

Review

Applications of affinity chromatography in proteomics

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Abstract

Affinity chromatography is a powerful protein separation method that is based on the specific interaction between immobilized ligands and target proteins. Peptides can also be separated effectively by affinity chromatography through the use of peptide-specific ligands. Both two-dimensional electrophoresis (2-DE)- and non-2-DE-based proteomic approaches benefit from the application of affinity chromatography. Before protein separation by 2-DE, affinity separation is used primarily for preconcentration and pre-treatment of samples. Those applications entail the removal of one protein or a class of proteins that might interfere with 2-DE resolution, the concentration of low-abundance proteins to enable them to be visualized in the gel, and the classification of total protein into two or more groups for further separation by gel electrophoresis. Non-2-DE-based approaches have extensively employed affinity chromatography to reduce the complexity of protein and peptide mixtures. Prior to mass spectrometry (MS), preconcentration and capture of specific proteins or peptides to enhance sensitivity can be accomplished by using affinity adsorption. Affinity purification of protein complexes followed by identification of proteins by MS serves as a powerful tool for generating a map of protein–protein interactions and cellular locations of complexes. Affinity chromatography of peptide mixtures, coupled with mass spectrometry, provides a tool for the study of protein posttranslational modification (PTM) sites and quantitative proteomics. Quantitation of proteomes is possible via the use of isotope-coded affinity tags and isolation of proteolytic peptides by affinity chromatography. An emerging area of proteomics technology development is miniaturization. Affinity chromatography is becoming more widely used for exploring PTM and protein–protein interactions, especially with a view toward developing new general tag systems and strategies of chemical derivatization on peptides for affinity selection. More applications of affinity-based purification can be expected, including increasing the resolution in 2-DE, improving the sensitivity of MS quantification, and incorporating purification as part of multidimensional liquid chromatography experiments.

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Proteomics is the study of the total proteins from a particular cell line, tissue, or organism. This field includes three main approaches: expression proteomics, cell-map proteomics, and structural proteomics. Expression proteomics (also called “differential expression proteomics”) is the study of global changes in protein expression. Cell-map proteomics is the systematic study of protein–protein interactions [1,2]. Structural proteomics involves the determination of three-dimensional protein structures on a genomewide scale. Structures are determined via experimental techniques (crystallography and NMR) and computational studies. We do not address structural proteomics in this review.

The most commonly used experimental techniques in proteomics are two-dimensional protein electrophoresis (2-DE)¹ for separating proteins and mass spectrometry (MS) for the identification of separated proteins. However, affinity chromatography is also a powerful protein separation method. This method is based on the specific interaction between an immobilized ligand and the target protein to be separated. The process is termed “immunoaffinity chromatography” when an immune protein is used as the ligand. Although it is not

¹ Abbreviations used: 2-DE, two-dimensional electrophoresis; IgG, Immunoglobulin G; CSF, cerebrospinal fluid; IMAC, immobilized metal affinity chromatography; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; TAP, tandem affinity purification; TOF, time-of-flight; Con A, concanavalin A; ICAT, isotope-coded affinity tag.

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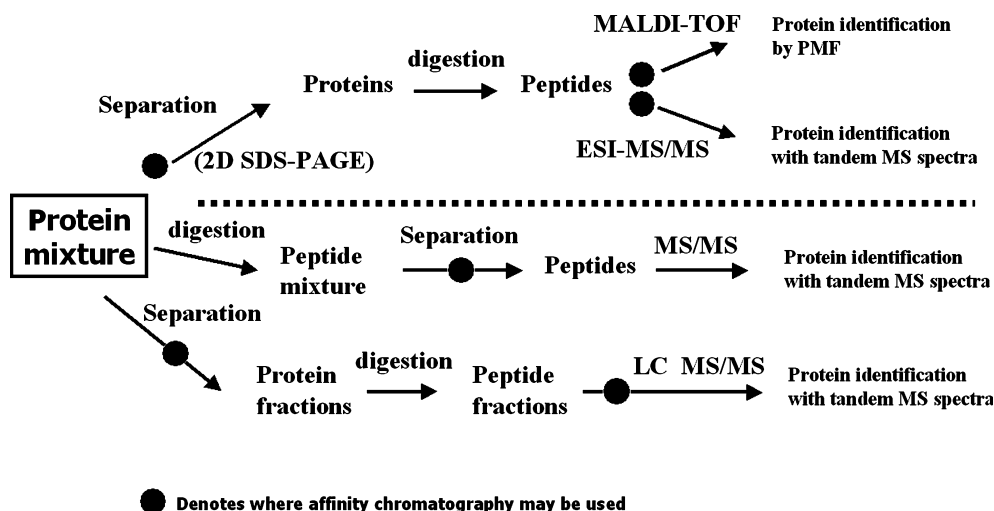


