P1. New Approaches with an Established Reversed Phase Resin – The Versatility of Amberchrom™ CG71

Jon Fisher
jonfisher@rohmhaas.com
Other Authors: Rashmi Agarwal and Marlin Kinzey

Healthcare
Dow Chemical Company
727 Norristown Road
Spring House, PA 19477-0904 U.S.A.

For over 20 years, Amberchrom™ reversed phase resins have been used for production-scale purification of biotherapeutics. Most of these processes involve the use of hazardous solvents for the reversed phase purification of peptides and oligonucleotides. However, Amberchrom™ CG71 has unique properties that allow it to be used in a gentler hydrophobic interaction mode for protein purification. Additionally, the use of nonflammable solvents such as hexylene glycol, give greater flexibility in manufacturing. Lastly, Amberchrom CG71 works well for the selective removal of detergents and color bodies from recombinant protein feedstreams. In this study we demonstrate the use of Amberchrom™ CG71M, an acrylic 75 micron resin, for protein purification with a variety of different conditions.

P2. New Aspects of Biomimicry: The Development of Novel, Low Molecular Weight Affinity Ligands for the Selective Capture, Purification and Analysis of Protein Superfamilies.

Milton Hearn
milton.hearn@sci.monash.edu.au
Other Authors: Simon Mountford, Roy Jackson, Anjali Bhagwat, Agron Mataj, Reinhard Boysen, and Chunfang Zhang

ARC Special Research Centre for Green Chemistry
Monash University
Building 75, Wellington Road
Clayton, Victoria 3800 Australia

In this presentation, we describe the development use of new families of biomimetic affinity ligands derived from heterocyclic compounds for the capture, purification and analysis of proteins produced by recombinant DNA technologies. In particular, from selected sub-libraries of compounds, generated via combinatorial synthetic strategies and principles, specific ligands have been identified via screening methods that make them eminently suitable, following immobilization, for use in the (pseudo)affinity or 'mixed mode' purification of the target protein(s). This presentation describes the framework employed for the design, synthesis and application of these new ligand systems as part of a quality-by-design (QbD) approach for the selective purification of different classes of genetically engineered proteins and monoclonal antibodies in particular. Central to these considerations has been the development of new tools for the immobilization of these new ligands to optimal surface density levels to ensure excellent capture and subsequent recovery, with preservation of biological activity. Moreover, the strategy pursued in these studies provides a powerful avenue to reduce the number of steps (unit operations) in the purification of a specific protein and removal of host cell protein (HCP) contaminants through the use of positive and negative adsorption strategies with columns in series. Examples of the application of this new technology with a variety of different protein superfamilies will be discussed.

This work was supported by the Australian Research Council.

P3. Multi-modal peptide separations (RP, AEX, Ion-exclusion, HIC, HILIC modes) with a single column containing a mixed-mode reversed-phase/weak-anion exchange material

Marek Mahut
marek.mahut@univie.ac.at
Other Authors: Michael Laemmerhofer, Raquel Nogueira, Wolfgang Lindner

Christian Doppler Laboratory for Molecular Recognition Materials
University of Vienna
Waehringerstrasse 38
Vienna, 1090 Austria

Analytical and preparative scale separation of peptide has become an important application area of liquid chromatography in fields like proteomics, synthetic peptide purification and purity control, and many more. The most frequent technique utilized for chromatographic separation of peptides is gradient reversed-phase HPLC. On contrary, ion-exchange chromatography alone is seldom used, but became an important separation mode in 2-dimensional concepts in proteomic research.

We recently presented mixed-mode separation materials, based on particulate and monolithic silica support, that were modified chemically with a chromatographic ligand consisting of a hydrophobic strand and a weak anion-exchange moiety. Besides, polar embedded groups such as amide and thioether functionalities were incorporated in the chromatographic ligand as well. These mixed-mode materials turned out to be highly useful for separation of synthetic peptides by a separation mechanism that is complementary to that of gradient RP-HPLC that is commonly adopted as the state-of-the-art technique in the field.

Depending on the employed conditions, a column packed with this material can exploit hydrophobic interaction, anion-exchange, ion-exclusion, and hydrophilic interaction as retention and selectivity principles. As a consequence, the column can be operated in the RP mode (neutral compounds), anion-exchange mode (AEX) (acidic compounds), ion-exclusion chromatography mode, hydrophobic interaction chromatography (HIC) mode and hydrophilic interaction chromatography (HILIC) mode as well. This allows a flexible adjustment of selectivity by tuning mobile phase conditions. These distinct separation mechanisms will be outlined by selected examples of peptide separations.

For some synthetic peptides the column showed excellent loading capacity in the RP/WAX separation mode and outperformed gradient RP-HPLC by factor of 10-100.

P4. Development of a Boronate Affinity Chromatography Method for Determining Glycation Content of HGS-X Bulk Drug Substance and Final Drug Product

Jeffrey Hanten
jeff_hanten@hgsi.com
Other Authors: Marla Estock, Michael Byrne

Analytical Sciences
Human Genome Sciences, Inc.
14200 Shady Grove Road
Rockville, MD 20850 USA

A method for determining glycation levels in HGS-X bulk drug substance (BDS) and final drug product (FDP) was developed using a boronate affinity chromatography column on a high-pressure chromatography system coupled with a fluorescence detector. The boronate affinity column binds the glycated protein species through an m-aminophenyl boronate ligand on the column resin, allowing the non-glycated form to flow through. The critical assay attributes were evaluated to provide optimal binding of the glycated species, providing adequate separation of the glycated and non-glycated forms. The optimized assay was qualified demonstrating specificity, a precision of = 3.7%, and an accuracy of 80 – 89% across the linear range with a suitable limit of quantitation. The method provides a robust alternative to less specific methods. The glycation levels of BDS and FDP measured by the boronate affinity chromatography method are in excellent agreement with those determined by liquid chromatography/ mass spectrometry (LC/MS). The method provides a fast and efficient way to quantify glycated protein species and has provided critical information for comparability of HGS-X BDS and FDP lots.

P5. Automated parallel chromatographic separations in process development.

Juergen Friedle
j.friedle@atoll-bio.com
Other Authors: Tim Schroeder

Marketing & Product Innovation
Atoll GmbH
Ettishofer Str. 10
Weingarten, B-W 88250 Germany

A miniaturized column system in standard microplate format, harbouring exchangeable arrays with up to 96 individual minicolumns, was adapted for automated operation in a modified commercial liquid handling workstation. For this purpose the eight channel liquid delivery system of the robotic workstation was reversibly connected to the columns, in order to allow uptake and loading of different volumes of samples and buffer solutions in the individual steps of the separation procedure.

Liquid flow in the columns was driven by positive pressure liquid displacement, like 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 a next separation step for a further chromatographic dimension or analysis like UV,MS, HPLC or SDS-PAGE.

The combined Atoll - Tecan robotic system allowed to perform automated high throughput small scale bio-chromatographic separations of protein samples by running up to eight individual columns simultaneously.In this example a full method development procedure for a therapeutic mAb is decribed. Loading crude feedstock to a series of different Protein A resins and elution at acidic pH lead to a favourite candidate. This was followed by negative AIEC with 8 different resins. The most suited candidate out of this experiment was optimized regarding protein binding by varying salt concentration and pH. To obtain information, if a flowthrough or bind/elute step is better suited, a parallel set of experiments was performed with 8 different CIEC materials eluting at increasing salt concentrations.

All experiments were first performed manually with PipetColumns, then transferred to automated RoboColumns and finally scaled to 10ml LC columns for proof of concept. Final decision on the optimum overall process could be drawn within 3 days!

P6. Recovery of whey protein isolate using open loop non isocratic SMB Technology

Deepti Sahoo
Deepti.Sahoo@biotek.lu.se
Other Authors: Jonatan Andersson, Bo Mattiasson

Department of Biotechnology
Lund University
Getingevägen 60 , 22241 Lund, Sweden
Lund, Sweden

The recovery of whey proteins using an open loop non isocratic simulated moving bed (SMB) technology was studied. A cation exchange chromatographic support, Streamline SP, was used. The SMB configurations were designed based on the results from a single column breakthrough experiment. The information obtained from the single column cycle (step volumes, flow rates, elution characteristics etc) was used for setting-up the SMB process (number of columns in each zone, switch time, volumes and flow rates). A comparative study on different process configuration was made to optimize yield and productivity of the system. The experimental set-up that was used contained 10 columns, a central multi-port valve, several pumps and equipment for collecting fractions. Different process configurations (with regard to column arrangements) were tested in order to optimize yield and productivity. The best process configuration showed a productivity increase of 20%, a product concentration increase of 2.7 times and a buffer consumption decrease of 3.3 times compared to what was previously reported in the literature for whey protein recovery using single column chromatography

P7. Developing Separations of Monoclonal Antibodies And Other Proteins Using Reversed-Phase UPLC®

Hillary B. Hewitson
hillary_hewitson@waters.com
Other Authors: Thomas E. Wheat, Uwe D. Neue, Paula Hong, Kenneth J. Fountain

Chemistry Applied Technology
Waters Corporation
34 Maple St.
Milford, MA 01757 United States

The analysis of biological macromolecules, such as monoclonal antibodies, has required improvements in sensitivity and chromatographic resolution to assure complete and accurate characterization. It is now possible to apply the principles of the UPLC® technology to this analytical challenge using a sub-2µm particle column with 300Å pores and a C4 bonded phase.

