that it only utilizes information about the precursor ion and collisionally-induced fragment ion masses. In the last year we have combined ion mobility methods and overtone mobility methods with an ion trap that is equipped with 157 nm photodissociation capabilities. This approach makes it possible to first separate isomers and then follow unique fragments that are formed with multiple MS analyses of the fragments that are generated by photo- or collision induced-dissociation techniques. The approach is at an early stage. Examples including the resolution of mixtures of carbohydrate isomers will be given. Advantages and disadvantages of the methods will be discussed

Lecture L11

The Chicken as an Experimental Model for Biomarker Discovery in Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) remains the most lethal gynecological cancer in the Western world due to a combined lack of effective therapeutics and screening strategies. The dearth of effective EOC screening strategies has been attributed to several factors including the heterogeneous pathogenesis of EOC, difficulty in obtaining significant numbers of early-stage human EOC tumors, and few experimental models. The chicken, however, is emerging as a promising animal model for studying EOC because the onset of disease is spontaneous with high prevalence rates (5-35%), the genotype and phenotype is similar to human EOC including CA-125 expression, the mutational frequency of the p53, and the up-regulation of HER-2/neu, and specific to our interests is the ability to collect longitudinal blood samples without sacrificing the animal. The strengths of the chicken as an experimental model of EOC forms the foundation for our efforts to study intra- and inter-individual biological variability with advanced mass spectrometry-based technology as a function of health and disease.

Blood was drawn from 148 age-matched B-strain chickens starting at 2.5 years of age every three months for one-year followed by tissue resection and preservation. Pathological assessment of the tissues confirmed that 7% of the birds developed EOC. A global shotgun proteomics LC-MS/MS study of selected longitudinal plasma samples and matched tissues identified several candidate protein biomarkers for further study. Plasma proteins serum albumin, vitellogenin-I, transthyretin, apolipoprotein A1, and a newly discovered form of ovomacroglobulin were quantified both within- and between 14 birds (5 = healthy, 3 = early-stage EOC, 2 = late-stage EOC, and 4 = cancers other than EOC) via protein cleavage isotope dilution mass spectrometry (PC-IDMS) and selected reaction monitoring (SRM). Concurrent to these efforts, a global untargeted LC-MS/MS-based metabolomics study of the same longitudinal plasma samples used in the SRM study was initiated to identify potential small molecule biomarkers and complement the existing proteomics datasets. The results of these studies will be discussed in the context of the many technical challenge that exist for biomarker discovery, the power of longitudinal plasma analysis and it's implications in biomarker discovery and diagnosis (i.e., personalized medicine and the index of individuality), and future directions for using the chicken as an experimental model for EOC.

Lecture L12

Accurate, High-Throughput Identification of Stable Protein Complexes using a Tagless Strategy

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We describe a novel method for identification of stable, soluble protein complexes in microbes. Our strategy is based on the premise that the great majority of such complexes will survive intact through a series of orthogonal chromatographic methods, with complex components having correlated elution profiles. A major challenge is the potential for false positives (FP) caused by co-elution of proteins that are not part of a complex.

The soluble components of biomass extract are separated using a series of chromatographic columns. The elution profiles of each protein across one or more chromatographic steps are measured with the aid of mass spectrometry and iTRAQ reagents (Dong et al., 2008, *J Proteome Res.* 7:1836-49). For every region of elution space where two proteins overlap, Pearson correlation coefficients are calculated between vectors of normalized relative protein amounts estimated using iTRAQ. These data are used to train a random forest classifier to identify true interactions in a manually curated gold standard (GS) set. We compare the discriminating power of these proteomic data to that of other high-throughput data, such as correlation of gene expression profiles.

As a pilot study, we demonstrate this technique in identifying a high-precision subset of stable protein complexes in *Desulfovibrio vulgaris*, a model bacterium potentially useful for bioremediation via immobilizing toxic heavy metals in soils at DOE sites. Our method is able to identify 66% of GS interactions present in our proteomic data at a 0 FP rate; using the same thresholds results in the prediction of 854 novel interactions.

This strategy is effective at identifying a subset of stable inter-protein interactions in a bacterial proteome at high precision. Advantages of this approach include not requiring a mutant library (needed for alternative tag-based approaches), and a low FP rate.