Fig. 1. Potential applications of affinity chromatography in proteomic studies. A dashed line in the figure divides the 2-DE-based and non-2-DE-based approaches. Solid circles indicate the places where affinity chromatography may be employed.

the leading technology, affinity chromatography plays an important role in proteomics. Affinity chromatography can be used as part of a traditional 2-DE–MS workflow. Furthermore, affinity chromatography can be used in the identification of protein–protein interactions [3].

Prior to analysis by MS, a mixture of proteins or peptides must generally be reduced in complexity, and proteins must generally be fragmented into peptides. As shown in Fig. 1, the approaches used in proteomics can be classified as either 2-DE- or non-2-DE-based depending on the order of these pre-MS steps [4]. The 2-DE-based approach, for example, attempts to reduce the complexity of a protein mixture using two-dimensional gel separation, followed by the tryptic digestion of separated proteins into peptides. Affinity chromatography can be employed to reduce the complexity of protein or peptide mixtures in both 2-DE- and non-2-DE-based approaches. It has generally been applied in three stages: (1) protein separation prior to 2-DE, (2) protein affinity chromatography prior to MS, and (3) peptide affinity chromatography prior to MS. Each of these stages will be discussed below.

Protein separation prior to 2-DE

Affinity chromatography can be used to selectively concentrate samples before 2-DE. Applications include removal of a protein or class of proteins that could interfere with 2-DE resolution, concentration of low-abundance proteins so that they can be visualized in the gel, or separation of proteins into two or more groups for further analysis. As shown in Table 1, most applications of affinity purification attempt to purify a small group of proteins from complex mixtures [5–25].

Affinity depletion

Sample preparation before 2-DE may include cell fractionation and the removal of high-abundance proteins to help detect proteins present at low concentration. Affinity depletion provides an effective means of removing a specific protein or group of proteins. A classic example is the depletion of albumin and immunoglobulin G (IgG) from serum or cerebrospinal fluid samples by adsorption on affinity resins [26–28]. Lollo et al. [29] recently used ProtoClear, a patented affinity matrix, rather than affinity chromatography based on the small molecule Cibracon Blue, to reduce albumin and IgG in samples of human serum. Removal of albumin and IgG from the serum led to significant improvement in the resolution of protein spots on two-dimensional gels. Comparing 2-D gels of serum cleared either by Cibracon Blue or by ProtoClear, Lollo et al. [29] also found that Cibracon Blue removed a number of proteins in addition to albumin which is generally undesirable.

According to Patterson [30], a collaborative project between Oxford GlycoSciences and Pfizer led to separation by 2-DE and the identification of proteins in cerebrospinal fluid (CSF) in a study aimed at identifying markers for Alzheimer's disease. The selective removal of albumin, IgG, transferrin, and haptoglobin was accomplished by affinity depletion and enabled the effective 2-DE analysis of plasma and CSF samples. The study involved 512 samples from 228 patients. It resulted in the annotation of 1131 protein spots and the identification of potential markers of Alzheimer's disease [30]. Murakami et al. [31] analyzed the minor proteins of human milk by using immunoabsorbents to remove three major proteins (alpha-lactalbumin, lactoferrin, and secretory immunoglobulin A). In another