The variables that are critical for developing a successful reversed-phase separation method for intact proteins were evaluated. These variables included mobile phase constituents, solvent selection, flow rate, separation temperature, and gradient slope. Peak shape, peak area, resolution, and carryover were measured for a variety of protein probes representing a wide range of molecular weight, acidity/basicity and hydrophobicity. Monoclonal antibodies and their subunits were also used in the evaluation. Results will show how simple adjustments to the separation conditions can cause desirable changes in the chromatographic result, including alterations in selectivity and improved recovery. In addition, the controlled separation experiments can be fitted to a theoretical model for the calculation of retention factors and column efficiency for a variety of protein separations.

The combination of the Waters® ACQUITY UPLC system and a new column packing material developed specifically for the reversed-phase separation of intact proteins can provide increased resolution, sensitivity, and speed.

P8. Techniques for Improving the Isolation of Synthetic Peptides

Jo-Ann M. Jablonski
jo-ann_jablonski@waters.com
Other Authors: Thomas E. Wheat, Kenneth J. Fountain

Chemistry
Waters Corporation
34 Maple Street
Milford, MA 01757 USA

Peptides have many biological functions and are essential for the research and development of biopharmaceuticals. Even though the synthesis and cleavage of the peptide is carefully controlled, numerous impurities are generated. Impurities include deleted and truncated sequences, cleavage adducts, incomplete deprotections, and modified amino acids. All of these contaminants must be removed from the target peptide for unambiguous results in future experiments. Several approaches are available to adjust the purification process for improved yield of pure material. Some peptide mixtures are difficult to dissolve and keep in solution throughout the isolation process. Solvents like dimethylformamide or dimethylsulfoxide are good for dissolving samples but their use can jeopardize the chromatographic purification. The patented at-column dilution technique, used in conjunction with temperature control and focused gradients, improves chromatographic resolution, column mass capacity, and purification system ruggedness by preventing sample precipitation. Controlling the temperature of the isolation at the large scale vastly improves the purification of the product by improving the peptide's solubility in the mobile phase and increasing efficiency. Focused gradients give better resolution of the peptide product from its closely-eluting contaminants without increasing run time. In this study, we illustrate the use of at-column dilution, temperature control and focused gradients for improving synthetic peptide isolation. Employing these techniques ultimately lead to improved process efficiency and peptide products with higher purity and increased yield.

P9. A Novel Column for Analyses of N-Linked Glycans of Glycoproteins

Beth L. Gillece-Castro
Beth_Gillece-Castro@waters.com
Other Authors: Thomas E. Wheat, Kim Van Tran, Joomi Ahn,
Jonathan Turner, Diane Diehl

Waters Corp.
34 Maple St.
Milford, MA 01757 United States

Glycosylation is a post-translational modification of proteins. The sugar chains on glycoproteins can mediate biological activity, and are therefore associated with the safety and efficacy attributes of many biopharmaceuticals. The relative amounts of the individual glycan structures must be monitored at all stages of research and development.

We have developed a glycan analysis solution for these applications. N-linked glycans are released from glycoproteins and are labeled with 2-aminobenzamide (2-AB). The derivatized oligosaccharides are separated using the new ACQUITY UPLC® BEH Glycan Separation Technology column containing 1.7 micron particles. A fluorescence detector with a low volume flow cell is specific for the 2-AB label, and enhances sensitivity beyond the predicted improvement from narrow peaks. The Waters ACQUITY UPLC instrument provides the operating characteristics to realize the resolution, sensitivity and speed benefits of a sub-2 micron particle packing material.

Labeled IgG glycans are separated during a 35 minute gradient in HILIC mode. One sample that contains mono-sialylated, neutral (G0F, G1F, and G2F), and high mannose oligosaccharides, was used during development and QC to guarantee resolution of relevant components. Specifically, G0F is well separated from Man5, and can be baseline resolved in less than 15 minutes. The isomers of G1F are also baseline resolved. The chromatogram of a mixture of high mannose oligosaccharides demonstrates separation of isomeric mannose 7 structures.

This separation chemistry can be used in other applications requiring oligosaccharide analysis. Glycomics experiments often employ a standard measure of retention based on a series of dextran polymers. The 2-AB dextran polymer series had resolution from one glucose unit (GU) to more than twenty-two GUs during a 50 minute gradient. The same column and mobile phase can be used for neutral and charged oligosaccharides. The acidic glycans from the protein bovine fetuin are model glycans for the structures present on drugs such as erythropoietin. The chromatographic separation of the fetuin mono-, di-, tri-, and tetra-sialylated structures is clearly demonstrated. Further, isomers of the disialo-triantennary structure are separated by two minutes. In conclusion, the UPLC Glycan Separation Technology columns, when operated on the ACQUITY UPLC instrument with fluorescence detection, provide a high resolution, reproducible, and rapid method for separating and profiling glycans from glycoproteins.

P10. Preparative isolation and purification of peptides by countercurrent chromatography

Yanbin Lu
luyanbin@zju.edu.cn

College of Food Science and Biological Engineering
Zhejiang Gongshang University
149 Jiao gong Road, Experimental Building 2#, room 301
Hangzhou, ZHEJIANG PROVINCE 310035 PR CHINA

Counter-current chromatography (CCC) was developed by Ito in the late 1960s. The stationary phase used in CCC was liquid, without solid phases, which relies on the partition of a sample between two immiscible solvents to achieve separation. The relative proportion of solute passing into each of the two phases is determined by the respective partition coefficients. Therefore, CCC benefits from great advantages when compared with the traditional liquid-solid separation methods: it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination. In addition, CCC is a preparative technique with high recovery, acceptable efficiency and the ease of scaling-up. Regarding the recent numerous literature about CCC development and applications, it is obvious that CCC is a promising preparative separation technique, extremely useful for the separation and purification of natural products.

Recent advances in peptide countercurrent chromatography may be attributed to three major factors: (1) the use of a new two-phase solvent system with a low viscosity, (2) development of pH-zone-refining CCC; (3) the combined use of ion-pair (exchange) CCC. The solvent system composed of tert-butyl methyl ether/n-butanol/acetonitrile/water at various volume ratios permitted the application of high-speed CCC, which provides efficient separation of peptides in an preparative mode.

P11. Terminal-specific, solid-phase PEGylation of rhEGF using self-cleavable intein tag

Junghye Kang
vanini@hanyang.ac.kr
Other Authors: Hyun Kyoung Byun, E. K. Lee*

Chemical Engineering
Hanyang University
Sa-3 dong, Sangnok-gu
Ansan, Kyeonggi-do 425-791 Korea

Chemical conjugation of activated PEG (polyethylene glycol) to a protein, i.e., PEGylation, is an established process to improve the pharmacokinetic properties of injectible biopharmaceutics. However, it is important to control the site of PEGylation to maintain the bioactivity of a protein by avoiding the masking effect of active sites by PEG molecules. In this study, we suggest solid-phase, terminal-specific PEGylation by using affinity tagging with intein. Inteins are self-splicing proteins that can effectively link affinity tags to a certain domain of a protein, allowing the tag to bind to a solid phase and exposing the opposite side to the mobile phase.

We have developed an integrated process for expression-refolding-PEGylation-purification. rhEGF (recombinant human epidermal growth factor) was used as a model protein and the intein with chitin binding domain (CBD) was used as a model intein system (purchased from New England Biolab, Inc., USA). The first step was to express a fusion protein composing of rhEGF-intein-CBD complex in E. coli as inclusion body. Since the C terminus of EGF was responsible for receptor binding, the intein was fused to the C-terminus allowing PEG to attach to the N-terminus during a PEGylation step. After intermediate refolding, the fusion complex was bound to the chitin resin and pre-purified through the chitin column. Methoxy PEG aldehyde was then introduced to the column to PEGylate specifically the exposed side, i.e., N-terminus amine, of the bound rhEGF. After cleaving out the intein-CBD domain by thiol addition, we would obtain side-specifically PEGylated rhEGF.

The PEGylation site was determined by mass spectrometry, and the bioactivity of the PEGylate was compared with the native and randomly PEGylated EGF. It indicated the merits of the integrated strategy for terminal-specific, solid-phase PEGylation of therapeutic proteins with medical significance. In this presentation, the experimental details and the results will be discussed focusing the process characteristics of the integrated process.

P12. Purification of coagulation factor VIII by chromatographic methosds - Influence of the salt concentration on the size o f the factor VIII/von Willebrand factor complexes.