Lecture L14

An Integrated Glycomics Approach Towards an Assay for Cancer Biomarkers

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We have developed an integrated platform to discover glycoprotein biomarkers in serum. This platform has been applied to several cancers for early detection. In this talk we will discuss a novel work-flow for detection of cancer in hepatocellular cancer, ovarian serous cancinoma and pancreatic cancer. This work-flow uses a combination of microarray and mass spec based technologies. We first deplete the 14 highest abundance proteins and analyze the high abundance proteins based on MALDI-MS of their glycans. Glycan markers for HCC and pancreatic cancer have been identified based on changes in haptoglobin and macroglobulin fucosylation. We extract the remaining proteins and search for gross changes in their glycan structures using lectin arrays to investigate differences in carbohydrate content on serum glycoproteins. This procedure has been performed on sets of samples from HCC and cirrhosis patients where the lectin arrays indicate that the intensity for AAL and LCA was significantly higher in HCC, indicating an elevation of fucosylation level. Then serum from 10 HCC samples and 10 cirrhosis samples were used to screen the altered fucosylated proteins by a combination of isotopic labeling, lectin extraction and LC-MS/MS. Using this method a set of 9 potential glycosylated proteins showing differences in expression were identified for further analysis. Using these target markers 27 HCC and 27 cirrhosis serum samples were used for lectin-antibody arrays to validate the change of these fucosylated proteins. C3, CE, HRG, CD14 and HGF were found to be biomarker candidates for distinguishing early HCC from cirrhosis. Further work using a phase 1 validation set based on elisa and lectin-elisa assays showed that HGF and HGF+CD14 could provide an AUROC of 0.79 and 0.8 matching that of the currently used marker AFP but with improved sensitivity in detection. These markers are currently undergoing a phase 2 blinded serum marker validation. This integrated platform using a combination of microarray and mass spectrometry techniques has also been recently used to identify a panel of markers for ovarian serous carcinoma and pancreatic cancers which are now undergoing initial validations.

Lecture L15

Making Glycomics Quantitative

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Glycosylation is one of the most common post-translational modifications encountered in eukaryotic systems. One of the analytical challenges facing scientists in the characterization of glycoproteins involves the ability to identify and quantify changes in the attached glycans. This topic is of importance to a variety of researchers ranging from those involved in the batch-to-batch analysis of recombinant glycoproteins to those involved in glycomics.

One of the most accurate methods currently available for quantification involves the use of isotopic labeling followed by mass spectrometric detection. To this end, we have developed a range of *in vivo* and *in vitro* isotopic labeling strategies for glycan quantification. Two of these approaches use isotopic variants of methyl-iodide ($^{12}CH_3I/^{13}CH_3I$ and $^{13}CH_3I/12CH_2DI$) to label the glycans during permethylation. The third *in vitro* procedure incorporates ^{18}O during PNGaseF deglycoslation. Because this labels only one site per glycan, the reproducibility is significantly improved compared to the permethylation approaches. A second advantage is that ^{18}O labeling allows glycans from two samples to be combined prior to clean-up and permethylation, therefore eliminating errors resulting from parallel sample processing. The *in vivo* approach relies on the hexosamine biosynthetic pathway that uses the side-chain of glutamine as its sole donor source of nitrogen for aminosugars in the production of sugar nucleotides, and offers all of the benefits associated with SILAC, including the investigation of glycan turnover rates.

Even with these developments, it is not possible to quantify individual glycans present in a mixture of isomeric glycoforms. To solve this issue, we have been developing novel MS compatible separation strategies using uPLC and a variety of mobile/stationary phases, particularly reversed-phase and HILIC. To date, we have been able to demonstrate baseline separations of glycans that differ only in the position of sialic acid attachment, and are working on resolving branching isomers.

This presentation will summarize both of these efforts, and will discuss our overall strategies for the quantification of individual glycans released from both simple (individual recombinant proteins) and complex (glycomic) samples.

Lecture L16

Characterization of the Unusual Glycosylation of Maize-Derived Recombinant Bovine Trypsin

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Trypsin, a pancreatic serine protease, is an important protein used in applications including cell culture and pharmaceutical protein production. Due to safety and regulatory concerns, there is high demand for non-animal sources of trypsin. Recombinant bovine trypsin has been produced in transgenic maize as an alternative to the animal-derived reagent.

We have undertaken a series of analyses to comprehensively determine the biochemical properties of maize-derived trypsin. The recombinant trypsin was found to be identical to the native protein, with the exception that two primary molecular weight forms were observed in the maize-derived product by SDS-PAGE. MALDI analysis of chemically-released glycans and ESI-MS of the intact protein revealed that the recombinant trypsin contained seven major glycoforms. LC-MS/MS analysis indicated that all glycans were attached to a single tryptic peptide containing several potential glycosylation sites.