Table 1
Examples of affinity chromatography placed before 2-DE

Affinity ligand on support	Target protein	Biological sample	Reference
Heparin	Cell surface proteins	<i>Helicobacter pylori</i>	Utt et al., 2002 [5]
Soybean lectin	Glycoproteins	<i>Campylobacter jejuni</i>	Young et al., 2002 [6]
Heparin/streptavidin	Heparin-binding proteins/biotinylated proteins	<i>Mycoplasma pneumoniae</i>	Ueberle et al., 2002 [7]
RNA ligands	Complex (RNA-binding) proteins	HeLa nuclear extract	Tian, 2002 [8]
Microcystin	Phosphatases	Nuclear extracts of Jurkat cells	Guo et al., 2002 [9]
Avidin	S-glutathionylated proteins	ECV304 endothelial-like cells	Lind et al., 2002 [10]
N-acetyl-D-glucosamine	N-acetyl-D-glucosamine (GlcNAc)-specific lectins	Rat liver	Lefebvre et al., 2001 [11]
Glutathione	Glutathione binding proteins	<i>Caenorhabditis elegans</i>	Van Rossum et al., 2001 [12]
Champedak (<i>Artocarpus integer</i>) lectin-M	Glycoproteins with N-linked oligosaccharides	Human serum	Hashim et al., 2001 [13]
Glutathione-S-transferase (GST)-S5a fusion protein	Polyubiquitinated proteins	Mammalian tissues	Layfield et al., 2001 [14]
Le(a) trisaccharide (alpha-L-Fuc[1,4]-beta-D-Gal[1,3]-D-GlcNAc)	Fucose-binding protein	Bull sperm and seminal plasma	Ignatz et al., 2001 [15]
Ni ²⁺ nitrilotriacetic acid	SsrA-tagged proteins	Ribosome of <i>Escherichia coli</i>	Roche and Sauer, 2001 [16]
Tetanus toxoid (TT)/protein G	Anti-TT antibodies/total immunoglobulins (Ig)	Human serum	Layer et al., 2000 [17]
Galactose (on Sepharose)	Galactose-specific lectin	African yam bean	Machuka and Okeola, 2000 [18]
3-(4-Mercaptobutylthio)-1,1,1-trifluoro-2-propanone	Cutinases	<i>Monilinia fruticola</i> (Wint.) honey	Wang et al., 2000 [19]
Heparin	Basic proteins	<i>Haemophilus influenzae</i>	Fountoulakis et al., 1998 [20]
Monomeric actin	Actin-binding proteins	Mouse C2 myoblasts and myotubes	Coumans and dos Remedios, 1998 [21]
MLK2N-GST fusion protein	MLK2-SH3 domain binding proteins	Lysates of human breast tumor cells	Rasmussen et al., 1997 [22]; 1998 [23]
Monomeric actin on divinylsulfone-activated agarose (Mini-Leak)	G-actin-binding proteins	Human skeletal muscle	Coumans et al., 1997 [24]
<i>Biomphalaria alexandrina</i> -derived lectin	Fucosylactose determinant-bearing glycoprotein	Extracts of adult male worms	Mansour, 1996 [25]

study, an immunoaffinity column that contained β -casein- and bovine IgG-specific IgG-immobilized Sepharose was used to remove the major proteins, including β -casein and IgG, from bovine milk, and low-abundance milk proteins were identified [32]. The main disadvantages to affinity depletion are that other proteins with lower affinity may also be removed and that the sample may be diluted when a buffer or water is used to wash away unabsorbed proteins.

Similar to affinity depletion, the technique of “affinity extraction” involves the use of affinity chromatography to isolate a specific protein or group of proteins from a sample before analysis by a second method [33]. The second analytical method can be one-dimensional or two-dimensional gel electrophoresis. The main application of affinity extraction is to remove specifically interfering molecules from samples. Examples include the use of immobilized protein A and anti-mouse immunoglobulin supports for removing human anti-mouse antibodies before a sample is analyzed by immunoassay [34] and the use of immobilized anti-human immunoglobulin or protein A supports to

adsorb selectively enzyme-immune complexes from patient samples [35].

