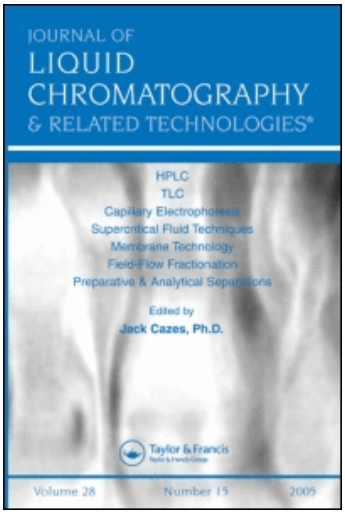

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Subproteomics in Analytical Chemistry: Chromatographic Fractionation Techniques in the Characterization of Proteins and Peptides

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Subproteomics in Analytical Chemistry: Chromatographic Fractionation Techniques in the Characterization of Proteins and Peptides

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ABSTRACT

A review of liquid chromatography (LC) techniques for the subfractionation of complex protein mixtures into "subproteomes" is presented. Fractionation of protein mixtures into general subclasses based on physical, chemical, and biological attributes of proteins is an effective way of classifying and characterizing complex proteomes. Classical chromatographic techniques, such as reverse-phase, ion-exchange, and size exclusion chromatography (SEC) are discussed, as well as a variety of affinity chromatography techniques such as immobilized metal affinity chromatography (IMAC) and lectin affinity chromatography. Applications of these methods to proteomic endeavors are highlighted. Multidimensional chromatography methodologies are examined for combining

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orthogonal chromatographic formats interfaced with mass spectrometric (MS) detection. The versatility of liquid chromatography for proteomic analysis makes LC a valuable tool for the protein characterization of living systems.

Key Words: Multidimensional chromatography; Affinity chromatography; IMAC; Lectin affinity; SELDI; Proteomics.

INTRODUCTION

With the sequencing of the human genome for the most part completed, and many other organism's genomes being defined, many scientists have turned to the task of unraveling the complexity of the products of these genomes, proteins. The DNA sequences in the genomes of organisms provides the blueprint for the manufacturing of a wide variety of protein products, but the genome does not give to us a complete picture of proteins that are made. Not all proteins contained within the genetic material of cells are expressed and those that are expressed may undergo a variety of post-translational modifications that cannot be predicted by gene sequencing. Since it is the proteins within an organism that provide the chemical, structural, and biological processes of a living organism, an extensive effort is being made to better understand the function and interactions of expressed proteins within a living system.

The name "Proteomics" has been given to the study of overall protein content expressed, or in some cases not expressed, by an organism's genome. Although the study of proteins is by no means a new undertaking, Proteomics, coined by Marc Wilkins in the early 1990s,[1] is a term used to describe the entire protein complement of an organism and how it relates to the overall biology of an organism. In Proteomics, we are concerned with getting the whole picture of proteins expressed by a living system, their levels of expression, the post-translational modifications made to proteins that are expressed, their regulation, and interaction, along with how these aspects change within an organism as the result of external and/or internal stresses. Modifications to the proteins generally happen post-translationally and do not correspond to DNA sequence information. Many of these post-translation modifications have been implicated in disease or stress states, such as alterations in oligosaccharide structures on proteins that have been demonstrated in some cancer types.[2] Phosphorylation is another type of post-translational modification that has important biological significance in cellular regulation and signaling pathways.[3,4]

Subproteomics is a term that has been applied to selected subsets of proteins isolated based on subcellular location of a protein.[5,6] Protein

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fractionation by isolation of subcellular organelles, such as the golgi apparatus, mitochondria, and ribosomes, has been a necessary task to help elucidate the complexities of living systems. As the subject organism gets more complex, the task of defining its complete proteome becomes a much more difficult task. It is apparent that subproteomics could be expanded beyond a protein's cellular location to include fractionation based on structural features of proteins. This would allow for further fractionation of samples, even of organelles, into structural classes, such as glycoproteins and phosphoproteins, or functional classes, such as enzymes. Subfractionation of complex protein mixtures into subproteomes based on these attributes could offer greater insight into post-translational modifications of these proteins as well as other structural variants.

The tool most widely used for the characterization of the proteome is two-dimensional gel electrophoresis (2DE).[7] Two-dimensional gels most commonly separate protein mixtures on the basis of isoelectric point or pI in the first dimension, and molecular size in the second dimension. Although this technique is a powerful research tool for investigating protein expression in complex mixtures it still has some disadvantages. Two-dimensional gels are relatively slow and labor intensive and do not lend themselves readily to automation. Quantitation methods for 2D gels based on staining techniques are relatively poor. Variants of some structures are not resolved unless new charges are incorporated in the molecule or significant changes in molecular weight have occurred, as in glycovariants.[8]

Multidimensional liquid chromatography (MDLC) approaches are attractive alternatives to 2D gels.[9–14] By using orthogonal separation techniques in a chromatographic format, a dramatic increase in peak capacity and resolving power can be achieved.[15] Multidimensional liquid chromatography techniques are becoming commonplace and when applied to proteomics research, MDLC has some distinct advantages over 2D gels. The number of dimensions that can be compounded for sample separations exceeds that of gel electrophoresis. There are a variety of separation mechanisms that can effectively be employed to reduce the complexity of the samples taken for analysis by fractionating the sample into subproteomes. Solubility issues that arise in 2D gels are not as serious a problem in liquid chromatographic (LC) methods, as a wide variety of stationary phases and solvents can be readily changed to suit the needs of the sample. Liquid chromatographic methods are easily automated, which is something of a problem with 2D gels. 2D gel electrophoresis is still a labor-intensive technique, although automation of some aspects of the technique has occurred, such as mapping gel differences and spot excision with robotics. Another advantage of MDLC techniques is that once a particular protein of interest or biomarker has been found, it is relatively easy to fine-tune a chromatographic system to rapidly isolate the

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analyte of interest, leaving unwanted materials behind. Finally, chromatographic methodologies are more readily interfaced with mass spectrometry (MS) for protein identification and quantitation. Quantitation by isotopic labels such as those demonstrated by Aebersold's ICAT technique,[16,17] and other isotopic labeling techniques[10,18–22] allow for much better relative quantitative information than that which can be obtained by dye or silver stained 2D gels.