A novel approach utilizing an immobilized non-specific proteolytic enzyme, pepsin, to further process the glycopeptide was employed to generate a series peptides that enabled location of the site of glycosylation by LC-MS/MS. The glycans were determined to be attached to a nonconsensus N-glycosylation site on the protein.

This presentation will highlight the strategies used for characterization of recombinant trypsin, with a focus on the elucidation of its unusual glycosylation.

Lecture L17

Separation of Virus and Virus-like Particles by Chromatography

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Modern vaccines and virus based delivery vehicles are produced in cell culture, while traditional vaccines are produced in the amnion fluid of eggs. Baculoviruses are produced in SF9 cells and Hi5 cells. They are important hosts cells for production of virus like particles by overexpressing respective virus antigens at the surface of the baculovirus as previously shown for influenza virus. A laboratory scale process for separation of baculovirus virus with polymethacrylate monoliths so-called CIM-disks and CIM tubes have been developed with an overall yield of up to 70%. Virus is loaded at intermediate salt concentration and eluted by a step salt gradient. Loading volume depend highly on the harvesting time of the virus or VLP. Early harvest can be loaded up to 500 column volume breakthrough whereas late harvest can be loaded only up to 40 column volume breakthrough. A protein free virus or VLP can be obtained when the capture step with monoliths is connected to size exclusion chromatography. Thereby it is also possible to separate virus from VLP and DNA. The viruses and the VLP can be purified to homogeneity with a high yield of infectious virus as measured by plague assays. A full purification cycle starting from clarified culture supernatant can be accomplished within a day. Furthermore we will address the so-called flow entrapment of viruses in monoliths. Our findings show, that show this is not the case our virus.

Lecture L18

Purification of Cell Culture-Derived Influenza Virus using Continuous Simulated Moving Bed Chromatography

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For influenza vaccine production batch chromatography is one of the most popular methods for purification. At present, the recovery and the scalability of the conventional batch processes are reasonably good. However, a continuous approach, like countercurrent simulated moving bed (SMB) chromatography, could improve the productivity considerably. This technology is already well established for the separation of enantiomers and fine chemicals. So far, however, only few studies described the application of SMB chromatography for the purification of biological macromolecules such as viruses out of complex bioreactor harvests.

In the presented study, a three column open loop SMB chromatography using a size exclusion matrix (Sepharose 4FF) was investigated for purification of cell culture-derived human influenza virus A/PR/8/34 (H1N1). Several different operating conditions were tested and compared to a conventional batch process in terms of their separation performance and levels of impurity. Batch chromatography resulted thereby in a viral recovery of 74% based on HA activity and a protein and host cell DNA depletion of 59% and 40%, respectively. The SMB process led to comparable results: About 70% of HA activity were found in the product fraction, while protein and DNA were reduced by about 55% and 30%, respectively. However, overall productivity of SMB chromatography was estimated to be about six times higher compared to the batch mode. In combination with additional purification steps for further reduction of contamination levels, SMB chromatography seems therefore a promising method for large scale vaccine manufacturing.

Lecture L19

Thermodynamics of Adsorption of PEGylated Lysozymes on Hydrophobic Chromatographic Resins

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PEGylation of pharmaceutical proteins can lead to improved biocompatibility, stability and residence time of the drug in the body. Usually the protein is already purified before PEGylation, which means the covalent binding of polyethylene glycol (PEG) molecules to a protein. Therefore, the purification task after PEGylation is reduced to a separation of the PEGylated isoforms or a fraction with a certain PEGylation degree from the unPEGylated protein.

The PEGylation changes the hydrophobicity of the proteins which leads to different interactions in hydrophobic chromatography systems. Therefore, hydrophobic interaction chromatography (HIC) is a promising technique for the separation of the isoforms.

Lysozyme is a protein which is often used as a model protein due to its well-known structure and surface properties. Lysozyme can be PEGylated at three lysine residues. The outcomes of this are mono-, di- and tri- PEGylated isoforms.

Thermodynamics of protein adsorption on hydrophobic resins are studied by measuring the specific enthalpy of adsorption with isothermal titration calorimetry (ITC) in combination with corresponding adsorption equilibrium isotherm measurements. The adsorption of the proteins on hydrophobic resins is influenced by many parameters, like temperature, type of salt and ionic strength. These parameters are systematically varied in the experiments with the goal of achieving a better understanding of the chromatographic process. The measurements of the adsorption equilibrium isotherms are carried out using a fully automated robotic liquid handling station. For the calorimetric measurements, two high precision isothermal titration microcalorimeters are used.