Elisabeth Cheng
echeng@butantan.gov.br
Other Authors: Daniela Jinzenji, Roberta Rodrigues de Carvalho, Claudia Iwashita, Isaías Raw, Elizabeth Angélica Leme Martins

Center of Biotechnology
Instituto Butantan
Avenida Vital Brasil 1500
São Paulo, São Paulo 05503-900 Brazil

Coagulation factor VIII (FVIII) is a glycoprotein, which deficiency or absence causes Hemophilia A. Treatment consists in infusions of FVIII concentrates. Due to the low concentration in plasma (150 ng/mL) and the lability of this protein, the development of purification processes continues to be a challenging task. Production of the most of the licensed plasma-derived factor VIII concentrates employs cryoprecipitation as the first step of purification, which requires expensive equipments such as centrifuges and cold rooms. Alternatively, direct chromatography of plasma has been found to be particularly advantageous for fine and rapid plasma fractionation. Gel filtration of plasma is effective as initial step purification, because it takes advantage of the fact that FVIII circulates in plasma in large complexes with the von Willebrand factor (vWF). However, the need of large columns and the low loading capacity makes this strategy expensive and time consuming, even using industrial gel filtration resins. In order to concentrate FVIII from the plasma sample, we studied the use anion-exchange column prior to gel filtration. Gel filtration of the ion-exchange column eluate in Sepharose 4FF or 6FF results in 2 peaks containing FVIII activity. In the first peak, FVIII can be obtained with higher purification factor, while in the second peak, FVIII/vWF complexes coelute with lower molecular mass proteins, including the FVIII (in)activators. The relative size of the peaks depends on the salt concentration in the elution buffer used in the ion-exchange chromatography. Using citrate buffer, we varied the concentration of NaCl and CaCl2. Our results show that variation of NaCl concentration has some influence on the size of the FVIII/vWF complexes, but variation of CaCl2 concentration is critical. Therefore the effectiveness of the gel filtration depends on the salt concentration in the buffer used as the mobile phase employed in the ion-exchange step.

P13. PAT-based Buffer Dilution and Mobile Phase Preparation On Demand

Michael Li
Michael.li@technikrom.com
Other Authors: Thomas Malone, Francis Bach, Lou Bellafiore

Process Science
Asahi Kasei TechniKrom
1855 Elmdale Avenue
Glenview, IL 60026 USA

Biopharmaceutical manufacturing requires inorganic buffer solutions, beginning with upstream cell culture or fermentation media and continuing through chromatography and filtration steps, ending with formulation. Buffer-make up variability has always been a significant contributor to increased costs in production scale biopharmaceutical processes. Furthermore, capacity constraints such as lack of physical capacity to accommodate large buffer tanks restrict downstream process throughput and capability. Overcoming human error and space restrictions in buffer preparations as well as the current industry bottleneck in downstream purification steps has required a paradigm shift that permits the preparation of tens of thousands of liters of dilute buffers in a footprint of less than 35 ft^2 and on demand reproducibly.

Due to the variability of mobile phase compositions conventional drug production within the pharmaceutical industry predominantly consists of post chromatographic quality control. In general, Out of Specification (OOS) product fractions are pooled and reworked. This post-process approach to quality control is time-consuming, increases the cost of goods sold, and offers little process understanding and control.

Manufacturing science philosophies, such as "6-sigma," are instrumental in achieving high product quality and manufacturing efficiency. In accordance with the FDA's "cGMPs for the 21st Century" initiatives, analyses reveal that improved control of a key variable leverages a large improvement in final product quality and profitability. By gaining real-time process control, excessive rework and discarded product can be avoided.

In this presentation, we examine the operational requirements and designs for a process-ready PAT-based buffer preparation system, which is capable of making reproducible and robust in-line buffer dilution from concentrates. The accurate buffer and mobile phase blending should afford better yield and product recovery and lower manufacturing cost.

P14. A UPLC-ELSD Method for Direct Quantification of Polysorbate

Joseph Liu
joseph.liu@imclone.com
Other Authors: Stephen Tracy, Joel Goldstein, Rick Crowley

Formulation Department
ImClone Systems
33 ImClone Drive
Branchburg, New Jersey 08876 U.S.A.

PURPOSE:To develop a simple and robust method to directly quantify Polysorbate 20 and Polysorbate 80 in protein formulations using a reverse phase-ultra performance liquid chromatography (RP-UPLC) with an evaporative light scattering detector (ELSD).

METHODS: The ELSD is a universal detector that identifies any analytes less volatile than the mobile phase regardless of their spectroscopic properties. Several polysorbate solutions with known concentrations are prepared in water to build a standard curve for determining the polysorbate concentration of unknowns. Following centrifugation for 5 minutes at 13200 rpm, a 50 ?L of each polysorbate standard solution or unknown sample is injected into a Waters Acquity UPLC system with a Waters Acquity UPLC BEH 1.7 ?m C18 column. Resolution of polysorbate is achieved with an acetonitrile gradient from 55% to 98% (0.6 mL/min) in 10 mM Ammonium Bicarbonate (pH 7.8). A Waters ELSD with optimized settings is used for the peak detection. The polysorbate peaks of unknown samples are then integrated and quantified using the polysorbate standard curve.

RESULTS and CONCLUSIONS:
The standard curves of Polysorbate 20 or Polysorbate 80 were found to be accurate from 0.002 % to 0.04 % with a coefficient of regression > 0.98. This method was applied to quantify the concentration of Polysorbate 20 and Polysorbate 80 in the formulated protein samples without further sample preparation. The limit of detection for Polysorbate 20 and Polysorbate 80 in formulated protein solutions is 0.001 % and the limit of quantification for Polysorbate 20 and Polysorbate 80 in formulated protein solutions is 0.002 %. This method has displayed more than 90 % of accuracy and precision at determination of the concentration of Polysorbate 20 and Polysorbate 80 in formulated protein solution. Consistent recovery of Polysorbate 20 or Polysorbate 80 from various sample solutions with and without protein (from 0 mg/ml to 36 mg/ml) indicates that this method is protein independent.

This method offers an extremely fast and accurate way to directly detect and quantify polysorbate without any notable sample preparation. It is protein independent and samples are resolved within 12 minutes using less than 5 milliliters of acetonitrile per sample, making this method an efficient option for detecting Polysorbate 20 and 80. This method may also be applicable for the detection of Polysorbate 40 and 60 as well.

P15. 3 micron SEC packings

Xueying Huang
xhuang@sepax-tech.com

Sepax Technologies, Inc.
5 Innovation Way
Newark, Delaware 19711 USA

This paper will present recently developed 3um silica particles for size exclusion chromatography packing materials, smallest particle size for commercial SEC products. Their pore sizes are controlled at 100, 150 and 300A. Those silica particles are surface modified with a proprietary technology to be compatible with biological molecules which have negligible non-specific interactions. Those 3um SEC packing materials have the highest separation efficiency, which is doubled compared to 5um SEC packing materials. A number of applications have been developed in the areas of commonly used proteins, monoclonal antibodies, pegylated proteins, and other monoclonal antibody derivatives.

P16. Separation of proteins using ProSwift™ reversed phase 1 mm I.D. monolithic columns

Srinivasa Rao
Srinivasa.rao@dionex.com
Other Authors: Charanjit Saini, Kelly Flook, Yury Agroskin, Chris Pohl

R&D
Dionex Corporation
445 Lakeside Drive
Sunnyvale, CA 94085 USA

Reversed phase chromatography based on hydrophobicity is routinely used for separation of proteins. Monolithic ProSwift™ reversed phase columns are ideal for such analysis of proteins as they offer several advantages over other RP columns. First, ideally engineered porous structures of the monolith result in highly efficient separation of proteins. Second, due to low back pressures are generated, high flow rates can be used with comparatively little loss of resolution resulting in high productivity. Third, since monolithic columns are made of polymeric stationary phases, they are stable at pH values 1-14.

The recent introduction of the ProSwift analytical RP 1 mm column has attracted investigators for protein analysis as it can be used on a standard HPLC system with minor plumbing changes along with use of a micro detector cell to maximize the signal. It also consumes much less solvents when compared to standard dimension columns. To develop this new 1-mm high resolution column, as mechanical compression was not easily achievable, we grafted the monolith to the PEEK walls using a proprietary process. This eliminates flow by the column wall, which is needed for high efficiency chromatography. Unlike monoliths prepared in fused silica capillary formats (<500 μm) that are bound to the inner wall using a silyl methacrylate, which is susceptible to hydrolysis at elevated pH, the PEEK material used for the 1mm column which brings pH stability and solvent compatibility, pressure stability and thus brings overall improvement in chromatographic performance. The longer formats offer high loading capacity which is often a necessary requirement for separation of mixture of proteins on two dimensional chromatography platforms.

In this poster, we show various features of the new 1mm i.d. reversed phase ProSwift monolith columns, their applications and comparisons with other columns. We also show fast separations of proteins achieved within a minute using this column and demonstrate robustness of the process and ruggedness of the column.