Concentration of low-abundance proteins

Even the best two-dimensional gels can routinely resolve no more than several thousand proteins; therefore, only the most abundant proteins can be visualized by gel electrophoresis if a crude protein mixture is used. In this context, affinity-based protein purification prior to 2-DE may help to reduce sample complexity and permit the analysis of lower-abundance proteins. Consider the erythropoietin receptor which is moderately abundant and occurs at about 1000 copies per cell or less than 2 pmol (100 ng) in 1 L of cell culture. This protein cannot be visualized from whole-cell extracts but can be enriched by antibody-based affinity purification to yield a silver-stained band [2]. One of the commonly used methods to enrich for phosphorylated proteins or phosphopeptides is immobilized metal affinity chromatography (IMAC) using gallium- or iron-chelated affinity columns that can selectively bind to the negatively

charged phosphate groups [36–38]. It should be noted that affinity chromatography can enrich only a single low-abundance protein, or group of proteins, at a time. Therefore, one may require the use of multiple affinity columns and purifications to achieve a desired result.

Increasing the amounts of low-abundance proteins relative to the amounts of other proteins facilitates the analysis by 2-DE and MS. Lopez et al. [39] followed this approach in the study of low-abundance mitochondrial proteins. Separate profiles of calcium binding proteins, glycoproteins, and hydrophobic/membrane proteins were obtained by enriching these proteins using calcium-chelated, Con A-, and phenyl-immobilized minispin affinity columns, respectively. The resulting fractions were further separated and characterized by 2-DE and MS.

Segregation of total protein by using group-specific ligands

Proteins can be separated into two or more classes by affinity chromatography using group-specific ligands such as heparin [40], triazine dye [41], and metal ions (Cu^{2+} , Ni^{2+} , and Zn^{2+}) [42]. For example, mouse liver proteins were classified into metal-binding and non-binding proteins by immobilized metal ion affinity chromatography combined with 2-DE [42]. The 2-DE resolution was increased by the use of chromatographic prefractionation of protein samples. Jungblut et al. [42] attributed this increase in resolution to (i) the increase of the separation area of 2-DE (two gels instead of one) and (ii) the increase of the concentration of the less abundant protein species in the sample. However, the protein fractions separated by affinity chromatography do not necessarily represent functional protein classes. Proteins having different functions can bind to the same affinity support as a result of nonbiospecific interactions, such as ionic and hydrophobic interactions.

Protein affinity chromatography prior to MS

Protein separation in a non-electrophoresis-based study

Affinity chromatography can be employed after 2-DE for the purification of separated proteins. However, in many cases, affinity chromatography is applied before MS to samples that have not been subjected to gel electrophoresis. For non-2-DE-based studies, affinity chromatography can be used on both proteins and peptides for separation before or after the proteolytic digestion. For example, lectin affinity chromatography has been employed to isolate a specific class of glycoproteins and enrich the glycopeptides following digestion [43]. This approach is particularly useful for studying posttranslationally-modified proteins, e.g., glycoproteome and phosphoproteome.

Affinity chromatography has been used to reduce the complexity of peptide mixtures by isolating proteins of interest before proteolytic digestion. Prior to digestion of proteins and to analysis by LC-MS/MS, the total protein content from a sample may be separated by affinity methods. Furthermore, phosphorylated proteins can be enriched by immunoprecipitation with anti-phosphotyrosine, anti-phosphoserine, or anti-phosphothreonine antibodies [44]. The enrichment of phosphoserine/threonine-containing proteins from crude cell extracts has also been accomplished via a chemical replacement of the phosphate moieties by affinity tags [45].