In the present study different forms of PEGylated lysozyme are explored: mono-, di- and tri PEGylated lysozyme for 5 and 10 kDa PEG each. The adsorption of native lysozyme and these six forms of PEGylated lysozyme on Toyopearl PPG-600M, a medium hydrophobic resin, was studied with microcalorimetric and adsorption equilibrium measurements. The structure of the ligands of the chosen adsorber is similar to the structure of the PEG. Additionaly, the same measurements were carried out for pure PEG to get a better insight into the adsorption mechanism of the PEGylated lysozyme forms. The specific enthalpies of adsorption Δh_p^{ads} of the PEGylated lysozymes forms, native lysozyme and the pure PEG were determined with a sodium phosphate buffer with additional ammonium sulfate at different ionic strengths each. A molecular picture is discussed which describes the complex adsorption mechanism.

Lecture L20

Assessing solution stability of PEGylated proteins

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The therapeutic potential of proteins and peptides is increasing in the biopharmaceutical market. Conjugation of PEG (polyethylene glycol) to proteins, known as PEGylation, has been used to improve the in-vivo lifetime of protein drugs in the human body. Several FDA approved blockbuster products, such as PEG-Intron® and Pegasys®, have demonstrated the commercial importance of PEGylation. Thus there exist huge potential for increasing the therapeutic value of the promising class of biopharmaceutical proteins by PEGylation.

As for all biopharmaceutical drugs, the solution stability of such PEGylated drugs need to be assessed. PEGylation results in a new macromolecule with significantly changed physicochemical characteristics, like solubility and stability. Highly concentrated protein formulations are desirable, at the risk of uncontrolled aggregation, precipitation or crystallization. Solution stability studies normally span months for these protein drugs and tools to measure solubility and stability in a short time span would decrease the time to market for new drugs. So there is a strong desire for alternative methods to rapidly gather information on the phase behavior of promising target molecules. A promising alternative suitable for miniaturization and high throughput screening is the osmotic second virial coefficient, B_{22}. A general correlation between B_{22} and solubility and solution stability has been demonstrated. Experimental investigation in our lab has demonstrated the use of a chromatography based quick approach for assessing the solution stability of PEGylated proteins through B_{22} screening. This approach has been miniaturized allowing high throughput data generation, having the potential to ultimately bringing drug candidates quicker to the market. This presentation will show both experimental results and theoretical aspects of this chromatography based quick approach for assessing the solution stability of PEGylated proteins.

Lecture L21

Protein Adsorption and Transport in Macroporous and Polymer Grafted Ion Exchangers - a Side-by Side Comparison

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This work compares the physical properties and protein adsorption characteristics of two polymeric cation exchangers: UNOsphere S, which has an open macroporous architecture, and Nuvia S, which is based on a similar backbone matrix but which contains polymeric surface extenders. Both materials were obtained from Bio-Rad Laboratories (Hercules, CA) and a purified mAb was used as a model adsorbate. The characteristic pore sizes, determined by inverse size exclusion chromatography (iSEC) with dextran probes, were about 130 nm for UNOsphere S, and only about 12 nm for Nuvia S, indicating that the polymeric extenders occupy a substantial portion of the base matrix pores. Somewhat greater exclusion limits were, however, found for Nuvia S in 1M NaCl, suggesting that the polymeric extenders are not rigid but collapse partially at high ionic strength. mAb pulse response experiments under these non-binding conditions showed near complete exclusion on Nuvia S, consistent with the dextran behavior. Very large mAb equilibrium binding capacities were obtained, however, at low ionic strength (20 mM), approaching 330 mg mAb/ml of particle for Nuvia S compared to about 100 mg/ml for UNOsphere S. Much higher adsorption rates were also found for Nuvia S and the rate was nearly independent of protein concentration in solution, suggesting that the polymeric extenders enhance both adsorption capacity and diffusional mass transfer. Confocal laser scanning microscopy (CLSM) showed very sharp intraparticle protein concentration profiles for UNOsphere S, perfectly consistent with a pore diffusion mechanism, but diffuse concentration profiles for Nuvia S, consistent with a solid diffusion mechanism. The improved capacity and transport afforded by the polymeric extenders provide substantial potential benefits for bioprocess applications without sacrificing the desirable flow properties of the backbone matrix.