P17. ProSwift monolith 1mm I.D. ion-exchange columns for Protein separations

Yury Agroskin
yury.agroskin@dionex.com
Other Authors: Srinivasa Rao, Kelly Flook, Charanjit Saini, Maria Rey, Andy Woodruff, Jim Thayer, Chris Pohl

R&D
Dionex Corporation
1228 Titan Way
Cupertino, CA 94088 USA

ProSwift monolith columns offer several advantages over conventional porous columns. They include fast mass transfer, high loading capacity, stable resolution even at elevated flow rates and wide pH stability. These characteristics support versatile performance in a wide range of protein separations. ProSwift Ion Exchangers in 1mm format are well suited for two dimensional chromatography applications due to their high loading capacity and superior overall performance.

Earlier, we introduced weak anion and strong anion exchange monolithic phases with tertiary amine and quaternary amine functionalities respectively, and weak cation exchange columns with carboxylate functionality. These columns are polymethacrylate based polymer materials in 1 x 50 mm format and provide improved sensitivity and reduced solvent consumption compared to larger 4.6 mm I.D. columns. The ease of using these 1mm monolith columns at elevated flow rates on analytical HPLC instruments has attracted investigators interested in running samples under reduce run-time conditions that result in improved productivity. Very little adjustment is needed to adapt 1mm ProSwift columns to standard HPLC instrumentation.

Currently, a 1mm strong cation exchange column (ProSwift SCX) with sulfonic acid functionality is under development. In this poster we present data using anion and cation exchange phases including the new 1mm ProSwift SCX column for various protein separation applications. We will show applications using monolith anion exchange columns including separation of transferrin, pancreatin and other complex protein mixtures. We will also present application examples for 1mm ProSwift SCX columns including separation of snake venom proteins and monoclonal antibodies. We will discuss data for important column characteristics including dynamic capacity, ruggedness and reproducibility.

P18. Negatively cooperative binding of myoglobin and positive one of lysozyme/albumin to vesicles

Francisco Torrens
francisco.torrens@uv.es
Other Authors: Gloria Castellano

Institut Universitari de Ciència Molecular
Universitat de València
Edifici d'Instituts de Paterna, P. O. Box 22085
València, València 46071 Spain

The role of electrostatics is studied in the adsorption of cationic proteins to zwitterionic phosphatidylcholine (PC) and anionic mixed PC/phosphatidylglycerol (PG) small unilamellar vesicles (SUVs). For model proteins the interaction is monitored vs. PG content at low ionic strength. The adsorption of lysozyme–myoglobin–bovine serum albumin (BSA) is investigated in SUVs, along with changes of the fluorescence emission spectra of the cationic proteins, via their adsorption on SUVs. The structures of the attached lysozyme–bovine serum albumin (BSA) layer on the protein–SUV systems play a significant role. A model for both proteins, which composes two protein sub-layers with different structures–properties, is proposed. The partition coefficients are determined and the isotherm curves represented. Provisional conclusions follow. (1) On the protein–vesicle binding model protein and vesicle effects were analyzed. Myoglobin, DNC-melittin and melittin association to zwitterionic phospholipid vesicles can be described by a simple model, incorporating a water–membrane partition equilibrium, modulated by electrostatic charging of the membrane, as the protein accumulates at the interface. The surface potential induced in this way counteracts the association of further peptide. The effect can be satisfactorily treated by using a Gouy–Chapman approach. Further protein binding is difficult because the repulsion of like charges becomes the dominant mechanism. In terms of conventional binding mechanisms this would correspond to a negative cooperativity. On the other hand, lysozyme and albumin binding to zwitterionic and anionic vesicles follow a schematic simplified model of positive cooperativity, which represents the interaction between the protein considered as a dipole moment and the charged phospholipid headgroups taken as an isolated anion. (2) In mixed zwitterionic/anionic vesicles the charge effect on the protein binding model was analyzed. For lysozyme–anionic enough vesicles and myoglobin, the electrostatic repulsion between cationic ad-proteins dominates. (3) The salt effect on the protein binding model of mixed zwitterionic/anionic vesicles was analyzed.The cooperativity increases with ionic strength, meaning that the electrostatic repulsion between cationic ad-proteins decreases with increasing salt effect. (4) In anionic vesicles the effect of vesicle charge on protein binding shows that, with increasing anionic vesicles, the protein–protein electrostatic repulsion is decreasingly important vs. the protein–vesicle attraction. (5) For lysozyme–mixed zwitterionic/anionic vesicles and myoglobin, cooperativity increases with pH. With increasing pH and decreasing cationic character of the protein, the protein–protein electrostatic repulsion is decreasingly important against the protein–SUV attraction. The opposed is observed for lysozyme–zwitterionic vesicles.

P19. Characterization of extremely alkaline resistant New TOYOPEARL anion exchanger

Yuji Kubo
y_kubo@tosoh.co.jp
Other Authors: Hiroyuki Moriyama

Separation Center
Tosoh Corporation
4560 Kaisei-CHO Shunan
Yamaguchi, 746-8501 Japan

In the purification of proteins such as biopharmaceuticals, the alkaline resistance of the packing material is one of the crucial column performances. The packed column is usually washed with sodium hydroxide aqueous solution to reduce the risk of contaminating the product with harmful substances, ex. lipids, viruses, endotoxins, and cell debris. (Sanitization or CIP operation) Therefore, there is a strong market demand to improve the alkaline stability of the packing material. If the alkaline stability of the packing material is low, the resolution and the adsorptive capacity of the column decrease during recycling.

We developed a new anion exchanger TOYOPEARL resin to improve the life time of the packing material in an alkaline solution. This new TOYOPEARL resin has not only high alkaline resistance, but also very hydrophilic and high mechanical stability as well as a conventional TOYOPEARL. This resin is stable in 1mol/L sodium hydroxide soaking test for three months.

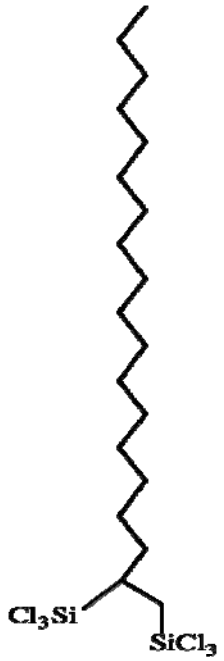
In this poster, we introduce the fundamental performance of this extremely high alkaline resistant TOYOPEARL anion exchanger.

P20. Pendant Dipodal Alkysilane Bonded Phases for Low pH HPLC

Barry Arkles
executiveoffice@gelest.com
Other Authors: Gerald Larson, Youlin Pan, YunMi Kim

Gelest Inc.
11 East Steel Rd.
Morrisville, PA 19067 USA

While octadecylchlorosilanes remain the dominant materials for preparation of bonded phases utilized in preparative chromatography, their stability is not robust in low pH phosphate buffer systems utilized for many biological separations. 1,2-bis(trichlorosilyl)octadecane, the first example of a pendant dipodal silane, has been synthesized. A variety of methods have been utilized to evaluate the stability of surfaces bonded with the new C18 and related bridged dipodals at different low pH regimes. Stability based on equilibrium studies has been predicted. Contact angle studies for up to 6000 hours at pH extremes were studied. Bonded phases on Kromasil were compared and indicate that at a minimum the dipodal phases are 3 times more stable than conventional silanes.



Pendant Dipodal C18 Silane

Conventional C18 Silane

P21. Observation of interaction between RNA and GFP-Protein by affinity capillary electrophoresis

Atsushi Taga
punk@phar.kindai.ac.jp
Other Authors: Ryosuke Satoh, Shunji Ishiwata, Shuji Kodama, Atsushi Sato, Kentaro Suzuki, Reiko Sugiura

School of Pharmacy
Kinki University
3-4-1, Kowakae
Higashi-osaka, Osaka 577-8502 Japan

Some RNAs playing anti-cancer functions by interacting with proteins have been discovered in recent studies. New technology for binding assay with brief operations is expected. We have already reported a technique to observe interaction between a mRNA and a purified protein by affinity capillary electrophoresis (ACE) with UV detection on the ISPPP 2008 held in Baden-Baden, Germany. The basic principle of ACE has been observed in some of our previous papers [1,2]. In this study we tried to assay binding between a mRNA as a ligand in a back ground electrolyte (BGE) and a green fluorescence protein (GFP) fusion protein in a lysate without any purification as a protein sample by using a lazar induced fluorescence (LIF) detection capillary electrophoresis system.

A capillary tube in which the inner wall was modified with carboxyl group to prevent protein adsorption was used. The carboxylated capillary of a product FunCap/CE-Type C was obtained from GL Science inc. A semiconductor laser emitting a light at 473 nm was used for excitation, and fluorescence intensity at 500-600 nm was measured to detect a protein sample of GFP tagged Rnc1 protein which can interact with Pmp1 mRNA in a BGE [3]. GFP-Rnc1 is weakly negatively charged under the neutral condition, and Pmp1 mRNA has phosphate group of strongly negatively charged residue, resulting GFP-Rnc1 migrates faster than Pmp1 mRNA to the cathodic end with electroosmotic flow migrating from the anodic end to the cathodic end. Therefore migration delay of GFP-Rnc1 can be observed by interaction with Pmp1 mRNA depending on molar ratio of binding type protein. As a result, interaction between GFP-Rnc1 and Pmp1 mRNA was observed using our system, and the strength of this interaction was estimated to be in the order of 108 as an association constant (Ka).