Preconcentration of protein samples for the study of low-abundance proteins prior to capillary electrophoresis can be accomplished by using affinity adsorption (extraction) [46]. The application of immunoaffinity chromatography for solid-phase extraction before analysis by MALDI- or by ESI-MS can be performed on the protein sample without proteolytic digestion. When samples are from nongel sources, the affinity purification can be achieved either on-line or off-line. In off-line mode, the affinity chromatography functions to extract the target protein. One example of this approach involves the Na^+ /galactose cotransporter (vSGLT) of *Vibrio parahaemolyticus*. This protein was tagged with C-terminal hexahistidine and has been purified to apparent homogeneity by Ni^{2+} affinity chromatography and gel filtration, as confirmed by ESI-MS [47]. An on-line coupling of affinity chromatography to ESI-MS has also been employed. An immunoaffinity column packed with immobilized anti-human transferrin was used on-line for the purification and identification of transferrin isoforms from diluted serum [48]. Much attention has been directed recently to the development and application of on-line sample preconcentration and microreactions in capillary electrophoresis using affinity sorbents based on chemical or biological specificity. The basic principle involves two interacting chemical or biological systems with high selectivity and affinity for each other [49]. For example, the presence of immunoreactive gonadotropin-releasing hormone in serum and urine can be determined by on-line immunoaffinity capillary electrophoresis coupled to mass spectrometry [50].

Studies of protein–protein interactions

An understanding of how cells work as systems depends on determining the complex network of cellular protein–protein interactions. Blackstock and Weir [1] refer to the study of protein–protein interactions as “cell-map” proteomics. Yeast two-hybrid and phase display are the two most commonly employed techniques. An alternate approach to studying protein–protein interactions, by purifying entire multiprotein complexes by affinity-based methods, has been

proposed. By applying an affinity tag, or tandem affinity purification (TAP) tag, Rigaut et al. [51] developed a generic approach for isolating interacting proteins and purifying protein complexes. After proteins that interact nonspecifically are washed away, the complex is eluted and analyzed by mass spectrometry. A cross-linking reaction and/or a gel electrophoresis step may be needed before mass spectrometry can be performed, as in the example of analyzing the yeast nuclear pore complex Nup85p [52]. Protein complexes were isolated using an affinity tag, and the purified protein complexes were then partially cross-linked, separated by SDS-PAGE, and identified by peptide mass mapping following tryptic digestion. Identification of cross-linked proteins allowed the spatial organization of proteins within the complex to be established.

A protein affinity chromatography approach may be superior to the yeast two-hybrid approach because it generates fewer false positives and is more amenable to high-throughput operations [53]. Affinity chromatography enables proteins that form complexes within a cell to be purified. The interesting proteins can be purified from, for example, a cell lysate along with associated proteins. Recombinant DNA technology is used to tag a 'bait' protein with a peptide fragment such as GST, epitope, or TAP tag. A large-scale approach can tag all bait proteins with these generic peptides. The TAP method purifies a protein complex that contains the TAP-tagged protein in two consecutive affinity steps on IgG- and calmodulin-coupled beads. According to Shevchenko et al. [54], TAP gives a higher yield of affinity purified proteins than other tags, with lower background of nonspecifically associated proteins.

Affinity purification of the protein complexes followed by mass spectrometric protein identification serves as a powerful tool for generating a map of protein-protein interactions and cellular locations of the complexes, according to a review by Blackstock and Weir [1]. All of the affinity purification approaches to identifying protein complexes are based on the use of protein tagging, either at the genetic level or at the level of proteins extracted from cells. In a study on the application of mass spectrometry for studying protein-protein interactions, Figeys et al. [3] demonstrated a FLAG-epitope-tagged affinity-based approach to purify protein complexes and reviewed the principles of mass spectrometry in relation to the identification of low femtomolar amounts of protein. For the recovery of lower-abundance epitope-tagged proteins and associated proteins, the immunoaffinity approach may be either a batch adsorption followed by elution or a chromatographic step wherein the cell extract is passed over a packed column of immobilized antibody. This antibody may be bound to protein A-immobilized beads, enabling affinity adsorption of the epitope-tagged protein complex from the cell extract [55].