Lecture L22

Ion Exchange Analysis of Proteins with Automated pH Gradients

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Complete analysis of proteins requires the combination of several analytical techniques. Where chromatographic separations are part of the analytical suite, it is often desirable to use multiple modes of separation and to preserve the biologically active structure of the protein. Among the chromatographic techniques that meet these objectives, ion exchange can be the most discriminating. Obtaining the best resolution with this technique requires optimization of both the column and the mobile phase. Among the options for adjusting the separation, the pH of the chromatographic eluents has the largest effect on selectivity. Efficient protocols have been developed for screening separations with a selection of columns over a series of buffers that differ in pH. This process has been automated in a system that combines the chromatographic instrument and the control software. In addition to these experiments, many investigators have tested pH gradients for protein separations. While useful separations have often been reported, such pH gradients have not been widely applied because they are difficult to control. Simple buffer mixing does not give the desired pH profile because pH is a logarithmic function. When the intended pH is further from the pK, pH changes become very large with small changes in buffer proportions. It is also complicated to control the pH profile when changing column dimensions or flow rate. We have developed and evaluated algorithms and a software user interface to simplify this process. The technology is implemented on a four solvent pump and the gradient table is programmed directly in units of pH and salt concentration. The system uses a “buffer system” that is freely defined by the user to calculate the required proportions of the four mobile phase reservoirs and to recalculate those proportions at each pump stroke. This recalculation permits accurate and precise pH gradients over the range of the buffer system, and it generates both linear and non-linear gradients of pH as programmed by the user. With this control over the buffer properties as delivered to the column, it is possible to identify the ways in which the ion exchange media alters the pH of the buffer as it passes through the column. Several examples of protein separations, including monoclonal antibodies, will also be shown. With the use of this accurate and precise control of pH gradients, new selectivity becomes available for optimizing selectivity in protein analysis methods.

Lecture L23

pISep Controlled pH Gradient Chromatography Can Outperform Isoelectric Focusing for Complex Protein Separations

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Abstract: Isoelectric focusing is the “gold standard” of one dimensional protein separation. It is generally thought that the high quality immobilized gradients now available enable a much more powerful separation based on proteins’ pI than classic salt based IEX or even standard chromatofocusing. We demonstrate, using a complex mix of polyclonal an-

tibodies with alkaline pIs, that wide range, tightly controlled pH gradients on ordinary high resolution ion exchange resins can resolve the proteins far better than IEF. We provide an underlying physical model explaining this as a consequence of the pH dependent interaction between the charged surface of the stationary phase and patches of charge on the surface of the macromolecules.

Lecture L24

Sample displacement chromatography – an alternative way for purification of high and low abundance plasma proteins

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Sample displacement chromatography (SDC) for preparative purification of peptides and proteins in reversed-phase mode was first introduced by Hodges et al. [1, 2]. When this chromatographic separation mode is applied, during loading, there is competition among the sample components for the binding sites of the surface of the stationary phase, and under overloading conditions, weekly bound proteins were displaced by tighter bound ones. Veeraragavan et al. [3] applied the SDC method for purification of proteins in ion-exchange mode.

We used SDC in ion-exchange [4] and hydrophobic interaction mode for separation of proteins from human plasma. Both, high capacity, particle-based porous materials as well as monolithic anion- and cation-exchanger supports were applied. The composition of bound and eluted proteins was dependent on column loading. Under overloading conditions, the weakly bound proteins are displaced by stronger binding proteins, and we could show that SDC can be applied for isolation of high and low abundance proteins from human plasma in both small analytical and preparative scale.

In ion-exchange mode, under overloading conditions, human serum albumin (HAS) can be displaced from weak anion-exchanger column and highly enriched in the flow-through fraction. In the second step, the fraction containing tightly bound proteins can be eluted at higher ionic strength by use of a step gradient. This fraction contains highly enriched vitamin K dependent clotting factors and inhibitors, and inter-alpha inhibitor proteins. In the following step, the immunoglobulins can be removed from the flow-through fraction by cation-exchange chromatography, and highly enriched HSA can be collected in the final fraction containing non-bound proteins. Finally, highly enriched IgG fraction can be obtained under overloading the cation-exchanger column by sample displacement, and tightly bound proteins can be eluted in the next step by use of a salt step gradient.

Sample displacement chromatography of proteins from human plasma was also performed in hydrophobic interaction mode. Further low-abundance proteins could be isolated and identified by ESI-MS/MS and MALDI TOF MS. The use of hydrophobic interaction chromatography under sample overloading conditions for preparative isolation of some therapeutic proteins such as immunoglobulins and clotting factors and inhibitors was also presented. For this sake, a combination of columns according to the method developed by Mant and Hodges [1,2] was chosen.

In ion-exchange and hydrophobic-interaction mode, both, particle-based and monolithic supports can be used for SDC. However, if monolithic supports are used, sample displacement occurs much earlier, and SDC on monolithic supports can be used for enrichment of low abundance proteins such as clotting factors.