In this article we will be investigating a wide variety of LC approaches for the subfractionation of complex protein samples into subproteomes, allowing for a more manageable handling of mixtures of proteins. We will also present the use of MDLC systems for analyzing protein mixtures in a format comparable to 2DE. Many of these chromatographic methods, such as ion exchange chromatography and hydrophobic interaction chromatography are classical techniques that should not be overlooked for their ability to reduce the complexity of protein samples. Others are recently developed methodologies, such as aptamer chromatography and surface enhanced laser desorption ionization (SELDI) that bring new possibilities into the field. These methods may be applied to fractionate protein samples into subproteomes, specific classes of proteins defined by structural or chemical features of the proteins themselves. Liquid chromatographic methodologies in conjunction with fractionation techniques, such as centrifugation for the isolation of cellular organelles and MDLC approaches will assist scientists in exploring the protein expression of living systems, in exploring Proteomics.

CHROMATOGRAPHIC APPROACHES TO SUBFRACTIONATION OF PROTEOMES: SUBPROTEOMICS

Size Exclusion Chromatography

Size exclusion chromatography (SEC) or gel filtration chromatography (GFC) fractionates samples into subproteomes based on molecular size that can then be used to approximate molecular weight. Size exclusion chromatography may be carried out in different formats where proteins can be in their natural state, with complete multi-subunit assemblies of proteins being chromatographed intact, or done under a combination of denaturing and/or reducing conditions to break apart covalent and noncovalent interactions between subunits and cofactors of complex protein systems. The packings used in SEC chromatography consist of porous support materials with a defined pore size. Molecules are retained by their ability to enter the pores in the packing material. The amount of time they spend within an SEC column

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being proportional to how much of the volume inside the porous packing material is available to a particular analyte. Retention can then be correlated to molecular weight by comparison to a set of analytes of known molecular weight run under identical conditions. Only a certain range of molecular sizes can be fractionated by SEC. Molecules above a certain molecular size are completely excluded from the porous material and cannot be separated. Similarly, molecules below a certain molecular size have access to the entire pore volume and are not separated either.

Size exclusion chromatography has been used by a number of researchers as a front end or down stream fractionation technique to try to simplify complex protein samples for further separation.[12,23] Wang and coworkers used SEC separations to fractionate *E. coli* lysates into segments of >100,000, 100,000–10,000, and <10,000 dalton range before tryptic digestion.[12] Size exclusion chromatography was also used in the same experiment to observe the efficiency of in-line tryptic digestion by monitoring the increase in UV response of low molecular weight SEC fractions. Size exclusion chromatography has been used to fractionate human serum in the analysis of the glycoprotein prostate specific antigen (PSA).[24] Two-dimensional protein mapping has been described using restricted access media (RAM), a chromatographic format that combines SEC and other chromatographic separation techniques, such as ion exchange chromatography and reverse phase chromatography. The outer surface of the RAM material is coated with hydrophilic hydroxyl groups while the internal surface of the pores are coated with a chromatographic functionality, such as an ion exchanger or reverse-phase chemistry such as C_{18}.[25] Direct coupling of SEC to MS for protein characterization has also been demonstrated by Canarelli and coworkers, who separated a test mixture of horse heart myoglobin and soybean trypsin inhibitor by using a novel on-line microdialysis unit.[26] Size exclusion chromatography is an effective way to obtain low molecular weight fractions of proteins and peptides, namely those under 20 kD that do not run well on 2D gels due to the low molecular weight.[11]

Covalent Chromatography

Covalent chromatographic methods fractionate protein samples based on chemical reactions between select solutes of interest and a reactive chromatographic stationary phase within the column. A covalent bond is formed between solute and stationary phase to retain certain materials on the column. Other materials are then washed off, leaving behind the analytes of interest. The column can then be eluted by introducing another species that reacts with the analyte-stationary phase bond, releasing the analyte from the stationary phase. Of the functionalities present in proteins, thiols are best suited for

covalent chromatography due to the ease in forming and breaking disulfide linkages. Thiol-disulfide exchange is commonly used in electrophoresis by the addition of β-mercaptoethanol (BME) or dithiothreitol (DTT) to reduce the disulfide bridges within proteins and to break apart the three-dimensional structures, separating protein chains held together by these disulfide linkages. Figure 1 shows an outline of covalent chromatography using thiols present on the surface of proteins. Proteins containing active thiol groups, generally cysteine, are reacted with a solid chromatographic support containing reactive disulfides such as 2-pyridyldisulfide, thiosulfonates, or thiolsulfinites.[27] As a result of thiol-disulfide exchange, the protein becomes covalently bound to the chromatographic support. Proteins can be released from the support by the addition of an excess of a reactive thiol such as DTT. Chromatographic fractionations of proteins and peptides have been effectively accomplished through this methodology by Wang and Regnier.[13] Cysteine containing peptides were selectively fractionated from tryptic digests on thiopropyl Sepharose followed by reverse phase liquid chromatography (RPLC) with MS detection. This fractionation technique significantly reduces the complexity of peptides derived from the digestion of intact proteins.

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) is a chromatographic technique that may be used to fractionate protein samples based on interaction

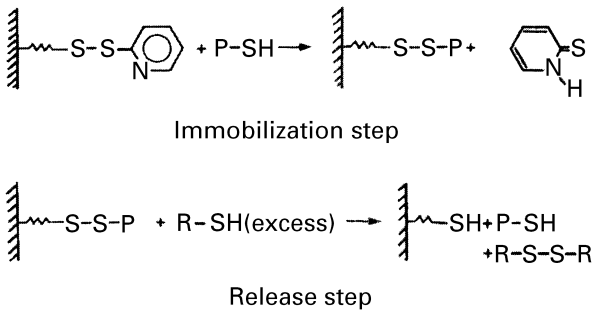

Figure 1. Outline of covalent chromatography using reactive thiol groups on the proteins surface. In the immobilization step, a free cysteine group on the surface of a protein undergoes thiol exchange with column. To elute the proteins bound to the column a reducing agent, such a dithiothreitol, is added in excess, releasing the bound protein. Reproduced from Ref.[27] with permission.

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of hydrophobic areas of a protein with hydrophobic ligands on the chromatography support. Although the chromatographic support is similar in many ways to reverse phase chemistries, the mechanisms of interaction between the protein and the hydrophobic packing material and loading and elution conditions are different. In HIC, the hydrophobic ligands on the support material differ from RPLC ligands in that they are short chain aliphatic or aryl moieties.[28,29] Protein solutions are generally loaded onto the column under a high salt concentration that disrupts the structured ordering of water molecules around the protein, causing them to interact with the hydrophobic portions of the packing material. Proteins are eluted from the column with a low salt mobile phase or with the addition of organic additives, such as ethylene glycol or isopropanol. Although HIC is generally used as a purification technique in large-scale processes, small-scale use of HIC in a membrane chromatography format has been used to separate a monoclonal antibody from bovine serum.[30] The chromatographic properties of HIC have also been demonstrated for a series of model proteins on a variety of different support materials.[31] The separation of trypsin inhibitor by tandem HIC columns of different hydrophobicities has demonstrated the possibility of improving the separating power of HIC.[32] Hydrophobic interaction chromatography can be an effective chromatographic format for the fractionation of proteins in a complex sample based on the hydrophobic nature of the analytes present.