Peptide affinity chromatography prior to MS

Two types of MS are commonly employed in proteomics: matrix-assisted laser desorption ionization with time-of-flight detection (MALDI-TOF) and electrospray-tandem mass spectrometry (ESI-MS/MS). Both methods identify a protein by measuring the masses of peptides generated from the protein. For example, Shevchenko et al. [56] performed MALDI mass spectrometric analysis of the peptide mixtures produced by in-gel tryptic digestion of a protein as a first screen. Up to 90% of proteins were identified by searching sequence databases against lists of peptide masses (protein-database search). The remaining proteins can be identified by the partial sequencing of several peptides from the un-separated mixture by nanoelectrospray tandem mass spectrometry followed by a search of a database of multiple peptide sequence tags (EST database search). Before analysis by mass spectrometry, the digest may be concentrated or purified with an affinity step. For example, the protein band excised from a gel may be digested by trypsin and purified on a metal resin column (an IMAC column) before analysis by tandem mass spectrometry [2].

The purification of peptides of interest by affinity chromatography is slightly different from that by protein affinity chromatography because of the difference in molecular sizes between protein and peptide. The ligand for a protein of interest is normally very specific and has a high affinity due to the unique three-dimensional structure of protein. In most cases, each protein affinity chromatography step involves the use of a specific ligand. For instance, Journet et al. [57] examined the separation of mannose-6-phosphate-containing proteins using an affinity support bearing mannose-6-phosphate receptor. A great variety of antibodies and other non-immunological ligands on solid supports are commercially available for protein affinity chromatography. In contrast, the availability of ligands for peptide separation is much more limited. As shown in Table 2, group-specific ligands including chelated metal ions and lectins such as concanavalin A (Con A) are useful for the affinity separation of peptides. Furthermore, one can use metal-immobilized columns for the capture of phosphopeptides through the interaction of their negatively charged phosphogroup and immobilized ions, including Fe(III) and gallium(III) [37,58]. The effects of nonspecific adsorption may sometimes be severe when these group-specific affinity ligands are used for peptide separation.

Preconcentration or capture of specific peptides

Peptides from the digestion of proteins contained within 2-DE spots are often assayed by either MALDI-TOF or LC-MS/MS. One way that affinity chromatography can be used in this process is the preconcentration

of phosphorylated peptides from the tryptic digestion of phosphoprotein spots by using immobilized Fe(III) [58]. For samples not from 2-DE, affinity purification of peptides using a proper immobilized ligand is also frequently performed before directing the purified peptides to LC-MS/MS analysis. For example, immobilized anhydrotrypsin can be used to purify the peptides of potential carboxypeptidase E substrates from CpEfaf/CpEfaf mice [59]. Alternatively, immobilized avidin may be used to purify serine and threonine phosphopeptides from tryptic digests of biotinylated proteins [60] or biotinylated cysteinyl peptides may be isolated from the enzymatic digestion of proteins [61].

Affinity (or immunoaffinity) chromatography can be used to extract peptides from the proteolytic digestion before they are analyzed by MALDI or ESI-MS. Similar to protein affinity separation, the affinity purification of peptides can be performed either off-line or on-line. In off-line studies, the affinity chromatography involves a solid-phase extraction of the target peptide. Spahr et al. [61] employed an affinity selection method to capture cysteinyl peptides and thereby simplify the mixture. Both the captured cysteinyl and the noncysteinyl peptides are analyzed by LC-MS/MS to increase the number of proteins identified [61]. Affinity chromatography has also been connected on-line to ESI-MS [62].