[1] R. S. Hodges, T. W. L. Burke, C. T. Mant, J. Chromatogr. A 444 (1988) 349.

[2] C. T. Mant, R. S. Hodges, J. Chromatogr. A 972 (2002) 101.

[3] K. Veeraragavan, A. Bernier, E. Braendli, J. Chromatogr. 541 (1991) 207.

[4] M. Brgles et al. J. Chromatogr. A 1218 (2011) 2389.

Lecture L25

Use of Mixed Mode Ligand Libraries to Achieve High Resolution Chromatographic Purification and Analysis of Recombinant Proteins

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Over the past several years, the design, synthesis and application of mixed mode chromatographic separation media has generated considerable interest. A central consideration driving these developments has been the need to demonstrate additional selectivity options with these new systems, both in larger scale applications dealing with the purification of biomacromolecules from crude feedstocks and well as the high resolution analysis of these biomolecules, particularly when they contain extensive post-translational modifications. In this presentation, strategies will be described for the use of libraries of mixed mode chemical ligands, immobilised onto suitable support materials, for use in these application fields as multimodal affinity chromatographic materials. In particular, robust, miniaturised high throughput screening methods have been developed based on robotic liquid handling systems to identify particular ligand candidates for specific application endpoints with a variety of recombinantly generated proteins, including industrial enzymes, reporter (diagnostic) proteins and monoclonal antibodies. These screening procedures enable relevant equilibrium binding characteristics (qm, Ka and ? values) as well as adsorption and desorption kinetic values (kon and koff) to be rapidly obtained both by miniaturised batch (tank) adsorption methods as well as with miniaturised packed bed systems and relative selectivity options determined. The information acquired from these screening studies is then utilised to guide further design and selection of optimised ligands with greater selectivity for a particular protein structure and to enable the progressive scaling of these chromatographic systems from the analytical level through to the larger process application. To illustrate the potential of this approach, exemplars will be described based on our experiences with a variety of recombinant proteins expressed by either prokaryotic or eukaryotic host cells in culture.

Lecture L26

Isolation and characterization of therapeutic antibody charge variants using ion exchange displacement chromatography

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Monoclonal antibodies (MAbs) are therapeutical proteins with important applications in many disease areas. MAbs charge variants separated by cation exchange chromatography are potential critical quality attributes and need to be well characterized.

Purification and isolation of closely-related antibody charge variants have been traditionally performed using HPLC with salt gradient elution at relatively low sample loading. In this report, we have demonstrated the isolation and enrichment of charge variants of a monoclonal antibody IgG1 using cation exchange displacement chromatography. We successfully achieved the high-throughput separation of acidic, main and basic charge variants with high recovery (>70%) and purity (>90%) by using a commercially available stationary phase in conjunction with a commercially available displacer. In addition, we have isolated and enriched a trace methionine-oxidized variant of the monoclonal antibody allowing a secondary means of identification of this variant while providing sufficient enrichment for further analysis, stability tests and potency determination. Further characterization of the displacement trains by SEC indicate the possibility of enrichment of high and low molecular weight species. Glycan analysis of the displacement fractions indicates minimal variation in glycan distribution patterns among a wide spectrum of charge variants. These results provide a case study demonstrating the utility of ion exchange displacement chromatography as a viable approach to isolate and enrich antibody charge variants for enhanced molecular characterization.

Lecture L27

HIC with a combination of chaotropic and kosmotropic salts for mAb aggregate purification

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Aggregates are a major concern regarding safety and efficiency of therapeutic monoclonal antibodies (mAbs). On the one hand, aggregates may lose biological activity. Contrariwise, severe side-effects may occur when applied to a patient. Soluble aggregates can accumulate because of increasing fed stock titers or acidic virus removal. Besides Ion Exchange Chromatography, which is frequently used, HIC can possibly remove aggregates due to their hydrophobic nature. However, reasons for the previous depreciation of HIC are the comparably low dynamic binding capacity and the extensive need for rather kosmotropic salts like Ammonium Sulfate. Disposal of Ammonium Sulfate is for environmental reasons cost-intensive.

In our try to overcome these problems, we investigated the influence of more chaotropic salts in combination with kosmotropic salts regarding purity, selectivity and recovery for 3 different kinds of hydrophobic interaction resins. According to the Hofmeister series, chloride has a destabilizing influence on the water structure. For practical reasons, we chose a combination containing the Protein A elution buffer ion Citrate. This enables smooth integration of our new method into downstream processing. We could show that selectivity is altered by the use of the citrate & sodium chloride combination, which led us to the attempt establishing a flowthrough protocol. Also, recovery and purity can be increased, significantly.