Reversed Phase Liquid Chromatography

Reversed phase liquid chromatography is one of the most well known separation modes available to analytical chemists.[33] In the field of proteomics, RPLC is generally used as the final chromatographic step before a sample is introduced into the MS. This may be done in an online fashion by electrospray ionization[9,34–36] or by the collection of fractions to be spotted onto plates for matrix assisted laser desorption ionization (MALDI).[10,13,34,37,38] In most cases, separations done on reversed phase columns are protein samples that have already undergone proteolytic digestion to give peptides, which are more easily separated by RPLC. This is not always the case, however. An example being that of Cochet et al., who used RPLC as the primary fractionation step for the human erythrocyte membrane proteins Glycophorin A and Glycophorin B.[39] Liu and coworkers have investigated RPLC fractionation of yeast ribosomal extracts.[40] Reversed phase liquid chromatography is generally chosen as the final separation method before MS analysis due to the ability to remove salts, chaotropic agents, and other chemical entities that are not compatible with the MS. The ability to focus proteins and peptides at the head of an RPLC column in MDLC separations, due to the mostly aqueous nature of

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other separation modes, makes RPLC columns ideal as an analyte trapping column. This aids in the fractionation of complex mixtures by having analytes focused into a tight band before gradient elution of analytes. This is especially true when column effluent is sent directly to the MS via electrospray ionization. Column effluent can be diverted to waste by valve switching to remove salts and other unwanted species that are soluble in aqueous media. Once the interfering substances have been washed off the column, the MS can be brought on line and the gradient elution of analytes begun.[9] In most cases, mobile phase additives are required to improve reversed phase separations. Commonly used additives to the mobile phase include trifluoroacetic acid (TFA), formic acid, ammonium acetate, and ammonium hydrogen carbonate. These additives are usually combined with the mobile phase in concentrations ranging from 0.025% to 0.25%. They may improve separations of proteins and peptides but may also have a deleterious effects on MS detection, especially in ESI-MS. Trifluoroacetic acid, when used as a mobile phase additive, improves separations of proteins and peptides, giving larger peak capacities but at the same time TFA suppresses MS ionization, resulting in lower signals and higher detection limits. Garcia and colleagues have investigated the ion-suppression effects of a series of common additives to mobile phases and have found that there is a balance to maintain between ionization efficiencies and separations.[41] While formic acid gave the best results of the additives tested for ionization efficiencies, TFA yielded the best separations. Similar concerns have been noted by Liu and coworkers, who chose the increased efficiency of a TFA containing mobile phase over one with formic acid that lead to wider peaks and loss of peak resolution.[40]

Ion-Exchange Chromatography and Chromatofocusing

Ion-exchange chromatography is probably the most widely used method for protein fractionation.[42] Nearly all proteins contain amino acids or carbohydrates with groups that can be positively or negatively charged. It is these charged groups that give rise to a protein's electrical properties that allow for separations in gel electrophoretic methodologies, such as isoelectric focusing (IEF). Here proteins are separated based on isoelectric point (pI), the pH at which a polypeptide chain has an overall net charge of zero, and discontinues movement within an electric field. In ion-exchange chromatography, proteins that are positively or negatively charged or that have areas of charge density on their surface, bind to the packing material within the column or membrane by electrostatic interactions. Column packing materials generally consist of charged moieties, such as positively charged quaternary and tertiary amines or negatively charged carboxylic acids and sulfonates

covalently attached to the packing materials. The sample is placed on the column in a buffered solution and elution of analytes off the column is typically done with a non-buffering salt such as NaCl in a step or linear gradient fashion. Elution of columns by pH changes and other methods such as affinity elution are also done.[27]

Chromatofocusing is a technique that uses conventional ion-exchange columns to selectively fractionate proteins based on pI. Chromatofocusing was first described by Sluyterman and Elgersma[43] and is analogous to the first dimension separation of 2DE, IEF, but in a LC format. In chromatofocusing a pH gradient is formed within the ion-exchange column by using a single eluent at a pH different than the equilibration buffer used to load the sample. Proteins are eluted off the ion-exchange column and focused at their pI. Chromatofocusing has been used by Keenan and Holmes to study apo- and holo-vitamin D-binding protein from pig plasma.[44]

Ion-exchange chromatography has been widely used as a frontal fractionation technique for MDLC methods. Mobile phases used for ion-exchange are aqueous buffers with varying salt concentrations, and these can be easily removed by running eluents onto a trapping column or directly onto the RPLC column used to introduce samples to the MS. The trapping or RPLC column can be washed to remove buffers, salts, and other additives before being brought in-line with the MS detector or fraction collection device. Automated setups using this approach have been demonstrated by a number of research groups.[9,14,40,45,46] Other numerous examples exist of single and MDLC techniques for protein characterization where ion-exchange has been used as a separation dimension.[11,26,30,36,37,47–52] Ion-exchange chromatographic techniques offer a convenient way to fractionate protein samples for further characterization in an on-line automated fashion.

Affinity Chromatography Techniques

Affinity chromatography techniques offer chromatography users a wide variety of possible interactions to exploit for subfractionation of complex protein samples. Affinity interactions of proteins and peptides can be relatively general in nature, as in immobilized metal affinity chromatography (IMAC) where certain groups such as cysteine, histidine, and phosphate may be retained by the chromatographic media, or extremely specific as in antibody–antigen recognition or enzyme–substrate interactions. The wide variety of interactions that can be used gives chromatographers a powerful tool to selectively fractionate complex protein mixtures based on molecular function, structural motifs, and/or post-translational modifications.