Study of the site of posttranslational modifications

Glycosylation and phosphorylation are common posttranslational modifications. Affinity chromatography of glycopeptides is possible because target glycans can be recognized by lectins even after proteolysis. For the enrichment of phosphopeptides, antibodies and some IMACs are available for affinity separation. However, affinity chromatography procedures such as IMAC and the use of phospho-specific antibodies have typically suffered from high levels of background binding on nonphosphorylated peptides. To overcome this problem, proteins are often digested with trypsin, and the resulting peptides are then converted to methyl esters, enriched for phosphopeptides by IMAC, and analyzed by LC-MS [63]. As reviewed by Adam et al. [64], two chemical methods, the base-catalyzed phosphate elimination method [45,65] and the phosphoramidate modification method [66], are available for measuring the phosphorylation state of proteins. The former involves the affinity purification of tagged peptides, while the latter involves the solid-phase capture of thiol-modified phosphopeptides by reaction with iodoacetyl groups immobilized on glass beads.

Wells et al. [67] recently proposed a mass spectrometry-based method for identifying sites modified by O-GlcNAc and for studying glycoproteins. The method relies on mild beta-elimination followed by Michael addition with dithiothreitol. The modified peptides can

be enriched efficiently by affinity chromatography, and the sites can be mapped using tandem mass spectrometry. The researchers indicated that this methodology could also be applied to mapping sites of serine and threonine phosphorylation. They proposed an additional chemical method based on beta-elimination of the phosphate group and nucleophilic addition of an affinity tag to the resulting dehydroalanine residue [67].

Quantitative analysis via affinity tag

Although the 2-DE-MS/MS approach already permits relative quantification of proteins in the two-dimensional gel, quantitative proteomics is becoming even more accessible due to the use of stable isotope labeling [68]. A class of small-molecule reagents, isotope-coded affinity tags (ICATs), has been developed for the quantitative analysis of a proteome [69]. Griffin et al. [70] described an approach to the quantitative analysis of complex protein mixtures using a MALDI quadrupole time-of-flight (MALDI QqTOF) mass spectrometer and ICAT reagents. The ICAT reagent is designed to react with sulfhydryl groups in every protein. It possesses a deuterated or C13 linker and a biotin tag [69]. For practical applications, cysteinyl residues are derivatized with “light” or “heavy” ICAT reagents. The samples are combined and digested, and the biotin-tagged peptides are isolated by affinity chromatography. The isolated peptides are then identified and quantified by LC-MS/MS [69]. According to Gygi et al. [69], this method is suitable for high- or low-abundance proteins. The proteins in intact cells in a culture need not be labeled, and the quantitative process may be automated for high-throughput analyses. They suggested that this approach is useful for the proteomewide identification and quantitative profiling of proteins, and widespread application of the technique is expected in the field of quantitative proteomics [71].

Alternate separation techniques for proteome analysis have also been investigated with the aim of developing new platform technologies. These alternate techniques are one-dimensional or multidimensional HPLCs integrated with MS. Many approaches involve the quantification of proteins containing specific groups. Conrads et al. [72] described the combined use of isotopic ^{15}N -metabolic labeling and a cysteine-reactive biotin affinity tag to isolate and quantitate cysteine-containing polypeptides (Cys-polypeptides) from *Deinococcus radiodurans* and from mouse B16 melanoma cells. Ji et al. [73] presented a proteomic analysis method using affinity chromatography for the separation of signature peptides before MS analysis. Peptides are quantified using LC-ESI-MS and isotopically labeled internal standards [73]. Glycoproteins can be studied by the affinity selection and MALDI-MS identification of glycopeptides from tryptic digests [74]. In another work,

Riggs et al. [75] addressed the issue of automating the multidimensional chromatography of signature peptides as an approach to proteomics. According to their strategy, peptides are automatically reduced and alkylated in the autosampler of the instrument. Trypsin then digests all proteins in the sample on an immobilized enzyme column, and the digest is directly transferred to an affinity chromatography column [75].