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P22. Micropreparative liquid chromatographic purification of PEP-19 protein from low amounts of brain tissue

John Flensburg
john.flensburg@ge.com
Other Authors: Lars Haneskog, Per Svenningsson, Per E. Andrén

GE Healthcare Bio-Sciences AB
Björkgatan 30
Uppsala, SE-751 84 Sweden

John Flensburg[1], Lars Haneskog[1], Per Svenningsson[2] and Per E. Andrén[3]

[1]GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

[2]Dept. of Physiology and Pharmacology, Section of Translational Neuropharmacology, Karolinska Institute, SE-171 77 Stockholm, Sweden

[3]Dept. of Pharmaceutical Biosciences, Medical Mass Spectrometry, Uppsala University, P.O. Box 583 Biomedical Centre, SE-751 23 Uppsala, Sweden

PEP-19 (Purkinjecell protein 4; PCP 4) is a 6.8 kDa brain protein with a pI value of 6.2 that belongs to a family of proteins involved in calmodulin-dependent signal transduction by binding calmodulin via an IQ motif in a calcium-independent manner (1,2). PEP-19 expression has recently been shown to be decreased in an animal model of Parkinsons´s disease (3).

The objective of the present study was to purify biologically active PEP-19 from low mg amounts of mouse brain tissue (to be used to investigate potential protein binding partners using surface plasmon resonance sensor chip technology). An extract was prepared from brain tissue by a combination of grinding and ultrasonic disrupture. The clarified supernatant was used as starting material for anion exchange chromatography on Mono Q™ PC 1.6/5 at pH 8.0. A shallow NaCl gradient was necessary to separate PEP-19 from impurities with similar size and charge as the target protein. The second purification step consisting of gel filtration using Superdex™ Peptide PC 3.2/30 resulted in a chromatogram with a single peak corresponding to PEP-19, well separated from lower molecular weight impurities. The retention volume of PEP-19 indicated that the protein is a dimer. The identity of PEP-19 in chromatographic fractions was confirmed by nanoscale LC-MS/MS, and SDS-PAGE was used for assaying purity. From 15 mg of mouse brain tissue approximately two µg of PEP-19 (purity about 95 %) was obtained.

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P23. Analysis of Global Isoform Distribution of Human NGAL Protein

Ryan Bonn
ryan.bonn@abbott.com
Other Authors: Tracey Rae, Larissa Harwick, Svetoslava Stamenova,
Kevin Rupprecht, and Jeffrey Fishpaugh

Analytical Chemistry
Abbott Laboratories
100 Abbott Park Road, BLDG. AP-20
Abbott Park , Illinois 60064 USA

Elevated levels of Neutrophil Gelatinase Associated Lipocalin (NGAL) in urine have been established as a promising diagnostic indicator for acute kidney injury (AKI). Although significant structural information on the human NGAL protein has been revealed from findings with various recombinant expression systems and native protein isolated from neutrophils, little has been published regarding the NGAL protein species excreted in human urine. Development of a quantitative diagnostic immunoassay for NGAL in urine prompts an understanding of the native protein analyte and its structural similarity to a biosynthetic recombinant protein used to calibrate the assay.

Here we present findings from an analysis of native human NGAL isoforms found in urine (huNGAL). Size-fractionation of unprocessed human urine specimens with gel-permeation HPLC have shown that the predominant form of huNGAL from this matrix correlates to the monomeric apparent molecular weight, although oligomeric species are detected in some specimens. When observed, NGAL-containing oligomers are shown to occur through inter-protein disulfide linkage, as treatment with a reducing agent converts all NGAL activity to monomeric size in Western blots. We also demonstrate disparate isoelectric point profiles employing standard IEF and 2DE analysis comparing recombinant NGAL from multiple sources as well as partially purified native (huNGAL). All isoforms from reduced and denatured huNGAL were found to reside in the molecular weight region corresponding to the predicted mass of the protein. However, upon the development of 2D standardization techniques, the few discrete huNGAL isoforms span a surprisingly wide range in charge distribution (pI 5.9-9.1) given that the calculated theoretical pI of the native polypeptide is 9.0. Moreover, charge distribution profiles vary greatly among recombinant sources of NGAL leading to important questions regarding which pI and sample treatment is most valuable when constructing calibrator proteins for immunoassays.

P24. Purification and Analysis of Urine Human NGAL

Svetoslava Stamenova
svetoslava.d.stamenova@abbott.com
Other Authors: Tracey Rae, Ryan Bonn, Carol Ramsay, Luis Vargas, Jeffrey Fishpaugh and Kevin Rupprecht

Diagnostic Analytical Research
Abbott Diagnostic Division
100 Abbott Park Rd
Abbott Park, Illinois 60064 USA

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a 25 kDa protein currently being investigated for use as a biomarker for renal injuries. NGAL is a glycoprotein expressed primarily in neutrophils and various epithelial cells. It is also present in serum and urine.
Urine huNGAL exists primarily in a monomeric state though oligomers occur as well. To further investigate the role of NGAL in inflammatory disorders, a reliable method for concentration and purification of this low abundance urine protein is needed.
We have developed an affinity purification method that utilizes a monoclonal anti-huNGAL antibody covalently coupled to cyanogen bromide activated beads. Elution of huNGAL is accomplished with a low pH buffer. Utilizing this method we purified urine huNGAL that is essentially free of all other urine proteins. The method leads to at least a thousand fold increase in huNGAL starting concentration. The affinity purified huNGAL was analyzed using a variety of analytical tests including SDS-PAGE, Western Blot, GPC-HPLC and 2DE. The isolated material was >98% pure by SDS-PAGE and reactive to two monoclonal antibodies having known epitopes and a polyclonal anti-NGAL antibody. GPC-HPLC revealed dimeric forms as well as monomers. The two dimensional electrophoresis demonstrated that urinary huNGAL consists of a broad distribution of 5-6 isoforms, similar to recombinant huNGAL. The huNGAL from healthy urine was also identified by LC/MS/MS of a tryptic digest of the major band from SDS-PAGE. A Mascot data base search of the MS/MS data gave a positive match for huNGAL.
In summary, we have developed a robust and reproducible method for concentration and purification of urine huNGAL, which can be used as a valuable tool for future elucidation of NGAL expression, processing and function and its role in inflammatory disorders.

P25. Protein Purification based on "Mixed Mode" Chromatographic Systems

Milton Hearn
milton.hearn@sci.monash.edu.au
Other Authors: Anjali Bhagwat, Simon Mountford, Eva Campi, W. Roy Jackson

ARC Special Research Centre for Green Chemistry
Monash University
Building 75, Wellington Road
Clayton, Victoria 3800 Australia

A new class of chromatography media has been developed for protein purification based on immobilising low molecular weight chemical ligands with "mixed mode" selectivity. To achieve robust immobilization, various experimental parameters such as reaction time, solvent and temperature were investigated to optimise the coupling of these chemical ligands onto the activated solid support. These adsorbents were evaluated for their static as well as dynamic binding capacities for the purification of human IgG and other proteins at different conditions of pH, buffer and salt concentration. In particular, isothermal adsorption studies reveal that this new class of chemical ligands shows readily "tunable" selectivity in their affinity chromatographic interactions with proteins. As consequence, the opportunity arises to utilize the same ligand system in more than one mode, depending on the choice of mobile phase composition, to achieve orthogonality in the separation of protein mixtures. Examples of these new chromatographic systems will be demonstrated utilizing feedstock's derived from fermentation or cell culture.

P26. Acetonitrile-free HPLC Analysis and Preparative Purification of a Biopharmaceutical Peptide

Linda Lloyd
Linda.Lloyd@varianinc.com
Other Authors: Stephen Ball, Keeley Mapp

Varian Inc
Essex Road
Church Stretton, Shropshire SY66AX UK

The use of peptides as biopharmaceuticals is one of the major growth areas in current drug development. The purification of the synthetic peptides uses high performance columns, packed with either silica reversed phase media and acetonitrile based eluents. Recently there has been growing interest in the use of polymeric materials, including the poly(styrene/divinylbenzene) based PLRP-S, for these purifications due to their unsurpassed chemical and physical stability and the separation selectivity of these materials media is also independent of particle size, so enabling easy scale up from analytical to prep scale. These two factors can be exploited to improve the economics of a peptide purification by reducing the method development time and increasing column lifetime. However, there is now a greater influence on the cost of a purification which must be considered when developing a peptide purification method

Following recent developments in the chemical industry, and resulting global shortage in acetonitrile, the cost of this solvent has significantly increased and is now at the level where many processes have become uneconomic. New purification methods must be developed to reduce the consumption of acetonitrile. We have investigated the suitability of alternative solvents for the purification of synthetic peptides, method development, packing dynamic axial compression columns and the prep-scale purification.