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Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography was introduced in 1975[53] as a chromatographic method used to separate proteins on the basis of interactions with a metal bound to a chelating agent, covalently attached to a suitable chromatographic support. Chelating groups such as iminodiacetate (IDA), nitrilotriacetic acid (NTA), and *tris*(carboxymethyl) ethylene diamine (TED) are commonly used to immobilize the metal ion on the chromatographic support.[27,54] These chelating groups only partially occupy the coordination positions of the metal ions, leaving free spaces in the coordination sphere to interact with certain exposed groups on the surface of proteins. Figure 2 shows a schematic of the IMAC process. Chelating groups are first immobilized on a support material. The column is next charged with the metal to be used, binding to the chelating groups that only partially fill the metal coordination sphere, the other positions being filled by solvent or buffer molecules. Proteins are then loaded onto the column and bind to the metal by displacing the solvent molecules coordinated to the metal ion. Proteins interacting with

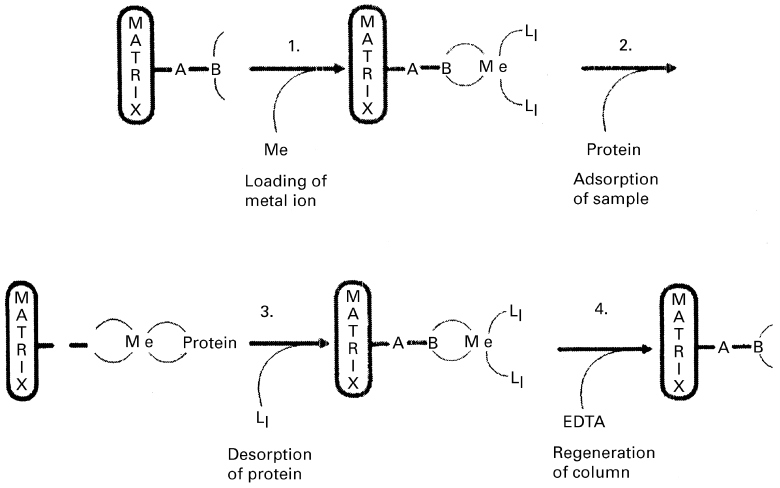

Figure 2. Outline of IMAC. A is a linker group that covalently bonds the chelating group, B to the support material. Metal ion, Me, is loaded onto the column and is partially bound to the chelating group with some coordination positions around the metal occupied by buffer or solvent molecules. Protein is loaded onto the column and binds to the metal ion by displacing solvent or buffer molecules. The protein is next eluted by addition of a competitive ligand in excess, such as imidazole. The column can be regenerated for use with the same or different metal ions by treatment with EDTA. Reproduced from Ref.[27] with permission.

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the immobilized metal include those with histidine, cysteine, tryptophan, and phosphate groups displayed on the surface of the protein. Proteins can then be eluted off the column with the addition of competing ligands, such as histidine or imidazole. The column can then be regenerated by washing with EDTA, then applying a metal ion solution to the column. Immobilized metal affinity chromatography can achieve an effective fractionation of complex protein samples based on the surface functionalities of the proteins.

A wide variety of different metals have been immobilized on these chelating columns, and they have shown a considerable range of selectivities for the fractionation of protein and peptide samples. Andersson[55] has explored the use of Cu(II), Zn(II), and Cd(II) immobilized on IDA and TED Sepharose for the fractionation of human serum proteins, along with a seven column tandam Zn(II)-IDA set-up to fractionate 4 mL of human serum. It was shown that the Zn(II)-IDA was particularly useful for the isolation of hemoplexin and α_2-macroglobulin. Human α-acid glycoprotein variants were characterized by Herve and coworkers using a Cu(II) charged IDA Sepharose column.[56–59] Comparisons of the binding affinity of phosphopeptides with different immobilized metals were conducted by Posewitz and Tempst.[60] In a "microtip" format using electrophoresis gel loading tips packed with an IMAC support charged with different metals, they found Fe(III), Ga(III), and Zr(IV) retained the highest percentage of phosphopeptides. Mercury ions have also been investigated as a metal for IMAC separations by Gelunaite and coworkers.[61] Hg(II) ions immobilized on an IDA support were shown to have specific selectivity for free thiols on the surface of proteins, however elution conditions to prevent column leaching of Hg(II) ions remains a serious consideration, especially considering the metal's toxicity.

Immobilized metal affinity chromatography has also been used as a fractionation technique in a multidimensional chromatographic approach for the selection of proteins and peptides containing phosphate,[4,18,35,62] histidine,[62–64] cysteine,[63] tryptophan,[63] and even whole cells.[51] Recombinant proteins have been purified by tagging a hexahistidine tail on to recombinant proteins to effect purification following expression.[34,65] The ease of use and ability to easily change the immobilized metal ion makes IMAC an effective technique for subproteomics work. Fractionations can be accomplished by using different immobilized metal ions to impart different selectivities for the analytes of interest.

Lectin Affinity Chromatography

Lectins are low molecular weight proteins that bind carbohydrate residues with high affinity and selectivity. Lectins have the ability to recognize carbohydrate residues within oligosaccharides, specific combinations of oligosaccharide

structures, or carbohydrate residues within a specific position in antennary complexes displayed on glycoproteins. Lectins can be used to fractionate complex protein mixtures into a wide range of subproteomes for analytical analysis. These subproteomes include various glycoslyated proteins such as antibodies, blood plasma proteins, and other glycoproteins.[37,48,66]

Pillate and coworkers have used the lectin *Jacalin*, a lectin with a binding specificity for the Gal-$\beta 1 \rightarrow 3$-GalNac disaccharide, to investigate interactions of *Jacalin* with serum proteins.[66] New forms of C4b-binding protein and hemopexin were identified, as well as several unidentified proteolytic enzymes. Hortin and Trimpe also used *Jacalin* immobilized on agarose to survey proteins in human plasma that affinity bound to the column.[67] Elliott et al. has used Concanavalin A (Con-A) affinity purification to isolate α_1-acid glycoprotein from human plasma to investigate differences in oligosaccharide structure in patients with rheumatoid arthritis.[68] Sumi and colleagues have used a combination of lectin affinity columns in the study of prostate cancer.[24] In this work a series of lectin columns consisting of Con-A, phytohemagglutinin E (PHA-E), phytohemagglutinin L (PHA-L), pea lectin, and wheat germ lectin were used to characterize the nature of the oligosaccharide heterogeneity in prostate specific antigen, a marker for prostate cancer. Lectins have also been used for the mapping of *N*-glycoslyation of human serotransferrin by Fu and Halbeek.[69] A series of lectin columns consisting of Con-A, *Sambucus nigra* agglutinin (SNA) and *Phaseolus vulgaris* leukoagglutinin (LPHA) were used for the characterization of glycosidic linkages within the serotransferrin protein. Lectin affinity has also been used as a fractionation step in a multidimensional chromatography approach by Geng, Regnier, and coworkers for the selection of glycoproteins and peptides using Con-A and *Bandeiraea simplicifolia* lectin (BS-II) in a signature peptide approach to proteomics.[10,62,70]

Lectin's affinity for glycoproteins and their variants offers an excellent way in which this important class of proteins may be selectively isolated from heterogeneous protein mixtures. The ability for lectins to impart different selectivites based on the types of saccharides present, as well as the different antennary structures that can be formed by the glycoconjugates attached to proteins, make lectins a valuable tool for the further characterization of glycoslyated proteins. Figure 3 shows different antennary structures found on α-acid glycoproteins. It is this heterogeneity within the oligosaccharides that effect separation of lectin based columns.