Prospective

For the purpose of high-throughput analysis, miniaturization of proteome analysis techniques is of increasing importance. “Proteomics on a chip” is a promising field [76]. Approaches involve either the microfabrication of microchannels to prepare samples, interfaced to MS for protein analysis, or the microfabrication of devices for coupling CE to ESI-MS. Affinity chromatography on a chip coupled with electrophoretic and/or MS methods would be an important advance. Fig. 2 shows two simple approaches to miniaturizing affinity chromatography on a fabricated microfluidic chip. Packing with affinity microbeads or *in situ* synthesis of a monolithic affinity polymer can be used to form an affinity separation bed in the the microfluidic channel. Affinity chromatography on a chip may perform a concentration/desalting function to increase the sensitivity of MS for protein identification. Proteins with posttranslational modifications selected by the affinity method may also be helpful in identifying modified proteins by mass spectrometry and eventually for the discovery of their cellular functions.

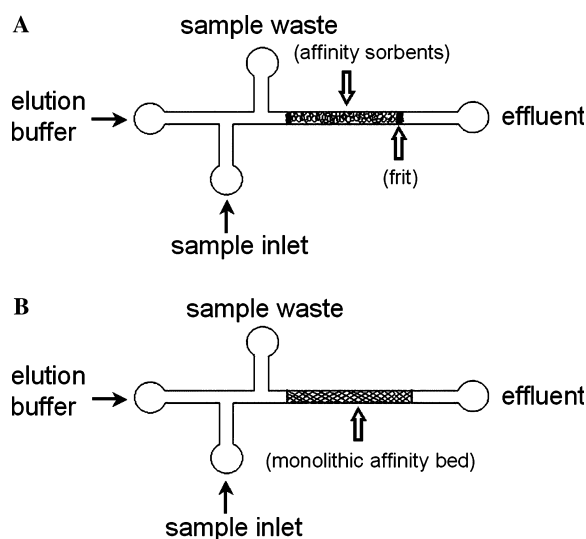


Fig. 2. Two approaches for miniaturizing affinity chromatography on fabricated microfluidic chips. (A) The use of a packed bed of beads for separation; (B) the use of a monolithic polymer bed for separation.

In the exploration of protein–protein interactions, the binding kinetics between interacting proteins could be thoroughly studied by affinity chromatography. A specific protein in a cell can contact different proteins in different cellular spaces or in different periods of a “life cycle,” so the interactions of a multifunctional protein with other cellular proteins can be very complex. All of these interactions may be studied by immobilizing this protein as the “bait.” The use of affinity tags makes the separation more general, but the introduction of additional tag genes could alter the activity and natural level of expression of the studied protein. ICAT reagents still are limited in that they attach only to the cysteinyl residue of proteins. Consequently, an opportunity exists to design an alternative tool to perform the affinity purification of protein complexes in the cell. Different approaches for affinity-based purification and concentration may be developed for different classes of protein complexes. For example, the affinity ligands and protein solubilization strategies appropriate for purifying phosphorylated proteins, glycoproteins, and their associated proteins should be different from those for other protein complexes. One important development is the use of *in vivo* cross-linking of protein complexes. The development of new types of affinity ligand-tagged proteins will also be useful. As an example, the use of the immobilized bis-arsenical fluorescein dye, FIAsH, for the affinity chromatography of CCXXCC-tagged protein has recently been developed [77].

Applications of affinity-based purification for increasing the resolution in 2-DE and sensitivity in mass spectrometric quantification will continue. Although many strategies involve the use of one-dimensional or multidimensional HPLC as alternatives to 2-DE, affinity chromatography is difficult to implement alone as a one-dimensional assay of proteins. This situation is a result of its nature, which is strictly to distinguish bio-specific proteins from other proteins. However, affinity chromatography using group-specific ligands can be very effective for separation of subproteomes, e.g., Protein A or Protein G for antigens and antibodies, Con A for glycoproteins, and human brain lectin for oligoclonal IgG antibodies. Short columns packed with nonporous microbeads are particularly useful for this micropreparative purpose [78,79]. Separated proteins from each subgroup are then directed to another one-dimensional gel electrophoresis and mass spectrometric characterization.

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