This study illustrates the use of a ethanol throughout the entire purification process, from the analytical method development to the packing of a 1” ID dynamic axial compression column and the prep-scale purification and analysis of a synthetic peptide.

P27. Investigation into the Alternatives to Acetonitrile for the Reverse Phase Analysis of Peptides

Linda Lloyd
Linda.Lloyd@varianinc.com
Other Authors: Stephen Ball, Keeley Mapp

Varian Inc
Essex Road
Church Stretton, Shropshire SY66AX UK

Due to well-documented developments in the chemical industry recently, there is now a global shortage of acetonitrile, the solvent of choice for most HPLC applications. Acetonitrile has been popular since the 1970's due to its low UV cut-off, low viscosity and good selectivity properties based on its relative polarity, hydrogen bonding and dipole properties.

As a result, laboratories world-wide are facing the prospect of having to re-develop many routine HPLC procedures. Exacerbated by the increased price of acetonitrile, methods of reducing consumption are more urgent. Many methods have focussed on reducing the solvent used per separation by using smaller particle size media packed smaller ID columns to run at lower flow rates. This is acceptable for small analytical separations but is not feasible for purification.

We have been investigating a range of alternative solvents that could be used for the reversed-phase HPLC analysis of peptides, which keeps the efficiency of the separation and selectivity of the media to maintain resolution. The data shown will demonstrate that there are a number of alternative solvents suitable for this application, and highlights some of the advantages/disadvantages of each.

P28. A new approach for fast separation of intact proteins with high overloading by using non-porous packings in RPLC

Xindu Geng
xdgeng@nwu.edu.cn; wang_phy@163.com
Other Authors: Fei Wang

Institute of Modern Separation Science
Northwest University
Xi'an, Shaanxi Province 710069 PR China

The purpose of the investigation is to present a new approach for fast separation of intact proteins in mg scale using 1μm non-porous RPLC packings in chromatographic cake (cake). Based on the fact that the resolution of protein separation is basically independent of column length and the diameter of the cake is larger than its length, the pressure of cake should be lower than that of the conventional column having the same packed bed volume. A kind of very small particle packings packed into the cake can be employed to separate proteins with high efficiency, operated under a lower column pressure. When the chromatographic cake (7.5 mm x 10 mm I. D.) was packed with 1μm non-porous RPLC-ODS packings and acetonitrile-water was employed as a mobile phase, 1μg and 40μg of seven standard proteins could be completely separated in one minute, while 0.50mg of the same seven proteins were also basically, completely separated in one minute. All experiments were carried out by conventional liquid chromatograph and the cake pressure was less than 210 bars under the flow rate of the mobile phase, 5.0mL/min. Based on this fact, a smaller cake packed with less particle size than 1 μm may be employed to separate proteins much sooner for analytical purpose, while a larger cake may be used for purification of a larger amount of proteins for preparative target. The explored approach would be expected to use in the "top-down" strategy in proteomics and for fast purification in usual biochemistry laboratory.

P29. A Newly Developed Hydrophilic Polymer-based Ion Exchange Resin for the Analysis and Purification of Various Biological Molecules

Ernest Sobkow
ejsobkow@ymcamerica.com
Other Authors: Noriko Shoji, Akiko Matsui, Masakatsu Omote, and Naohiro Kuriyama

YMC America, Inc.
941 Marcon Blvd., Suite 301
Allentown, PA 18109 USA

The recent development of the biopharmaceutical industry has been remarkable. Shortening the development time and reducing costs have become increasingly important. The development of efficient, economical and selective purification methods is required for successful commercialization of biopharmaceutical products. To meet these demands, we have developed new polymeric resins named YMC-BioPro series, which are specially designed for ion exchange (IEX) separation and purification of proteins, peptides and nucleic acids. YMC-BioPro series includes columns packed with 5 micron porous and non-porous polymer for analysis and laboratory scale purification, and bulk materials of 30 and 75 micron porous polymer for capture and purification. All materials are based on the same hydrophilic polymer beads with low nonspecific adsorption. Compared to conventional materials available in the market, the BioPro series shows higher binding capacity and higher recovery of biomolecules.

Analytical BioPro columns are 5 micron spherical, mono-dispersed beads, with optimal packing technology. These columns provide high theoretical plate number and symmetrical peak shape. Excellent resolution is achieved from the high column efficiency coupled with the excellent selectivity of QA (quarternary ammonium) and SP (sulfopropyl) ion exchangers. The 75 micron porous polymer resins have increased binding capacity and low pressure drop and are ideal for capture and intermediate purification steps. BioPro Q75 and S75 has similar retention selectivity to 5 micron porous type BioPro QA and SP, respectively, and allow for predictable scale-up from analytical to preparative separation

In this poster, we will show the benefits of YMC-BioPro series and some example cases of superior separation of important biomolecules, such as monoclonal antibodies and DNA.

P30. Affinity capture of cell culture-derived influenza virus particles

Lars Opitz
opitz@mpi-magdeburg.mpg.de
Other Authors: Michael W. Wolff, Sylvia Lehmann, Anke Zimmermann, Jonas Hohlweg, Udo Reichl

Bioprocess Engineering
Max Planck Institute for Dynamic of Complex Technical Systems
Sandtorstrasse 1
Magdeburg, Saxony Anhalt 39106 Germany

Human influenza vaccines are traditionally produced in embryonated chicken eggs. However, due to several disadvantages of this method, mammalian cell culture based influenza vaccine production processes are currently being established demanding new virus purification methods.

Our study provides comprehensive results from three different strategies to capture Madin-Darby canine kidney (MDCK) cell culture-derived influenza virus particles (A/Wisconsin/67/2005, A/Puerto Rico/8/34, B/Malaysia/2506/2004) based on affinity and pseudo-affinity adsorption.

First, an affinity based capture step, lectin-affinity chromatography (LAC), was developed. This method was investigated concerning the selection of lectins and matrices, viral recoveries and contaminant depletion as well as process robustness. LAC showed a high degree of contaminant reduction, in particular host cell dsDNA depletion. The results from LAC represent a high potential of an affinity capture step at the beginning of a downstream process for production of viral vaccines.

Influenza viruses have an affinity to sulfated carbohydrates, such as heparin or sulfated cellulose. Hence, column based Cellufine® sulfate is often used in industrial influenza virus purification. The main disadvantage of this method is the limited flow rate, due to high back pressure, leading to suboptimal process productivity. To overcome this drawback, we have developed a second capturing method for influenza viruses based on sulfated reinforced cellulose membranes (SCM).
Compared to commercially available cation exchange membrane adsorbers and column based Cellufine® sulfate resin, these membrane adsorbers achieved high product recoveries and contaminant reduction. In addition, the SCM allow an increased flow rate during capturing leading to superior productivity compared to conventional bead chromatography. Hence, this method is an economic alternative for industrial influenza vaccine production.

The third purification strategy was capturing of influenza viruses by immobilized metal affinity chromatography (IMAC) using zinc modified membrane adsorbers. This method shows valuable purification results for the influenza virus strain A/Puerto Rico/8/34. However, IMAC depends highly on the primary structure of viral envelope proteins as well as the accessibility of certain amino acids. Hence, IMAC is an interesting capturing method of A/Puerto Rico/8/34 in a laboratory scale, but is only of limited interest for industrial vaccine production processes.

P31. Particulate Formulation of Influenza Virus Antigen in the Form of ISCOMs

Tina Kröber
kroeber@mpi-magdeburg.mpg.de
Other Authors: Michael W. Wolff, Katrin Hebel (Department of Pediatrics, Otto-von-Guericke University, Magdeburg, Germany), Monika C. Brunner-Weinzierl (Department of Pediatrics, Otto-von-Guericke University, Magdeburg, Germany), Udo Reichl

Bioprocess Engineering
Max Planck Institute for Dynamics of Complex Technical Systems
Sandtorstraße 1
Magdeburg, Saxony-Anhalt 39106 Germany

Immunostimulating complexes (ISCOMs) are typically 40 nm spherical cage-like particles used as delivery system for vaccine antigens. They are composed of antigen, cholesterol, phospholipid and the built-in adjuvant saponin (Quil A). ISCOMs have been shown to induce strong antigen-specific cellular or humoral immune responses to various antigens in a variety of animal species.

Here, the production of ISCOMs containing the hemagglutinin antigen of influenza virus and the development of an in vitro testing system for these complexes is reported. Therefore, human influenza A virus was propagated in Madin Darby canine kidney (MDCK) cells, the supernatant clarified and the virus particles chemically inactivated (β-propiolactone) and concentrated (tangential-flow ultrafiltration). For crude purification of the concentrates, size exclusion chromatography was carried out to obtain starting material for ISCOM preparation. ISCOMs were produced by mixing Quil A with detergent (Mega-9)-solubilized influenza antigen, cholesterol and phospholipid. On removal of the detergent by dialysis the characteristic cage-like complexes were formed spontaneously. The ISCOMs were separated from free excess reaction components by a sucrose density gradient ultracentrifugation and evaluated in terms of particle structure and size via transmission electron microscopy (TEM) and dynamic light scattering (DLS). Finally an in-house developed assay was used to characterize the in vitro stimulatory properties of ISCOMs on blood-derived human dendritic cells (DC) in comparison to inactivated influenza virus concentrates.