Protein A and Protein G

Proteins A and G are bacterial proteins isolated from *Staphylococcus aureus* and group G *Streptococcus*, respectively. Both of these proteins bind to the Fc regions of immunoglobulins from many mammalian species. Proteins A

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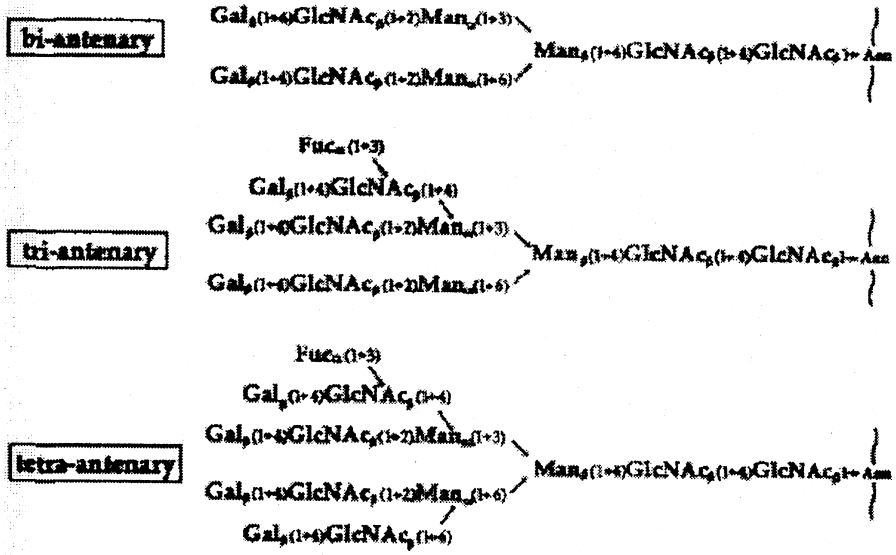

Figure 3. Structure of the major oligosaccharide moieties incorporated into α-acid glycoprotein. Heterogeneity within glycoproteins oligosaccharide structures can dramatically alter protein function and reactivity. Selectivity in the separation of glycoproteins by lectins is determined in large by the nature of these glyco structures. Reproduced from Ref.[87] with permission.

and G do show cross reactivity with different species of mammals and may not show any binding to immunoglobulins of certain species. Proteins A and G are commonly immobilized on suitable chromatographic media, such as Sepharose, and used in affinity purifications of immunoglobulins and immunoglobulin subclasses.[42,71] They may also be used to fractionate serum by removing immunoglobulins to analyze proteins of lower abundance within the serum samples. Riggin and Regnier have done work to determine antibodies to human growth hormone with a detection limit in the femtomole range.[72] Protein G has also been shown to bind serum albumin. In the area of proteomics these proteins may be used to selectively isolate immunoglobulins for further characterization, particularly in screening for antibodies to certain but unknown antigens for which no antigen template has been developed.

Aptamers

Aptamers are oligonucleotides ranging from approximately 15–60 nucleotides long that show specific binding to various molecules, including proteins

and small molecules.[73] Aptamers can be synthesized from RNA or DNA molecules in a high throughput combinatorial chemistry fashion. These molecules form three-dimensional structures that have binding efficiencies and selectivities that are very similar to antibodies[74] but overcome many of the drawbacks associated with antibodies, such as the ability to withstand harsh elution and sanitation conditions. Figure 4 depicts the three-dimensional arrangement of nucleotides in a structure called a G-quartet that was used by Rehder and McGown to effect a capillary electrochromatography separation of bovine B-lactoglobulin. Aptamers hold the promise of being able to synthetically fashion specific chromatographic supports that are both specific and selective for a particular target molecule. Aptamers also offer a method to fractionate diverse classes of proteins based on interactions with certain structural motifs displayed on a protein's surface.[75]

Aptamer affinity chromatography has been demonstrated by Drolet and coworkers for the purification of human *L*-selectin-Ig fusion protein expressed in Chinese hamster ovaries from the media broth.[76] The aptamer employed was selected from a combinatorial library synthesis routine for its specific binding to the fusion protein. Anti-peptide aptamers were also designed by combinatorial methods to bind a peptide fragment from synthesized human immunodeficiency virus type 1 Rev (HIV-1 Rev). Xu and Ellington[75] have shown aptamer binding of a specific epitope region of the HIV-1 Rev and systematically modified that sequence to examine the effects on binding. It was shown that the

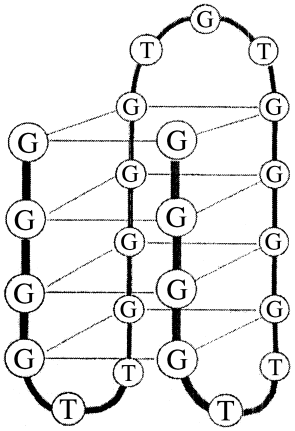

Figure 4. G-quartet structure of a DNA aptamer. This three-dimensional structure is assumed by a thrombin-binding aptamer and in naturally occuring telomeric DNA. The three-dimensional structure of aptamers give them unique selectivity for proteins and peptides. Reproduced from Ref.[79] with permission.

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aptamers recognized specific three-dimensional conformations of the peptide, as opposed to binding through electrostatic attraction to positively charged sequence runs. Hamaguchi et al. have demonstrated that aptamers can be used as molecular beacons for protein detection,[73] as well as the binding of RNA aptamers that bind and inhibit the ribosome inactivating protein Pepocin.[77] Kotia and coworkers have used aptamers as a chromatographic stationary phase for the separation of amino acids[78] and also to separate bovine lactoglobulin isoforms in open tubular capillary electrochromatography.[79] Aptamers have also been synthesized to specifically bind the nonstructural protein 3 (NS3) from hepatitis C virus. The use of combinatorial chemistry for aptamer synthesis may open up the possibility of specifically designed chromatographic stationary phases that can be used to recognize specific structural classes of proteins and fractionate them accordingly.