Lecture L28

Probing the Mechanisms of Protein Retention and Transport in Polymer-Derivatized Ion-Exchange Media

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Polymer-derivatized ion-exchange media are used operationally much like conventional ion-exchange adsorbents, with loading at low salt and elution at high salt. However, these similarities mask important mechanistic differences between the two types of media. This presentation will describe measurements based on a set of methods not widely applied to chromatographic systems in an effort to obtain clearer mechanistic insights. These include measurements of salt partitioning, adsorption energetics and different approaches based on confocal microscopy. These results, taken together, indicate that widely used assumptions regarding ion-exchange mechanisms should be applied to polymer-derivatized materials with caution, but that design of these materials can benefit from numerous additional degrees of freedom relative to those available for conventional materials.

Lecture L29

Chromatographic Dimensionality: A Separations Metric with Wide-ranging Implications for Bioseparations

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A large part of analysis in the life science and biomedical studies is the quest for identifying biomarkers of disease states. It has been observed that many suspected compounds are present either near the detection limit or below the detection limit. A recent study by Enke and Nagels[1] using mass spectrometry suggests that the number of compounds found in a natural substance analysis fits a log-normal distribution of intensities (concentrations). The log-normal basis, which is a power-law distribution function, can be further developed to obtain a relationship expressing the difficulty of finding trace substances (biomarkers) in an analysis scheme.

In this talk I will bridge the concept of chromatographic dimensionality[2], the distribution of natural substance intensities via Enke and Nagels[1] and a recent study using a power-law basis for a chromatographic statistical overlap theory.[3] It will be shown that we may be asking too much of instrumental analysis, even with multidimensional chromatography and multidimensional mass spectrometry, to find biomarkers at the trace and ultra-trace levels without specifically knowing where to look.

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[2] Schure, M. R. The dimensionality of chromatographic separations, Journal of Chromatography A, 1218, (2011) 293-302

[3]Schure, M. R., Davis J. M. The statistical overlap theory of chromatography for power-law (fractal) statistics, accepted for publication in the Journal of Chromatography A

Lecture L30

Quantitation of Intact Proteins in Complex Biological Matrices by Multi Dimensional Liquid Chromatography

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A multidimensional liquid chromatography based method was developed for quantitation of intact proteins. Model proteins, myoglobin and serum albumin were simultaneously resolved and quantitated from cell lysate using a strong anion-exchange chromatography and reversed-phase chromatography as the first and second dimension respectively. The method validation consisted of evaluating linearity, precision, and accuracy. A linear relationship (R(2)>0.99) between the concentrations of the two proteins and peak areas was observed over the concentration range; 12.0-120.4µg/mL and 8.5-85.4µg/mL for serum albumin and myoglobin, respectively. As a function of total protein in the lysate, this corresponded to 1.7 µg/mg to 17.2 µg/mg for serum albumin and 1.2 µg/mg to 12.2 µg/mg for myoglobin respectively in this method. The average RSD of peak areas for intra-day and inter-day analyses were 5.9% and 9.4% for myoglobin and 6.2% and 10.1% for serum albumin respectively. Over the linear range, the recoveries ranged from -15.4 to 9.0% for serum albumin and -2.5 to 9.4% for myoglobin. The system presented in this work is amenable for quantitation of expression levels of multiple target proteins. To our knowledge, this represents the first multidimensional liquid chromatographic method demonstrating the applicability of simultaneous quantitation of multiple proteins from complex biological mixtures in a single run.

Lecture L31

Antibody Purification Via Affinity Chromatography That Utilizes the Nucleotide Binding Site

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Described here is a novel method of small molecule affinity chromatography for purifying antibodies through a highly conserved domain in the antibody Fab fragment. Based on antibody crystal structure overlays and taking into account antibody symmetry there are at least two conserved nucleotide binding sites per antibody. An in silico screening of small molecules was carried out to select for a nucleotide analog with relatively high affinity for the nucleotide binding sites, monovalent K_d = 1-10 μM based on various IgG and IgE antibodies tested. These sites allow for a selective multivalent interaction with the surface immobilized nucleotide analog to purify antibodies from complex protein mixtures such as ascites fluid and cell lysate. Antibody capture was accomplished by injection of samples while running equilibration buffer (50 mM sodium phosphate pH 7.0) and elution of the antibody was achieved by running a gradient of mild elution buffer (2M NaCl in 50 mM phosphate pH 7.0). The column exhibited a capture efficiency of >96%, with an antibody purity of >93% using Rituximab as a proof of concept pharmaceutical antibody. This small molecule affinity purification method is further tested varying: injection concentrations, volumes, wash/bind times, elution gradients, antibody/protein-contaminant combinations, and varying injection buffers. The benefits can be listed as i) reduced column cost, ii) increased column capacity, iii) increased life-time for the column due to more durable materials, iv) resin and capture molecule stable to harsh regeneration steps to provide for a more pure product, and v) purified antibody retains its bivalently active state. This new small molecule affinity purification method moves to eliminate the need for immobilized protein based purification techniques that incorporate Protein A or Protein G affinity columns.