P32. Alternative Approach for Characterization of Impurities and Site-specific Modifications in Protein Drugs

Martin Gilar
martin_gilar@waters.com
Other Authors: Hongwei Xie, John C. Gebler

Biopharmaceutical Sciences
Waters
34 Maple St.
Milford, MA 01757 USA

Sequence variants and posttranslational modifications (PTMs) such as glycosylation, deamidation, and oxidation are common in recombinant protein pharmaceuticals. They potentially affect the safety, activity and stability of protein drugs. Effective monitoring of these variants and PTMs requires sensitive and reproducible methods. Traditionally, LC-MS and multiple tandem mass spectrometry (either DDA-MS/MS or targeted MS/MS) measurements are required for elucidation of such impurities and covalent modifications. In this study, we have evaluated an alternative approach for mapping protein digests, employing LC coupled with Data Independent Acquisition (DIA) Mass Spectrometry with alternated high-energy and low energy scanning (LC-MSE). The peptide precursor and fragmentation information acquired in MSE mode was utilized for effective identification of peptide sequences, and site-specific modifications within a single LC run. The stoichiometry of PTM modifications was inferred from the relative ratio of the MS signal intensities between modified and unmodified peptides.

Nearly complete sequence coverage was obtained for yeast enolase (96%), ADH (98%) and IgG (>97% for both heavy and light chain) proteins. Minor protein contaminants were found to be detected in enolase at levels between 1.4% and 13.4% (normalized to enolase 1). M-oxidation and N-deamination sites were characterized and quantified. Synthetic peptide standards were utilized to confirm the identified deamidations in “PENNY” motif peptide of IgG heavy chain, and to distinguish aspartic and isoaspartic isoforms of N-deamidations.

P33. Effects of particle porosity and surface chemistries on the separation of larger molecules

Rob Freeman
rfreeman@sge.com
Other Authors: Dan DiFeo, Hans Jurgen Wirth, and Andrew Gooley

SGE Analytical Science
2007 Kramer Lane
Austin, Texas 78758 USA

Liquid chromatography of biological samples is characterised by the presence of medium and large size molecules, typically peptides and proteins. Diffusion plays a crucial role in transporting these large molecules to binding sites inside the stationary phase pore structure. In adsorption chromatography, the limiting effect of the pore diameter is further enhanced by adsorbed analyte molecules partially blocking the pore structure.

Biological samples also tend to be very complex - many interesting aspects revolve around post-translational modification such as phosphorylation and glycosylation. Typically, the glycosylated or phosphorylated peptides resulting from enzymatic digests of targeted proteins are relatively hydrophilic and may not be retained under normal reversed phase chromatography conditions. Furthermore, in the case of phosphorylation, these peptides can form strong nonspecific interactions with metal ions on the contact surfaces.

To address the problems associated with biological analysis of peptides and proteins, we developed a range of columns with inert, metal-free column hardware and stationary phases which combine high bonding density with the appropriate pore size for the various analytes. 200Å and 300Å pore size C18 silicas with virtually no residual silanol activity are used for peptides. An application showing the analysis of glycosylated peptides is presented. The range is completed with a 1000Å C8 column specifically designed for intact protein analysis. This column is demonstrated with the separation of liver membrane proteins.

P34. HPLC separation for peptide and its typical by-products with DAISOGEL.

Yuji Kirino
ykirino@daiso.co.jp
Other Authors: Akira Sugisaki, DAISO CO., LTD. and
Tetsuyuki Saika, DAISO FineChem USA Inc.

DAISO CO., LTD.
1-12-18, AWAZA, NISHI-KU
OSAKA, 550-0011 JAPAN

The selection of stationary phase is one of the most important considerations in developing a reversed-phase chromatography process. First, we optimized conditions using standard stationary phase. Second, screening stationary phases under the condition obtained at the first step. Usually, we need only small modification to re-optimize conditions from what we got at the first step.

Especially for peptides, it expects to generate some kind of by-products in a production process. This study shows some comparison with our stationary phases and other commercially available phases in developing GLP-1 purification. Also, we can show some examples for by-products generated typically in peptide synthesis, such as deletion peptide, Asp succinimide in ?-transition and methionine oxidation.

P35. Novel Silica Phases for Protein and Peptide Separation - DAISOGEL BIO grades

Akira Sugisaki
E-Mail: asugisak@daiso.co.jp Poster
Other Authors: Yuji Kirino, DAISO CO., LTD. and
Tetsuyuki Saika, DAISO FineChem USA Inc.

DAISO CO., LTD.
1-12-18, AWAZA, NISHI-KU
OSAKA, 550-0011 JAPAN

How do you find the best silica-based stationary phase for protein and peptide separation? Necessarily high loading capacity, extended basic or acidic durability is very important for the process separation. The most interesting question is to find a balance between the resolution (correlated to the surface area of the silica) and the actual CHROMATOGRAPHICALLY ACCESSIBLE surface area. The latter depends on the ratio of the solute size and the pore size of the silica.

P36. Purification of IgG from complex mixtures using a Fc-binding

Amith Naik
adnaik2@ncsu.edu
Other Authors: Stefano Menegatti, Haiou Yang, Patrick Gurgel and Ruben G. Carbonell

Chemical And Biomolecular Engineering,
North Carolina State University
2098 EB1, 911 Partners Way, Centennial Campus
Raleigh, North Carolina 27695 USA

Antibodies due to their high specificity and potency have emerged as an important class of therapeutic agents. Whole antibody product capture using Protein A is expensive, and low pH elution can result in the formation of aggregates and yield losses. There is significant interest in the development of new specific ligands for human immunoglobulin G (hIgG) that might be less costly and more robust.

Our group has developed a hexamer peptide affinity ligand –HWRGWV- for purification of IgG. The peptide ligand was identified from a combinatorial solid phase peptide library using a three-stage screening procedure involving radiolabelled IgG and Fc fragments. In addition to high IgG specificity, this ligand has the unique ability to bind to the Fc region of IgG which has been confirmed by MS analysis and molecular docking program. In this work we demonstrate the potential of the HWRGWV ligand to purify IgG from different complex sources such as CHO cell culture supernatants, skim milk, whey and plasma. The purity and yield obtained for the IgG purification from various mixtures are as follows: 97% purity and 80% yield was obtained for an FDA-approved and widely used monoclonal antibody from CHO cell culture supernatant, 94% purity and 85% yield for IgG from whey, 92% purity and 75% yield for IgG from skim milk and 77% purity and 82% yield for IgG from Cohn II+III paste of human plasma.

Our group is now completing other aspects of the characterization of the ligand HWRGWV. In particular, the stability of the affinity absorbent towards CIP (cleaning-in-place) conditions is considered in detail. The main problems under consideration are 1) the stability of the ligand itself towards the cleaning agents / sanitizing solutions, such as NaOH, and 2) the stability of the coupling chemistry of the ligand onto the chromatographic resin. HPLC and MALDI-TOF MS analysis have confirmed the chemical stability of the pure peptide in 0.1M-1M NaOH solutions for exposure time up to 1 hour.

P37. Separation of glycopeptides and their glycoforms using HILIC columns and UPLC-MS(E) system.

Martin Gilar
martin_gilar@waters.com
Other Authors: Hongwei Xie, Thomas E. Wheat, Beth Gillece-Castro

Biopharmaceutical Sciences
Waters
34 Maple St.
Milford, MA 01757 USA

Peptide mapping is typically performed in reversed-phase liquid chromatography mode (RP-LC). Resolution of tens to hundreds of peptides in peptide maps remains difficult even when using ultra performance liquid chromatography (UPLC) systems and columns. An especially challenging problem is the separation and relative quantitation of glycosylated peptides (the same peptide sequence, different glycoforms). Because RP columns retain peptides based on hydrophobicity, they are rather insensitive to the presence of hydrophilic glycans. Consequently, the different forms of glycopeptides elute at similar times, and baseline resolution of all glycoforms is rarely achieved. We have evaluated a novel HILIC column packed with 1.7 um particles for peptide mapping of monoclonal antibody (Herceptin). Due to the greater affinity of HILIC sorbents towards hydrophilic compounds, the separation mode appears to be promising for separation glycans and glycopeptide glycoforms. The glycopeptides are more strongly retained on HILIC sorbent compared to non-glycosylated peptides. In addition, glycoforms of peptides are separated with sufficient resolution, so they can be quantified. Data-independent mass spectrometry acquisition MS(E) was utilized to highlight the retention times of glycopeptides using the characteristic glycan ions (204.1 and 366.1 Da). In addition to UV detection, the MS signal of peptide precursors was utilized for relative quantitation of glycoforms, producing comparable results.