Antibodies

Immunoglobulins, by their very nature, are molecular species that recognize a particular three-dimensional structure or epitope of a molecular species with high specificity. Antibodies exhibit high binding constants towards their particular targets. Monoclonal antibodies are a collection of antibodies that recognize a single epitope region of a molecule, whereas polyclonal antibodies recognize different epitopic regions of the same molecule. The ability of antibodies to recognize some ubiquitous structural features, such as phosphorylated serine, tyrosine, and threonine residues, on the surface of proteins, makes them useful for the fractionation of protein mixtures into phosphoprotein components.

Immunoglobulins have found extensive use in biochemistry for the detection of specific molecular species in Western blotting techniques, tissue staining, and ELISA assays, where the antibody is labeled with a fluorescent tag that can be read by a photometric detection or conjugated with an enzyme that will react with a particular molecular species to yield a photoactive species.[80] Antibodies can also be used as a chromatographic support for the fractionation of complex samples. The high specificity of antibodies to their target molecules makes them particularly attractive for the isolation and characterization of low abundant species in complex mixtures.[81] Antibodies may be immobilized onto a chromatographic support through a number of different chemistries, ranging from crosslinking to Protein A immobilized columns to oxidation of the sugar moiety in the Fc region that can then be covalently coupled to the chromatographic support.[82,83] Polyclonal and monoclonal antibodies that are reactive towards hemoglobin variants have also been described;[47] although not used in a chromatographic format, they

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could easily be adapted for such use, especially with the interest in the human plasma proteome. Antibodies have been raised against a wide variety of molecular species and are commercially available from a number of vendors against a wide range of target molecules.

The use of antibodies in a chromatographic format is not without certain drawbacks, however. Antibodies are rather expensive, time-consuming to produce, and may bind to the target so tightly that harsh elution steps may be necessary to elute the material.[76] Although harsh elution conditions do not present any real challenges to the analytical chemist, as biological functionality of a protein is not required, it does have the effect of destroying the antibody within the column and would require a newly manufactured column for each assay. Still, however, antibodies are a very viable technique for investigating low abundance proteins in complex samples by pulling out specific proteins of interest for further characterization.

Bio and Synthetic Ligands

The interaction of proteins to a wide variety of synthetic and naturally occurring ligands has been exploited in chromatographic separations for quite some time. Synthetic dyes, cofactors for enzymes, boronate esters, and other naturally occurring substances, such as carbohydrates have all been used for the chromatographic separation of proteins.[84–86] Synthetic and naturally occurring dyes in particular have been become extremely useful in fractionating protein mixtures. Chromatographic separations using triazine dyes, such as Cibacron Blue F3GA, have been used as a chromatographic step to purify α-acid glycoprotein variants.[57,87] The ability to synthesize dye affinity ligands to a number of protein classes by a combinatorial chemistry approach has made synthetic dyes attractive ligands for chromatographic immobilization. Figure 5 shows the structures of two anthraquinone triazine dyes, Cibacron Blue 3GA and a synthetic analogue of the dye that has been modified with a *p*-aminobenzyl phosphonic acid group. The synthetic analogue shows selectivity for alkaline phosphatase. Synthetic dyes that recognize lactate dehydrogenase, alkaline phosphatase, and trypsin have all been described, and with the aid of bioinformatics and molecular modeling techniques, new synthetic ligands can be expected in the future.[84] Synthetic ligands for the separation and purification of immunoglobulins have been developed by Verdoliva and coworkers.[88,89] A synthetic dye affinity adsorbent for the binding of human serum albumin has been synthesized by Nakamura and coworkers.[90] Dye based artificial lectins have been synthesized by Palanisamy et al. using a triazine scaffold bis-substituted with 5-aminoindan that showed retention of α-*D*-mannose, mannobiose, and mannan, but not other

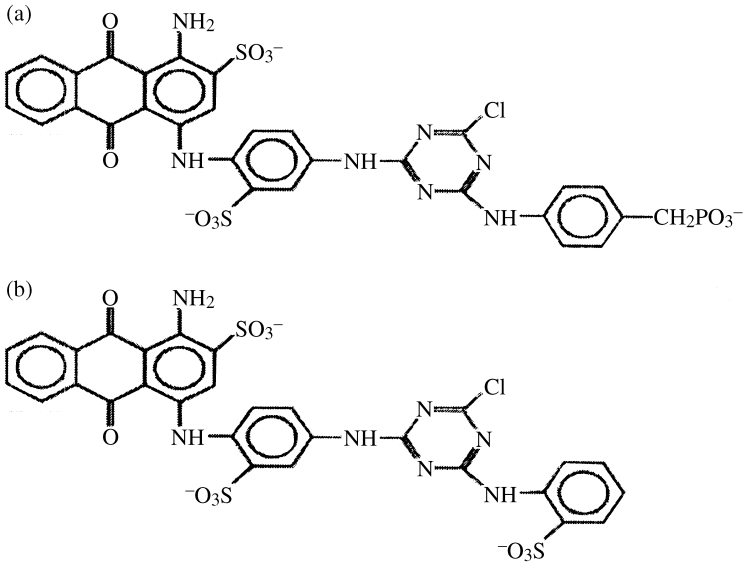

Figure 5. Structures of two anthraquinone triazine dyes, (a) a *p*-aminobenzyl phosphonic acid analogue of Cibacron blue 3GA used for the isolation of alkaline phosphatase from calf intestinal mucosa and (b) Cibacron blue 3GA. Reproduced from Ref.[84] with permission.

carbohydrates.[91] Boronate affinity using an immobilized *m*-aminophenyl boronic acid ligand, has been used for its affinity for *cis*-diol configurations for the separation of hemoglobin glycovariants. Figure 6 shows the interaction between *m*-aminophenyl boronic acid and *cis*-1,2 diols of a sugar residue in the oligosaccharide portion of glycoproteins. Boronate affinity chromatography has also been used for the separation of other glycoproteins, such as mistletoe lectin[92] and some nonglycosylated proteins such as β-lactamases, pepsin, and β-amylase.[86] Complex carbohydrate structures, such as heparin, immobilized on chromatographic supports, have been used for the purification of proteins.[93] The combination of synthetic and natural dyes, enzyme substrates, and other affinity ligands give chromatographers a wide variety of selectivities to pursue fractionation and description of complex protein samples.