Lecture L32

Separation and Structural Characterization of Glycosaminoglycan (GAG) Oligomers by LC-MS^n Using a Chemical Derivatization Strategy

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Structure analysis of glycosaminoglycan (GAG) oligomers, especially the identification of sulfation patterns, is required for a deeper understanding of their structure-function relationships. The major challenges for accurate determination of sulfation patterns for GAG oligomers include the inefficient on-line separation of positional sulfation isomers prior to tandem mass spectrometry analyses and the gas-phase losses of the sulfate groups upon collision-induced dissociation (CID). Here, we apply a chemical derivatization strategy involving permethylation, desulfation and peracetylation to label the original sulfation sites of the oligomers with acetyl groups. This method enables the online separation of isomeric GAG oligomers using regular reverse phase LC (RPLC), as well as structural characterization by sequential stages of tandem mass spectrometry (MS^n).

GAG oligomers were first reduced by sodium borohydride to eliminate the anomeric configurations at the reducing end, also to improve the LC separation. The triethylamine salts of reduced oligomers were obtained in order to allow dissolution in DMSO for subsequent permethylation. The permethylation step was performed according to the general sodium hydroxide method, which protect all the hydroxyl groups by replacing the hydrogen with methyl group. The fully permethylated products were then subjected to desulfation, followed by peracetylation to replace the sulfation groups with relative stable acetyl groups. By doing this, the oligomers not only have increasing retention on a reverse phase column, but also are more stable during the CID fragmentation. RPLC-MS^n analysis of derivatized oligomers was performed using a regular C18 column for separation and a linear ion trap (Thermo LTQ) for MS analysis.

We have demonstrated that isomeric chondroitin sulfate (CS) hexamers differ only in sulfation positions could be identified by MS^n analysis after derivatization. Briefly, derivatized hexamers were fragmentized into three distinguishable individual dimer units with each analyzed separately in a systematic, modular scheme. Combining such fragmentation scheme with on-line separation by standard RPLC, we successfully isolated and identified five isomeric species from the CS hexamers mixture. We also have applied the method to synthesized heparan sulfate (HS) oligomers, by utilizing trideuterated acetyl groups to label the original sulfation sites in order to differentiate between N-acetyled and N-sulfated amino sugars. Preliminary data shown that full derivatization and structural identification was achieved for 12 common types of HS disaccharides, as well as for certain larger oligomers. Investigation of the separation and analysis of HS oligomer mixtures by RPLC only or combined with ion-mobility spectrometry is currently underway.

Lecture L33

High Speed and High Resolution Separations of Peptides and Polypeptides Using Fused-Core Columns

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Since their introduction in 2006, columns packed with 90 Ångstrom Fused-Core® particles have been shown to be very effective for carrying out ultrafast and high resolution separations of small molecules with molecular weights up to 2000-5000 daltons. These columns deliver unexpectedly high efficiencies, which are comparable to those generated by sub-2-µm totally porous columns—at about 50% of the back pressure. Larger pore size, 160 Ångstrom, Fused-Core particles extend this molecular weight range to 15-20 kDa, making highly efficient, high resolution separations possible for many peptides, polypeptides, and small proteins. In addition, the stationary phase is prepared using sterically-protected C18 chains, which facilitate stable, reproducible separations up to 100°C. Such extended stability offers a significant advantage for the low pH mobile phases commonly used for such biomolecule separations.

The desirable properties of these 160 Å Fused-Core particles with their extra stable bonding chemistry will be highlighted for separations of various complex peptide samples. Example comparisons will be shown for these 160 Å superficially porous materials with totally porous 3 µm wide pore materials (300 Å), and with mid-pore sized (130 Å) sub-2-µm micron materials.

Finally, the effectiveness, of the addition of varying amounts of ammonium formate to formic acid (pH 3) to improve retention, peak shape and peak widths for basic peptides, without negative impact on MS detection, will be discussed.