P38. Development of Fused-Core HPLC Columns for High Speed Separation of Peptides and small Proteins

Wayne Way
wayne.way@sial.com
Other Authors: William H. Campbell, Paul Ross, Russel Gant

Supelco/Sigma-Aldrich
595 North Harrison Road
Bellefonte, PA 16823 USA

HPLC columns featuring 2.7 µm Fused-Core (superficially porous) particles with 90 Å pores demonstrate very fast separations of small molecules because of high efficiency and a flat van Deemter plot. These particles rival the efficiency of sub-2 µm totally porous UHPLC particles, but show only about one-half the backpressure. Fused-Core 2.7 µm particles with wider (~140 Å) pores are being developed to produce columns that have been optimized for the rapid separation of peptides and small proteins. The higher efficiency and lower pressure drop of Fused-Core particles allows preparation of longer columns with very large numbers of theoretical plates. This dramatically increases the peak capacity of the column system, which facilitates qualitative and quantitative HPLC and LC-MS analysis.

P39. 3 um Discovery BIO GFC Columns for High Resolution Protein Separations

Wayne Way
wayne.way@sial.com
Other Authors: Wayne K. Way, Hillel Brandes, and Hugh Cramer

Supelco/Sigma-Aldrich
595 North Harrison Road
Bellefonte, PA 16823 USA

Gel Filtration Chromatography (GFC) is a common technique for the resolution of biological macromolecules based on size and/or shape. The Discovery BIO GFC columns to date have been available only as a 5 micron particle size product. With the introduction of 3 micron particles, the advantages of higher efficiency with only a modest increase in pressure will be demonstrated. The relatively low flow rates that are generally optimal for gel filtration chromatography provide for the modest increase in backpressure. Comparisons will be made between 5 micron and 3 micron particle sizes.

P40. Methacrylate based polymeric monolithic columns for the fast and efficient quantification of Influenza A virus particles

Matjaž Peterka
matjaž.peterka@monoliths.com
Other Authors: Lidija Urbas[1], Peter Brne[1], Boštjan Gabor[1], Sašo Plevčak[1], Matjaž Peterka[1], Tony Brazzale[3], Miloš Barut[1],Boris Pihlar[2], Aleš Štrancar[1]

[1] *BIA Separations d.o.o., Teslova 30, Ljubljana, Slovenia*
[2]*Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, Ljubljana, Slovenia*
[3] *BIA Separations Inc., 1000 N West St. Suite, Wilmington, DE*

MBL
BIA Separations
Teslova 30
Ljubljana, Ljubljana 1000 Slovenia

Monolith is a stationary phase made of single piece of a porous material. Unlike conventional particle-shaped chromatographic supports, the pores in the monolith are interconnected and form a network of channels with the diameter of 1000 to 2000 nm. The binding sites in these channels are highly accessible for target molecules and since the predominant mass transfer depends on convection rather than diffusion, the dynamic binding capacity is flow independent. These characteristics make the monolithic supports suitable for fast separation and purification of large biomolecules such as proteins, DNA and viruses, which sometimes exceed 200 nm in size and thus have low diffusion constants.

In this work methacrylate based polymeric monolithic columns were optimized and characterized for their suitability of quantifying intact influenza A viruses, with the aim to develop a simple, robust and reliable analytical method. First a screening of different stationary phases (structure and surface chemistry) was performed in order to establish the optimal stationary phase for the binding of the virus. This was followed by the screening of different chromatographic conditions. The influence of mobile phase composition and pH on the recovery of the virus was investigated. The amount of virus in the flow-through and elution fractions was determined with the haemagglutination assay and the purity of the fractions with SDS PAGE.

P41. HPLC determination of supercoiled, linear and open circular forms of plasmid DNA in complex solutions by novel analytical bio-monolithic columns

Matjaž Peterka
matjaž.peterka@monoliths.com
Other Authors: Boštjan Gabor[1], Lidija Urbas[1], Matjaž Peterka[1], Peter Brne[1], Sašo Plevčak[1], Franci Smrekar[1], Tony Brazzale[3], Miloš Barut[1], Lucija Zupančič-Kralj[2], Aleš Štrancar[1]

[1] *BIA Separations d.o.o., Teslova 30, Ljubljana, Slovenia*
[2] *Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, Ljubljana, Slovenia*
[3] *BIA Separations Inc., 1000 N West St. Suite, Wilmington, DE*

MBL
BIA Separations
Teslova 30
Ljubljana, Ljubljana 1000 Slovenia

The recent developments in molecular therapies, such as nonviral gene therapy and DNA vaccination, have fostered the development of efficient plasmid DNA purification processes. The separation of supercoiled and open circular isoforms is one of the key steps in the large scale purification of pDNA vectors intended for therapeutic use. Furthermore, isoform separation, identification are also crucial to monitor manufacturing and to control pDNA quality during processing and in the final formulations [1].

Anion-exchange chromatography is fundamental in downstream processing of plasmids both as a process and analytical technique. Convective Interaction Media anion-exchange monolithic columns have already been successfully used for the industrial scale purification of pharmaceutical grade small 10 kb plasmid DNA [2]. In this work we report about the use of the newly developed monolithic analytical columns intended for plasmid DNA determination in terms of their analytical performance. Higher degree of sensitivity, precision and accuracy is necessary in order to determine the quality of clinical grade DNA intended for therapeutic use. The newly developed analytical columns are suitable for fast, efficient and robust HPLC determination of different pDNA samples from all steps of the manufacturing process – from bacterial fermentation to final product.

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P42. Binding Capacity and Binding Constants for Synthetic, IgG-binding Affinity Ligands by Batch Adsorption

Line Naomi Lund
LNmL@novonordisk.com
Other Authors: Niklas Borg, Marcus Degermann, Matthias Wiendahl,
Per-Erik Gustavsson, Roice Michael, Phaedria Marie St. Hilaire, and Arne Staby.

Affinity Purification Technology / Department of Biochemistry and Molecular Biology
Novo Nordisk A/S / University of Southern Denmark
Novo Nordisk Park
Måløv, - DK-2760 Denmark

Much emphasis is being put into optimizing monoclonal antibody purification as well as development of new ligands capable of competing with the commonly used Protein A ligands. Small affinity ligands based on streptococcal Protein A (e.g. MabSorbent® from Prometic Biosciences) and other mixed-mode alternatives (e.g. MEP HyperCelTM from Pall) are all ready on the marked. Attention has been put into developing small synthetic ligands that can resist harsh regeneration and sanitation conditions as well as being cheap to produce. When validating the ligands, purity obtained and capacity are two key factors to consider. Also important is the strength of the interaction between protein and ligand.

The purpose of our work is to determine the binding capacity and binding energies of two small synthetic ligands developed by Novo Nordisk A/S for monoclonal antibody purification. This is done by dynamic and static chromatography on a Tecan robot.

The ligands, both being ~1000 Da, have been found by screening ligand libraries by Encoded Bead Technology, a method owned by Novo Nordisk A/S. For comparison Protein A, Protein G and MabSelect Sure from GE Healthcare and MEP-HyperCell from PALL have been included in the study.

P43. Rapid Peptide Mapping Using Ultra High Pressure LC (UHPLC) and Superficially Porous Chromatographic Material

Taegen Clary
taegen_clary@agilent.com
Other Authors: Christine Miller, Maureen Joseph

Product Marketing
Agilent Technologies
5301 Stevens Creek Blvd.
Santa Clara, CA 95051 USA

A key component of protein analysis is peptide mapping with maximum sequence coverage, which typically requires long chromatographic runs, leading to throughput issues. Recent technology has enabled ultra high pressure separations and this work demonstrated rapid (sub 3 min) analyses using a new reverse phase technology. The stationary phase withstands up to 1200 bar pressure, thus allowing high flow rates and rapid gradients. Using LC/MS-based analysis, the resulting narrow chromatographic peaks require a high acquisition rate on the Q-TOF mass spectrometer in order to adequately sample the peaks. This work compares the new superficially porous material to a traditional silica based material. In addition, the new separation technology is compared with the best separation achieved on a high pressure (600 bar) LC system and an ultra high pressure (1200 bar) system. Excellent high speed separations with high coverage and accurate mass measurements using a standard protein digest as well as an antibody digest are demonstrated.

P44. Development and Qualification of Anion Exchange Chromatography Method for Charge Isoform Heterogeneity of a Protein

Kathy Yu
yuk@medimmune.com
Other Authors: Susanna Bilbulian, Ziping Wei, and Patricia W. Cash

Analytical Biochemistry
MedImmune, Inc.
One Medimmune Way
Gaithersburg, MD 20878 USA

Identification and characterization of charge isoform heterogeneity is an important aspect of biopharmaceutical product development. A robust and reliable analytical method for monitoring charge isoforms can help to ensure that the desired profile is maintained.

An anion exchange (AEX) chromatographic method has been developed to quantify charge isoform heterogeneity of a protein under non-denatured conditions. In this study, a Chrompack strong anion exchange column has been used to separate the charge isoforms of the product. The separation was achieved by optimizing mobile phase pH, sample amount loaded onto the column, ionic strength, and gradient. Specificity, repeatability, intermediate precision, and system suitability of the method have been evaluated. The qualification results demonstrate this method is specific and precise, and is a useful tool to resolve charge isoforms.