Surface Enhanced Laser Desorption Ionization

Surface enhanced laser desorption ionization is a relatively new technology now commercialized by LumiCyte and Ciphergen Biosystems. This

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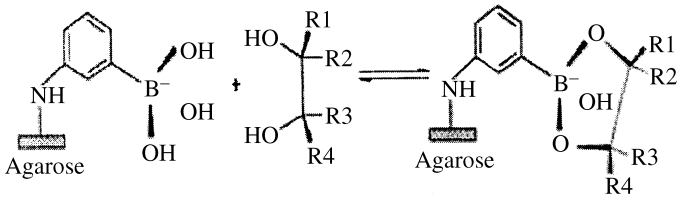

Figure 6. Affinity interaction between *m*-aminophenyl boronic acid and *cis*-1,2 diols of a polyhydroxyl compound under basic conditions. Glycoprotein containing sugar moieties with a 1,2 diol arrangement can be selectively retained by boronate columns. Reproduced from Ref.[25] with permission.

technology is similar to MALDI, and in many ways similar to DNA chip-based assays currently on the market. Figure 7 depicts the overall SELDI process.[101] In SELDI, a planar substrate is used as a support for chromatographic ligands that are immobilized on the surface of the chip [Fig. 7(A)]. These ligands can be conventional chromatographic matrices, such as ion-exchange and hydrophobic ligands or affinity matrices such as IMAC, antibodies, and other affinity supports. A complex sample is placed on the chip surface and allowed to incubate for a certain amount of time [Fig. 7(B)].

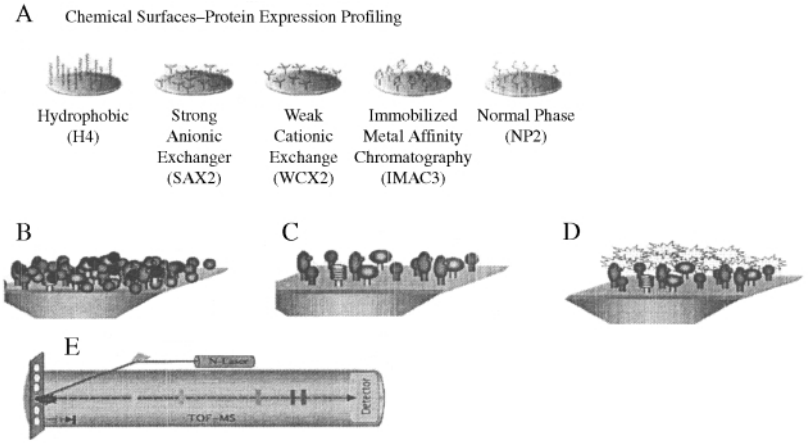

Figure 7. Overview of the SELDI process. (A) SELDI chip surface chemistry can be chosen for desired capture technique. (B) Sample is placed on the chip surface. (C) Non-retained materials are washed off the chip. (D) Matrix is added to the chip. (E) Samples are "eluted" off the chip surface by a laser pulse and detected by TOF-MS. Reproduced from Ref.[101] with permission.

Analytes selectively bind to the surface through affinity interactions and nonbonding species are removed by washing with buffer [Fig. 7(C)]. After washing, a MALDI matrix solution is added [Fig. 7(D)], and the chip is placed inside the sampling chamber of a time-of-flight MS (TOF-MS). Compounds are "eluted" off the chip by a pulse of laser light that ionizes the analytes into the gas phase and allows for TOF-MS detection [Fig. 7(E)]. In SELDI, chromatographic separation of analytes of interest within a sample occurs directly on the chip surface without the need for other chromatographic equipment. Samples can be fractionated according to specific protein classes by selecting the proper surface chemistry immobilized on the chip. Various protein classes such as glycoproteins, phosphoproteins, and enzymes may be affinity selected by lectin, IMAC, or enzymatic substrates bound to the surface of the SELDI chip.

Surface enhanced laser desorption ionization was first developed by Hutchens and Yip who described their surface enhanced neat desorption (SEND) and surface enhanced affinity capture (SEAC) processes.[94] Hutchens and Yip had used the technology on human glycoproteins, lactoferrin, affinity captured on agarose beads with single stranded DNA used for the affinity capture. The bead slurry was then washed and mixed with sinapinic acid on a glass probe, then irradiated with a laser. Since then, a number of articles have appeared on the use of what has now become SELDI, a chip based assay for the determination of proteins in a sample. Brockman and coworkers described an immobilized affinity chromatography set-up where monoclonal IgG anti-biotin antibodies were covalently attached to the MALDI probe. This allowed for the capture of biotinylated proteins for analysis.[95] Surface enhanced laser desorption ionization continues to be a useful tool for proteomics research, being used to screen for transcription factors on DNA immobilized chips, and to look at protein profiles to compare normal tissue samples to that of renal cell carcinoma.[96,97] The utility of SELDI should make this technology an important tool in the future of proteome research, with the ability to fractionate complex protein samples and to provide direct comparative profiles of disease and normal states. By simply changing the immobilized substrate on the surface of the chip, a number of diverse protein classes may be fractionated and analyzed.

Multidimensional Liquid Chromatography

Multidimensional liquid chromatography combines linear separations that are orthogonal in selectivity, to separate and fractionate complex mixtures of proteins and peptides. Multidimensional liquid chromatography approaches to proteomics can combine any of the previously discussed techniques in an

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analytical "train" to fractionate samples in a completely automated fashion. Some of the advantages of MDLC systems over 2D gels have already been discussed. The wide range of separations available, ease of automation, and compatibility with MS detection has all been previously mentioned. Another noticeable advantage of MDLC systems and LC systems in general, is the ability to work with larger sample volumes. Increasing the volume and physical amount of sample used for an analysis is difficult in 2DE. The volumes of sample used and the concentration of sample loaded are serious concerns in 2D gels. Overloading in 2DE causes protein precipitation and smearing of spots and bands within the gel. Although the same concerns over sample loading are also true for LC systems, it is a problem that is relatively easy to overcome by the scale-up of initial separations.

A number of different MDLC configurations have been presented in the literature for the fractionation and characterization of protein mixtures. Many of these methods have been combined with stable isotopic labeling to allow for relative quantitation between control and experimental states using MS detection. In most cases, some combination of ion-exchange, SEC, and/or affinity separation is used in the first dimension to fractionate the samples, followed by introduction of the sample onto a RPLC column for the final separation before on-line MS detection by ESI-MS or off-line detection by MALDI-MS.

Nadler et al. have used SEC combined with Protein A affinity for the analysis of IgG and its multimers.[98] Davis and coworkers[9] used an MDLC configuration that included strong cation exchange (SCX) and RPLC along with MS/MS detection for the characterization of human lung fibroblasts and human brain glioma cell culture media. Lectin affinity chromatography along with RPLC was used by Geng and colleagues to fractionate tryptic digests of nuclear glycoproteins of bovine pancreas cells for MS detection.[10]

Another MDLC set-up in which a biphasic column consisting of 10 cm of RPLC C_{18} packing in front of 4 cm of a SCX packing within a 100 μm fused-silica capillary was developed by Wolters et al. for shotgun proteomics techniques.[14] A quaternary mobile phase system consisting of acetonitrile, acetic acid, and water along with the volatile salt ammonium acetate was used. Peptides from a digested protein mixture were loaded onto the SCX portion of the column eluted onto the RPLC packing, that was then washed, and gradient eluted to the MS detector. Lui and coworkers[40] have described an MDLC configuration consisting of a single SCX column combined with two RPLC columns for the analysis of ribosomal extracts from yeast (Fig. 8). In this system, valve switching allows for one RPLC column to be developed while a second column receives effluent from a step gradient elution of the SCX column. The RPLC columns are switched back and forth to receive analytes in the next salt step. This configuration allows for a higher throughput of samples

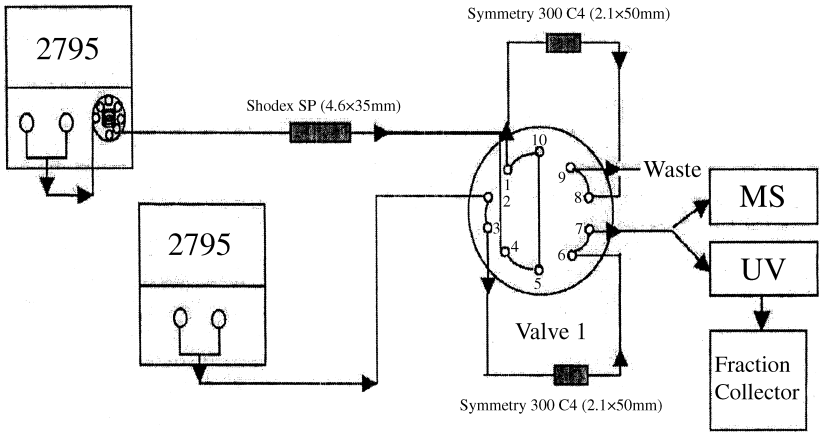

Figure 8. Multidimensional liquid chromatography system configuration used for the analysis of yeast ribosomal proteins. System consists of a SCX column connected to two RPLC columns through a switching valve. A step gradient is used to elute proteins off the SCX column and onto one of the RPLC columns. Once loaded, the valve is switched to allow the second column to receive the SCX effluent while the other column is gradient eluted to MS and UV detection along with fraction collection. Reproduced from Ref.[40] with permission.

by reducing the amount of down time the MS detector sees due to loading and washing of the RPLC column.

Multidimensional liquid chromatography configurations have also been developed that include on-line proteolytic digestion of protein mixtures (Fig. 9), that can be further separated by other chromatographic methods in an integrated and fully automated system before MS analysis.[12,99,100] Improved MDLC techniques will continue to advance the field of proteomics by completely automating sample fractionation, digestion, and separation of complex protein mixtures. Fully autonomous systems, with a wide variety of separation dimensions to fractionate protein mixtures according to different structural and functional classifications, will allow for higher throughput sample analysis.

CONCLUSIONS

In this article we have described a wide variety of chromatographic techniques that can be applied to the fractionation of protein samples into manageable sub-classes of proteins or subproteomes. All of the techniques

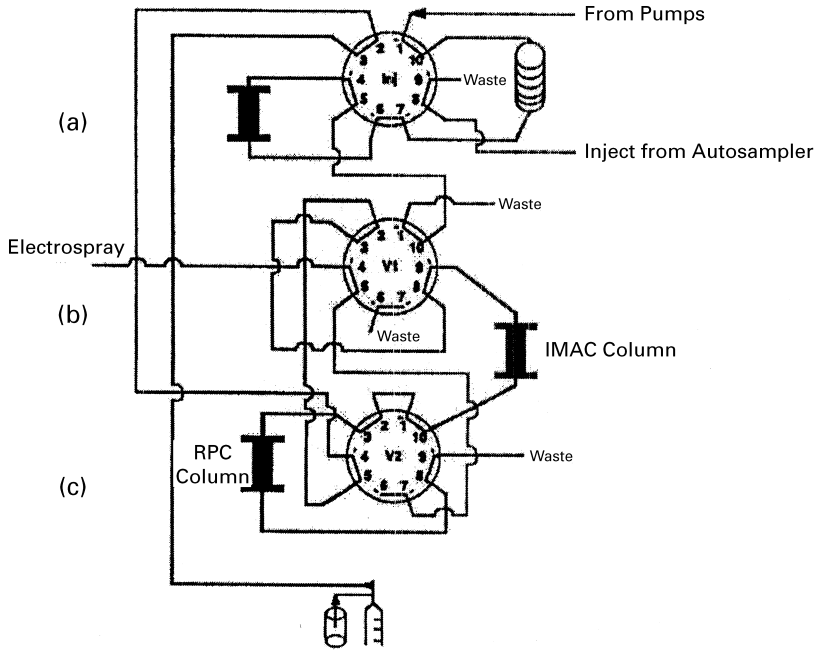

Figure 9. Overall plumbing configuration of a three column MDLC system for the characterization of phosphorylated milk proteins. This MDLC system incorporates, (a) trypsin digestion in an immobilized perfusion column, (b) affinity selection of phospho-peptides by a Ga(III) charged IMAC column, and (c) separation of affinity selected peptides by an RPLC column with final detection by ESI-MS. Reproduced from Ref.[18] with permission.

described above have utility in breaking down the complexity of samples to allow for better separation, characterization, and quantitation of biological samples. As scientists continue to reveal the inner workings of biological systems, these separation methodologies will allow for more involved separation systems that can be fully automated. Stand-alone automation will allow for more in-depth studies of the intricacies of biological systems. Multidimensional liquid chromatography systems that combine preparative and analytical scale separations can be used to probe low abundance proteins. New chromatographic stationary phases developed through combinatorial chemistry techniques, such as aptamers and biomimetic ligands, will offer new selectivities for fractionation of complex protein mixtures. Advances in specialized techniques, like SELDI, will allow for rapid profiling of samples for clinical testing, and thus allowing for a better

understanding of the biology behind many diseases and the specific roles that proteins play. The field of proteomics has opened up the seemingly endless task of characterizing the biology that we live with today. Chromatographic techniques for the fractionation of complex protein mixtures into subproteomes will allow for better characterization of the protein complement of advanced biological systems.

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